The Role of 12-Lipoxygenase in the Regulation of Platelet Function

by

Jennifer Yeung

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in the University of Michigan
2018

Doctoral Committee:

Associate Professor Michael Holinstat, Chair
Professor Steven McKenzie, Thomas Jefferson University
Professor Yoichi Osawa
Assistant Professor Jordan Shavit
Professor Alan Smrcka
Associate Professor Gregory Tall

Jennifer Yeung

jenniyeu@umich.edu

ORCID iD: 0000-0001-5279-025X

Dedication

I would like to dedicate this thesis to my orange tabbies (Rufus and Linus Yeung), nieces (Makayla Reese and Riley Mei Yeung), Benjamin Tourdot, and my loving family members (mom, dad, brother and Suki).

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Michael Holinstat, for the great support. It has been a great pleasure to have the opportunity to work with Dr. Michael Holinstat at both the University of Michigan and Thomas Jefferson University. I would like to thank Cardeza at Thomas Jefferson University members (Dr. Steven McKenzie, Joanne Vesci). I would like to acknowledge my thesis committee members, Dr. Jordan Shavit, Dr. Alan Smrcka, Dr. Greg Tall, Dr. Steve McKenzie, and Dr. Yoichi Osawa, for offering suggestions and providing guidance. Most importantly, I want to thank Dr. Reheman Adili, and Dr. Benjamin Tourdot for providing intellectual guidance, support, and feedback that helped my career. Lastly, I want to thank the current lab members for helping out along the projects especially the undergraduates (Angel Chen, Anet Szatkowski, and Madeline Jackson). I am also grateful for the sources of funding (American Heart Association Predoctoral Fellowship and NRSA F31 (HL129481)) that have supported my graduate studies and thesis work.

Table of Contents

Dedication ii

Acknowledgements iii

List of Figures vii

List of Tables xi

List of Appendices xii

List of Abbreviations xiii

Abstract xx

CHAPTER 1 Platelet Biology 1

1.1 Platelet structure 1
1.2 Overview of platelets in hemostasis and thrombosis 3
1.3 The role of collagen in hemostasis and thrombosis 4
1.4 Immune receptor mediated activation of platelet 10
1.5 Platelet activation and signaling mediated by G-protein-coupled receptors. 12
1.6 Central hypothesis aims 15

CHAPTER 2 Anti-platelet Therapy 17

2.1 Summary 17
2.2 Introduction 18
2.3 P2Y Receptor Antagonists 18
2.4 Glycoprotein (GP) Antagonists 23
2.5 Phosphodiesterase Antagonists 28
2.6 Thromboxane A_2 Receptor Antagonists 29
2.7 Thrombin Receptor Antagonists 30
2.8 Conclusion 31

CHAPTER 3 12-Lipoxygenase: A Novel Target for Anti-Platelet Therapeutics 33

3.1 Summary 33
3.2 Platelet Therapy in Cardiovascular Disease 34
3.3 12-LOX in the Human Platelet 36
3.4 12-LOX Substrates 39
3.5 The Biological Role of Metabolites 41
3.6 12-LOX Inhibitors 43
3.7 Conclusions 45
CHAPTER 4 Oxygenases and Their Derived Oxylipins Regulation of Platelet Function 49
4.1 Summary 49
4.2 Introduction 49
4.3 Cyclooxygenase 51
4.3 Lipoxygenases 55
5-Lipoxygenase (5-LOX) 55
12-Lipoxygenase (12-LOX) 58
15-Lipoxygenase (15-LOX) 61
4.4 Cytochrome P450 62
4.5 The interplay of oxygenases and generation of specialized pro-resolving lipid mediators (SPMs) 67
4.6 Discussion and Future Implications 70
CHAPTER 5 Protein Kinase Regulation of 12-Lipoxygenase-Mediated Human Platelet Activation 72
5.1 Summary 72
5.2 Introduction 73
5.3 Materials and Methods 75
5.4 Results 78
5.5 Discussion 88
CHAPTER 6 12-Lipoxygenase Activity Plays an Important Role in PAR4 and GPVI-mediated Platelet Reactivity 92
6.1 Summary 92
6.2 Introduction 93
6.3 Materials and Methods 95
6.4 Results 96

6.5 Discussion 110
CHAPTER 7 12-Lipoxygenase Modulates FcγRIIa Signaling in Platelets 115
7.1 Summary 115
7.2 Introduction 116
7.3 Materials and Methods 118
7.4 Results 122
7.5 Discussion 132
CHAPTER 8 Investigations of Human Platelet-type 12-Lipoxygenase: Role of Lipoxygenase Products in Platelet Activation 135
8.1 Summary 135
8.2 Introduction 136
8.3 Materials and Methods 137
8.4 Results 143
8.5 Discussion 156
CHAPTER 9 12-HETrE, a 12-Lipoxygenase Oxylipin of Dihomo-γ-Linolenic Acid, Inhibits Thrombosis via $G\alpha_s$ Signaling in Platelets 162
9.1 Summary 162
9.2 Introduction 163
9.3 Materials and Methods 165
9.4 Results 170
9.5 Discussion 181
CHAPTER 10 Discussion 186
Bibliography 213

List of Figures

Figure 1.1 Processes of platelet adhesion and activation to form a hemostatic plug or thrombus formation. 4

Figure 1.2 Immunoreceptor tyrosine-based activation motif (ITAM) receptors in platelets. 12

Figure 1.3 Overview of G-protein coupled receptors and signaling in platelet activation 15

Figure 4.1 Mechanisms of oxylipin regulation of platelet function. 51

Figure 4.2 Oxidation of arachidonic acid into their respective products by oxygenases. 56

Figure 4.3 PUFA oxidation by oxygenase. 60

Figure 4.4 PUFA oxidation by oxygenases. 63

Figure 4.5 Specialized pro-resolving lipid mediators (SPMs) constitute a wide array of lipids classes derived from the interplay of oxygenase activity on AA, EPA, and DHA. 67

Figure 4.6 LOX, COX, CYP 450 derived lipid mediators and SPMs can be divided into either pro- or anti-thrombotic classes based on their effects on platelet function. 70

Figure 5.1 Inhibitor selectivity. 79

Figure 5.2 PKC regulation of platelet aggregation. 80

Figure 5.3 PKC is an important determinant of platelet ATP secretion. 81

Figure 5.4 PKC activation rescues platelet aggregation downstream of 12-LOX. 82

Figure 5.5 Role of PKC in PAR-mediated integrin αIIbβ3 activation. 83

Figure 5.6 12-LOX regulates agonist-mediated ERK phosphorylation. 85

Figure 5.7 12-LOX inhibition attenuates calcium mobilization in human platelets. 87

Figure 6.1 12-LOX is an important determinant of platelet aggregation. 97

Figure 6.2 12-LOX activation is required for dense granule secretion in platelets. 100

Figure 6.3 12-LOX is not critical for agonist-mediated α-granule secretion..... 102

Figure 6.4 Inhibition of 12-LOX in whole blood attenuates platelet aggregation and adhesion. 104

Figure 6.5 12-LOX$^{-/-}$ mice have impaired platelet activation and hemostasis..... 106

Figure 6.6 COX-1 and 12-LOX regulation of PAR-mediated platelet activation. 110

Figure 7.1 12-LOX modulates FcγRIIa-mediated platelet aggregation. 123

Figure 7.2 Murine platelets require 12-LOX for normal FcγRIIa-induced platelet aggregation..... 125

Figure 7.3 FcγRIIa-mediated Rap1 and integrin αIIbβ3 activation are potentiated by 12-LOX. 126

Figure 7.4 Granule secretion mediated by FcγRIIa activation is regulated by 12-LOX. 129

Figure 7.5 The role of 12-LOX in regulating the FcγRIIa signaling complex. 130

Figure 7.6 12-LOX modulates early signaling components of the FcγRIIa pathway in human platelets. 131

Figure 7.7 Schematic model of 12-LOX role in the regulation of FcγRIIa pathway. 134

Figure 8.1 12-LOX product regio-specificity and substrate comparison. 145

Figure 8.2 Fatty acid metabolites regulate platelet aggregation. 148

Figure 8.3 Fatty acid regulation of platelet dense granule secretion. 150

Figure 8.4 Agonist-independent regulation of platelet activity by 12-LOX metabolites. 153

Figure 8.5 12-LOX metabolite regulation of Rap1..... 155

Figure 8.6 Fatty acid regulation of platelet-mediated clot retraction. 161

Figure 9.1 12-LOX is required for DGLA inhibition of platelet aggregation and thrombus formation. 172

Figure 9.2 12-HETrE inhibits platelet aggregation and thrombus formation. 174

Figure 9.3 Exogenous DGLA enhances platelet production of metabolites. 177

Figure 9.4 Hemostasis is not affected by 12-HETrE. 178

Figure 9.5 12-HETrE activates adenylyl cyclase-mediated signaling in platelets. 179

Figure 9.6 Proposed model of 12-HETrE inhibitory signaling in platelets. 185

Figure 10.1 The pro- and anti-thrombotic oxylipins of 12-LOX. 188

Figure A.1 Pleckstrin is a 47 kDa protein corresponding to one of the phospho-(Ser)-PKC substrate bands. 196

Figure B.1 12-LOX inhibitor selectivity towards 12-LOX. 197

Figure B.2 12-LOX inhibitors do not interfere with ATP luciferase assay. 198

Figure D.1 Charge remote allylic fragmentation. 201

Figure D.2 1D proton NMR of 13-HODE. 201

Figure D.3 2D COSY NMR of 13-HODE. 202

Figure D.4 1D Proton NMR of 13-HODE ester with Mosher (S) 202

Figure D.5 2D COSY NMR of 13-HODE ester with Mosher (S) 203

Figure D.6 1D Proton NMR of 13-HODE ester with Mosher (R). 203

Figure D.7 2D COSY NMR of 13-HODE ester with Mosher (R). 204

Figure D.8 13-HODE with labeled protons. 204

Figure D.9 Chemical Shifts of 13-HOTrE. 205

Figure D.10 1D Proton NMR of 12-HETrE. 205

Figure D.11 1D proton NMR of 12-HETrE ester with Mosher (S). 206

Figure D.12 2D COSY NMR of 12-HETrE ester with Mosher (S)..... 206

Figure D.13 1D Proton NMR of 12-HETrE ester with Mosher (R) 207

Figure D.14 2D COSY NMR of 12-HETrE ester with Mosher (R). 207

Figure D.15 12-HETrE with labeled protons. 208

Figure D.16 Chemical Shifts of 12-HETrE 208

Figure D.17 1D Proton NMR of 13-HOTrE. 209

Figure D.18 2D COSY NMR of 13-HOTrE. 209

Figure D.19 1D Proton NMR of 13-HOTrE ester with Mosher (S) 210

Figure D.20 2D COSY NMR of 13-HOTrE ester with Mosher (S) 210

Figure D.21 1D Proton NMR or 13-HOTrE ester with Mosher (R)..... 211

Figure D.22 2D COSY NMR of 13-HOTrE ester with Mosher (R)..... 211

Figure D.23 13-HOTrE with labeled protons. 212

Figure D.24 Chemical Shifts of 13-HOTrE. 212

List of Tables

Table 2.1 Approved antiplatelet drugs. 19

Table 2.2 Antiplatelet drugs under development 22

Table 3.1 12-LOX expression and function different species 38

Table 3.2 12-LOX inhibitors and targets 47

Table 4.1 Oxylipin regulation of platelets 54

Table 8.1 Primary product distribution. 144

Table 8.2 Primary Product Identification by Mass Spectrometry. 146

Table 8.3 Steady state kinetics of substrates. 149

Table C.1 Complete blood count was performed on hFcR/ALOX12-/- (n=4) and hFcR/ALOX12+/+ (n=3) mice. 200

List of Appendices

Appendix A: Protein Kinase Regulation of 12-Lipoxygenase-Mediated Human Platelet Activation 196

Appendix B: 12-Lipoxygenase Activity Plays an Important Role in PAR4 and GPVI-Mediated Platelet Reactivity 197

Appendix C: Platelet 12-LOX is Essential for FcγRIIa-Mediated Platelet Activation ... 200

Appendix D: Investigations of Human Platelet-Type 12-Lipoxygenase: Role of Lipoxygenase Products in Platelet Activation Charge remote allylic fragmentation 201

List of Abbreviations

10,11-DiHDPA	10,11-Dihydroxydocosapentaenoic acid
10,11-EDP	10,11-Epoxydocosapentaenoic acid
11,12-diHETE	11,12-Dihydroxyeicosatetraenoic acid
11,12-diHETrE	11,12-Dihydroxyeicosatrienoic acid
11,12-EEQ	11,12-Epoxyeicosatetraenoic acid
11,12-EET	11,12-Epoxyeicosatrienoic acid
11/14-HDHA	11/14-Hydroxydocosahexaenoic acid
11/14-HDPA	11/14-Hydroxydocosapentaenoic acid
12-HDHA	12-Hydroxydocosahexaenoic acid
12-HhTE	12-Hydroxy-5,8,10-14-heptadecatetraenoic acid
12-LOX	12-Lipoxygenase
12-LOX$^{-/-}$	12-LOX knockout mice; also known as ALOX12$^{-/-}$
12,20-diHETE	12,20-Dihydroxyeicosatetraenic acid
12(R)-HETE	12R-Hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid
12(S)-HEPE	12(S)-Hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid
12(S)-HETE	12(S)-Hydroxy-5,8,10,14-eicosatetraenoic acid
12(S)-HETrE	12(S)-Hydroxyeicosatetraenoic acid
12(S)-HETrE	12(S)-Hydroxy-8Z,10E,14Z-eicosatrienoic acid
12(S)-HpETE	12(S)-Hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid
12(S)-HpETrE	12(S)-Hydroperoxy-8Z,10E,14Z-eicosatrienoic acid
12R-LOX	12-LOX isoenzyme that generates R chiral products
12S-LOX	12-LOX isoenzyme that generates S chiral products
13-HODE	13-Hydoxyoctadecadienoic acid
13,14-DiHDPA	13,14-Dihydroxydocosapentaenoic acid
13,14-EDP	13,14-Epoxydocosapentaenoic acid
13(S)-HOTrE	13(S)-Hydroxy-9Z,11E,15Z-octadecatrienoic acid
13(S)-HpOTrE	13(S)-Hydroperoxy-9Z,11E,15Z-octadecatrienoic acid
14-HDoHE	14-Hydroxyl docosahexaenoic acid
14,15- DiHETE	14,15-Dihydroxyeicosatetraenic acid
14,15-diHETE	14,15-Dihydroxyeicosatetraenoic acid
14,15-diHETrE	14,15-Dihydroxyeicosatrienoic acid
14,15-EEQ	14,15-Epoxyeicosatetraenoic acid
14,15-EET	14,15-Epoxyeicosatrienoic acid
15-HETE	15-Hydroxyeicosatetraenoic acid

15-HETrE	15-Hydroxyeicosatrienoic acid
15-LOX	15-Lipoxygenase
15(R)-epi-LXA_4	5(S),6(R),15(R)-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid
15S-LOX	15S-Lipoxygengenase; also known as 15-LOX-1
15S-LOX type B	15S-Lipoxygengenase; also known as 15-LOX-2
16,17-DiHDPA	16,17-Dihydroxydocosapentaenoic acid
16,17-EDP	16,17-Epoxydocosapentaenoic acid
17-HDHA	17-Hydroxydocosahexaenoic acid
17,18- DiHETE	17,18-Dihydroxyeicosatetraenic acid
17,18-EEQ	17,18-Epoxyeicosatetraenoic acid
19-HETE	19-Hydroxyeicosatetraenoic aci
19,20-EDP	19,20-Epoxydocosapentaenoic acid
19,20-HiDPA	19,20-Dihydroxydocosapentaenoic acid
20-HETE	20-Hydroxyeicosatetraenoic acid
5-HETE	5-Hydroxyeicosatetraenoic acid
5-HT	Serotonin
5-oxo-ETE	5-Oxo-6,8,11,14-eicatetraenoic acid
5,6-diHETrE	5,6-Dihydroxyeicosatrienoic acid
5,6-EET	5,6-Epoxyeicosatrienoic acid
7,8-DiHDPA	7,8-Dihydroxydocosapentaenoic acid
7,8-EDP	7,8-Epoxydocosapentaenoic acid
8,15-diHETE	8,15-Dihydroxyeicosatetraenoic acid
8,9-DiHETE	8,9-Dihydroxyeicosatetraenoic acid
8,9-diHETrE	8,9-Dihydroxyeicosatrienoic acid
8,9-EEQ	8,9-Epoxyeicosatetraenoic acid
8,9-EET	8,9-Epoxyeicosatrienoic acid
$\alpha_2\beta_1$	Collagen receptor; also known as VLA-2
AA	Arachidnic acid
AC	Adenylyl cyclase
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
AERD	Aspirin-exacerbated respiratory disease
$\alpha_{IIb}\beta_3$	Glycoprotein IIb/IIIa or integrin
Akt	Serine/threonine-specific protein kinase (also known as protein kinase B (PKB))
ALA	alpha-linolenic acid
ALX	LXA_4 receptor
ATL	Aspirin-triggered lipoxin
BLT_2	Leukotriene B_4 receptor
Btk	Bruton's tyrosine kinase

CalDAG-GEF	Ca^{2+}-dependent exchange factor
cAMP	3',5'-cyclic adenosine monophosphate
cGMP	3',5'-cyclic guanosine monophosphate
CLEC-2	C-type lectin-like receptor 2
COX	Cyclooxygenase
cPLA2	Cytoplasmic phospholipase A2
CRP	Collagen-related peptide
CSK	C-terminal Src kinase
CTP	Cyclopentyl-triazolo-pyrimidine
CVD	Cardiovascular disease
CYP450	Cytochrome P450
$CysLT_1R$	Cysteinyl-leukotriene receptor type 1
$CysLT_2R$	Cysteinyl-leukotriene receptor type 2
$Cysltr1^{-/-}$	Cysteinyl-leukotriene receptor type 1 knockout mice
$Cysltr2^{-/-}$	Cysteinyl-leukotriene receptor type 2 knockout mice
DAG	Diacylglycerol
DES	Drug-elution stent
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
ECM	Extracellular matrix
EDA	Eicosadienoic acid
EDP	Epoxydocosapentaenoic acid
EEQ	Epoxyeicosatetraenoic acid
EET	Epoxyeicosatrienoic acid
EFA	Essential fatty acid
EP_1	Prostaglandin receptor
EP_2	Prostaglandin receptor
EP_3	Prostaglandin receptor
EP_4	Prostaglandin receptor
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FcγRIIa	Fc receptor for IgG (also known as CD32)
FcRγ	Fc gamma receptor
FDA	Federal Drug Administration
Gads	Grb2-related protein 2
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLA	Gamma-linolenic acid

GPCR	G protein-coupled receptor
GPIa	Glycoprotein Ia
GPIb-IX-V	Glycoprotein Ib-IX-V receptor complex
GPIb-V-IX	GPIb disulfide linked to two GPIb subunits to form GPIb, GPIX and GPV
GPIba	Glycoprotein Ibα
GPIX	Glycoprotein IX
GPO	Glycine-proline-hydroxyproline
GPR31	12(S)-HETE receptor; also known as 12-HETER
GPV	Glycoprotein V
GPVI	Glycoprotein VI
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
hFcR/ALOX12$^{-/-}$	FcγRIIA transgenic mice without 12-LOX
hFcR/ALOX12$^{+/+}$	FcγRIIA transgenic mice with 12-LOX
HIT/or HITT	Heparin-induced thrombocytopenia and thrombosis
ICAM-1	Intracellular adhesion molecule-1
Ig	Immunoglobulin
IL-6	Interleukin 6
$InsP_3R$	Inositol triphosphate receptor
IP	Prostacyclin receptor
IP_3	inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
IV	Intravascular
IV.3+GAM	Anti-FcγRIIA clone IV.3 antibody and goat anti-mouse antibody Fab'2
KGD	Lysine-glycine-aspartic acid
LA	Linoleic acid
LAMP-2	Lysosomal-associated membrane protein-2
LAT	Linker for activation of T cells
LEC	Lymph endothelial cell motility
LOX	Lipoxygenase
LT	Leukotriene
LTA_4	Cysteinyl-free leukotriene A4
LTB_4	Cysteinyl-free leukotriene B_4
LTB_5	Cysteinyl-free leukotriene B_5
LTC	Cysteinyl-leukotriene C
LTD	Cysteinyl-leukotriene D
LTE	Cysteinyl-leukotriene E
LTF	Cysteinyl-leukotriene F

LX	Lipoxin
LXA_4	Lipoxin A_4
LXB_4	Lipoxin B_4
MAC-1	Macrophage-1 antigen, also known as integrin αMβ2
MAPK	Mitogen-activated protein kinase
MaR	Maresin
MCP-1	Monocyte chemoattractant protein-1
MLC	Myosin light chain
MMP	Matrix metalloprotease
MYPT	Myosin phosphatase targeting subunit 1
NO	Nitric oxide
NSAIDS	Nonsteroidal anti-inflammatory drugs
p160ROCK	Rho-associated, coiled-coil-containing protein kinase 1
$P2Y_1$	Purinergic receptor for ADP
$P2Y_{12}$	Purinergic receptor for ADP
PAF	Platelet activating factor
PAR	Protease activated receptor
PAR1	Protease-activated receptor 1
PAR4	Protease-activated receptor 4
PCI	Percutaneous coronary intervention
PD	(Neuro)protectin
PD1	Neuroprotectin D1
PDE	Phosphodiesterase
PDE3	Type III-phosphodiesterase
PDX	Protectin DX
PDGF	Platelet-derived growth factor
PF4	Platelet factor 4
PG	Prostaglandin
PGD_1	Prostaglandin D_1
PGD_2	Prostaglandin D_2
PGD_3	Prostaglandin D_3
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
PGI_2	Prostaglanding I_2; also known as Prostacyclin
PGI_3	Prostaglandin I_3
PGJ_2	Prostaglandin J_2
PH	Pleckstrin homology
PI3K	phosphoinositide 3-kinase

PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein Kinase A
PKC	protein kinase C
PKG	cGMP-dependent protein kinase G
PLCγ2	Phospholipase C gamma2
PLCβ	Phospholipase C beta
PMA	Phorbol myristate acetate; diacylglycerol (DAG) mimetic
pMLC	Phosphorylation of myosin light chain
PPAR	Proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor gamma
PRP	Platelet-rich plasma
$Ptgir^{-/-}$	Prostacyclin receptor deficient mouse
PUFA	Polyunsaturated fatty acid
Rap1b	Small GTPase, Ras-related protein 1
RBC	Red blood cell
RhoA	Ras homolog gene family, member A
RIAM	Rap1-GTP-interacting adaptor molecule
Rv	Resolvin
RvD_1	Resolvin D_1
RvE_1	Resolvin E_1
SFKs	Src family kinases
sHE	Soluble expoxide hydrolase
SLP-76	Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa
SPM	Specialized pro-resolving mediator
STEMI	ST-elevation myocardial infarction
Syk	Spleen tyrosine kinase
TGF-β1	Transforming growth factor beta1
TIMI	Thrombolysis in myocardial infarction
TNF-α	Tumor necrosis factor-alpha
TPα	TxA_2 receptor
TRAP	Thrombin receptor activating peptide
TX	Thromboxane
TXA_1	Thromboxane A_1
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
U46619	TxB_2 mimetic
VASP	Vasodilator-stimulated phospoprotein

Vav1	GTP exchange factor
Vav3	GTP exchange factor
vWF	von Willebrand Factor
ω-3	Omega-3
ω-6	Omega-6
WT	Wild-type
$\alpha_v\beta3$	Integrin that is a receptor for vitronectin
Δ^{12}-PGJ_2	Δ^{12}-Prostaglandin J_2

Abstract

Platelets are small, anucleated cellular fragments derived from the megakaryocytes of the bone marrow. These small cellular fragments have a specialized role in maintaining hemostasis, a process that prevents blood loss, through the initiation of blood coagulation. However, excessive platelet reactivity can lead to a pathophysiological condition known as thrombosis. Platelet-mediated thrombosis is the primary underlying mechanism leading to cardiovascular life-threatening clinical events, such as myocardial infarction and stroke. Regulating excessive platelet reactivity is an essential aspect of antithrombotic therapy. A number of anti-platelet drugs have been developed to target specific signaling pathways or endpoints involved in platelet activation. Despite the effectiveness of current anti-platelet therapies, uncontrolled thrombosis or bleeding complications still persist. Therefore, elucidating the mechanisms involved in platelet activation is crucial for identifying the development of novel or alternative anti-thrombotic strategies.

Platelet 12-lipoxygenase (12-LOX), an oxygenase principally found to convert freed polyunsaturated fatty acids (PUFA) substrates from the membrane phospholipids following cellular stimulation, was identified to be a potential target for regulating platelet reactivity. Thus, the studies described in this thesis seek to assess whether 12-LOX activity modulated platelet responses or reactivity by using newly developed selective 12-LOX inhibitors, NCTT-956 and ML355. Although both NCTT-956 and ML355 blocked 12-LOX activity in platelets, ML355 was shown to be much more potent in inhibiting platelet activation. In support of the

pharmacological *ex vivo* studies, mice lacking 12-LOX (12-LOX$^{-/-}$) in platelets were protected from platelet-induced activation as well as thrombosis *in vivo*.

Additionally, the role of 12-LOX in immune-mediated platelet activation was also investigated in this thesis work. One form of immune-mediated platelet activation or thrombosis is heparin-induced thrombocytopenia and thrombosis (HITT), a life-threatening cardiovascular disorder. A key component of this disorder is the activation of FcγRIIa, a transmembrane receptor, by immune-complexes. I had shown that 12-LOX potentiated immune-mediated platelet activation and 12-LOX activity regulated early signaling effectors in the immune signaling pathways of platelet activation.

12-LOX predominantly generates 12(S)-HETE, an oxylipin or metabolite, from arachidonic acid (AA), since this is the most abundant ω-6 PUFA found in the lipid bilayer of cells. Dietary supplementation of plant- or fish-based oils can alter and enrich the contents of the lipid bilayer by which they shift 12-LOX substrate from AA to other PUFAs. Consumption of borage or primrose oil, which is enriched in ω-6 PUFA dihomo-γ-linolenic acid (DGLA), has been shown to be cardioprotective; however, its mechanism of action was unclear. In this thesis, I had also sought to elucidate the underlying mechanism by which DGLA could provide cardioprotection via the prevention of thrombosis. I had demonstrated that the oxylipin of DGLA generated by 12-LOX, 12(S)-HETrE, inhibited platelet activation and thrombosis *in vivo* in a Gα-signaling dependent manner. Altogether, my thesis work highlighted the importance of targeting 12-LOX in platelets or using its endogenous mechanism to generate oxylipins that have anti-platelet effects as potential therapeutic avenues for preventing unwanted platelet activation.

CHAPTER 1

Platelet Biology

Hemostasis is an intrinsic property of the vascular system that involves the interplay of platelets and the endothelial lining to 1) maintain blood in a fluid state, 2) close off damaged blood vessels, and 3) remove clots after restoration of vascular integrity. Extending beyond the classical roles of hemostasis and thrombosis, platelets are increasingly being recognized for other pathological disorders such as, inflammation, tumor metastasis, atherosclerosis, and non- or pro-infectious immunological functions.

1.1 Platelet structure

In 1882, Giulio Bizzozzero had established the phenomena of platelet morphology and their major contribution to thrombosis and hemostasis[1]. Bizzozzero had also demonstrated the difficulty in isolating platelets in blood removed from the vessel. Subsequently, he developed methods to isolate and preserve platelets as separate and intact structures, which led him to describe platelet morphology. To date, platelets are described as small, anucleated cytoplasmic fragments (approximately 2-5 μm in diameter and .5 μm thickness) derived from the megakaryocytes in the bone marrow.

Structurally, platelets can be conceptually divided into peripheral, sol-gel, and organelle zones[2]. The peripheral zone refers to the platelet plasma membrane that is coated with glycocalyx (glycoprotein-polysaccharide covering) that enables platelets to adhere to and aggregate with subendothelial matrix proteins and other platelets. Beneath the glycocalyx is the rigid lipid bilayer; however, additional membrane structure which aids in platelet spreading is provided by

the tiny folds of the platelet surface and the internalized membrane parts of the open canicular system[3]. The sol-gel zone contains organized microtubules, microfilaments, open canalicular, and the dense tubular systems. The cytoskeletal arrangement in this zone helps support platelet membrane contraction, as well as constriction to move secretory organelles (granules and dense bodies) to the platelet center prior to secretion of their vesicle contents through the open canalicular system[4-6].

The organelle zone contains three major types of secretory contents, α-, δ- granules, and lysosomes. While the α- and δ- granule contents do play important roles in hemostasis, thrombosis, and inflammation, the role of lysosomal contents in hemostasis and thrombosis is still unclear. The α-granules are the largest secretory organelles, ranging from 50 to 80 vesicles per platelet, and each is 200-500 nm in diameter. Contents of α-granules include cell adhesion molecules (integrin $\alpha_{IIb}\beta_3$, glycoprotein (GP)Ib-IX-V complex, GPVI, and P-selectin), chemokines (platelet factor 4 (PF4)/(CXCL4), growth or angiogenic factors (insulin-like growth factor 1, platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF-β1)), and clotting factors (fibronectin, thrombospondin, factor V, von Willebrand (vWF)). In humans, approximately 3 to 8 δ- granules per platelet with a diameter of 150 nm have been observed. Each δ-granule is filled with adenosine nucleotides (ADP, ATP), serotonin, ionized calcium, pyrophosphate, magnesium, and CD63. There are less than 3 lysosomes observed in each human platelet, and each lysosome ranges from 200-250 nm in diameter. Each lysosome is comprised of acid hydrolases, cathepsin D and E, lysosomal-associated membrane protein (LAMP)-2, and CD63.

1.2 Overview of platelets in hemostasis and thrombosis

Under steady state conditions, platelets circulate freely in a quiescent state in the blood without interacting with each other or the vascular endothelium. However, in the presence of endothelial damage whether from vascular injury or rupture of an atherosclerotic plaque, a chain of events is triggered, ultimately leading to platelet-rich clot formation. Depending on the initiating event, platelets may participate in normal hemostasis or pathologic intravascular thrombosis.

The molecular mechanisms underlying platelet function in hemostasis and thrombosis are well defined. Under high arterial shear rates (1000 – 10,000 s^{-1}), circulating platelets are rapidly decelerated and transiently interact with the damaged and exposed subendothelial connective tissue containing immobilized glycoprotein vWF bound to collagen (type I, III, and VI). vWF-collagen interacts with the platelet glycoprotein receptor (GP) Ib-V-IX complex, allowing platelets to translocate along the vessel wall and engage their receptors, glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$, with the subendothelial fibrillar collagen. This firm interaction also facilitates platelets to associate with fibronectin through its engagement with integrin $\alpha_5\beta_1$.

The engagement of platelet receptors with collagen also induces an "inside-out" cellular signaling cascade that leads to integrin $\alpha_{IIb}\beta_3$ activation[7,8]. This process involves intermediary proteins, talin and kindlin, which bind to the cytoplasmic domain of β_3 integrin to shift $\alpha_{IIb}\beta_3$ from an inactive to active state[9,10]. Active $\alpha_{IIb}\beta_3$ conformation increases its affinity for adhesive proteins, vWF, fibrinogen, fibrin, and fibronectin. These interactions are essential for the platelets to form stable aggregates with other activated platelets to promote thrombus growth (Figure 1.1).

Following firm adhesion and aggregate formation, platelets also release or locally generate soluble agonists, such as ADP, thromboxane A_2 (TxA_2), and thrombin, to mediate feedforward

autocrine and paracrine platelet activation via their respective G protein-coupled receptors (GPCRs). The activation of GPCRs initiates a series of intracellular signaling events, including generation of second messengers (DAG and IP_3). Eventually, the cascade of downstream signaling events culminates in the secretion of soluble factors, platelet spreading, and integrin activation. The secreted soluble agonists act on circulating platelets to be recruited and incorporated into a growing thrombus (Figure 1.1).

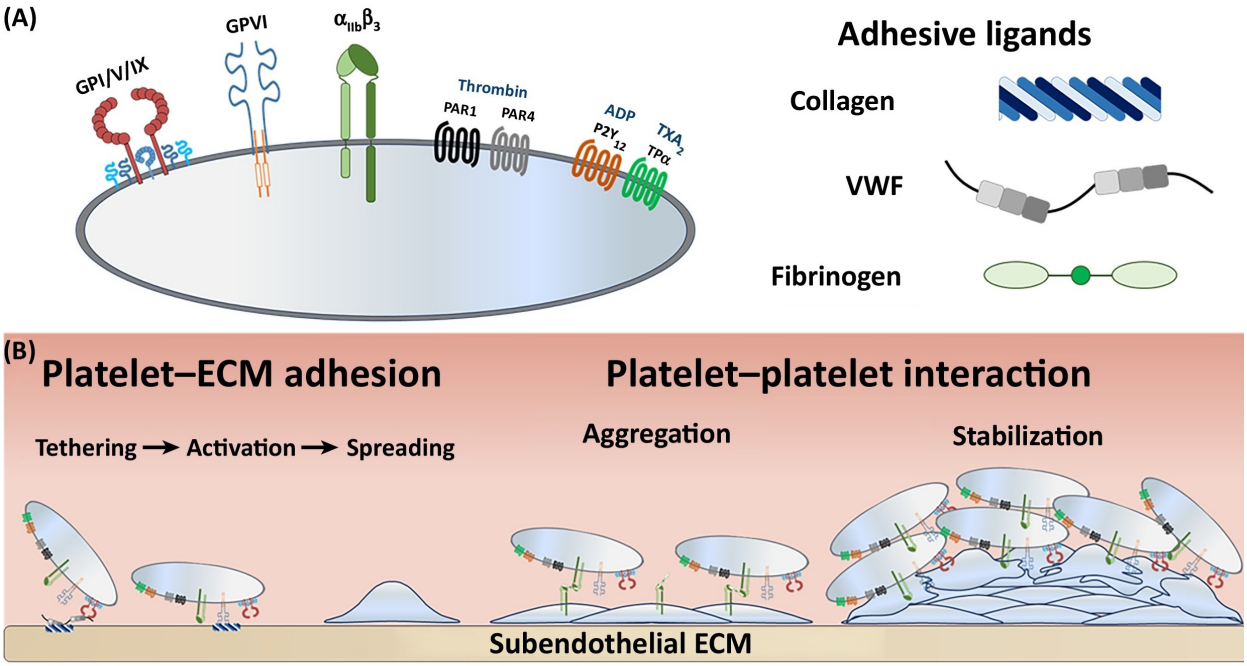


Figure 1.1 Processes of platelet adhesion and activation to form a hemostatic plug or thrombus formation.
Following vessel injury, platelets adhere to the vessel by "tethering" to the exposed collagen bound to von Willebrand factor (vWF) on the underlying subendothelial extracellular matrix (ECM) via glycoprotein receptor, GPIb-V-IX complex. Platelets are transiently immobilized at the injured site enabling platelets to engage their GPVI receptors with nearby fibrillar collagen, resulting in platelet activation, firm adhesion, and spreading mediated by their integrins. Platelet activation and firm adhesion are achieved by the synchrony of multiple surface receptors activation (i.e., thrombin, purinergic, and thromboxane receptors). Activated platelets also release granules to support a growing thrombus facilitated by platelet recruitment and aggregation[11]. Illustration is taken from (Tourdot et al, 2017).

1.3 The role of collagen in hemostasis and thrombosis

Collagen, a triple helical structure comprised of α-chains, is the most abundant structural protein in the extracellular matrix (ECM) to provide mechanical strength to the connective tissues. To

date, there are 42 different collagen genes that encode 28 different types of collagen α-chains. Collagen types I, III, IV, and VI have been observed to play a direct role in hemostasis through their interactions with platelet receptors. This section focuses on the signaling pathways of three main platelet adhesion receptors (GPIb, GPVI, and $\alpha_2\beta_1$) that indirectly or directly bind to subendothelial collagen.

GPIb-V-IX receptor complex-mediated platelet activation

vWF is a large multimeric glycoprotein that is found in circulation in its inactive conformation, platelet α-granules, subendothelial cell matrix, and the endothelial Weibel-Palade bodies. Damage to the vessel enables the A3 domain of vWF to bind to the exposed collagens from the subendothelial matrix [12,13]. As a consequence of this binding and under high arterial shear stress, vWF undergoes conformational change to expose the A1 cryptic binding site of vWF for the GPIb-V-IX complex (4 covalently linked type I transmembrane proteins: GPIbα disulfide-linked to two GPIbβ subunits to form GPIb, GPIX, and GPV)[14]. The interaction of GPIb-V-IX complex with vWF-collagen initiates platelet "tethering," which defines transient platelet adhesion. vWF/GPIb-V-IX complex interaction also induces intracellular platelet activation signaling events, leading to granule secretion, integrin activation and integrin-dependent stable platelet adhesion and aggregation[15].

Some studies have demonstrated that the initial vWF-induced activation of the GPIb-V-IX complex relies on the association of Src family kinases (SFKs) (Lyn and Fyn)[16] 14-3-3ξ, calmodulin, and phosphoinositide 3-kinases (PI3Ks) with the cytoplasmic tail of the GPIbα. These early complex interactions are deemed important for recruiting and activating spleen tyrosine kinase (Syk) and subsequent phospholipase Cγ2 (PLCγ2) phosphorylation[17], leading to enhanced intracellular calcium flux, as well as Akt activation leading to integrin $\alpha_{IIb}\beta_3$

activation[18,19]. In contrast, Syk has also been observed not to be required for GPIb-V-IX- and integrin-dependent stable platelet adhesion to vWF under high stress.

Studies have also reported that GPIbα can form a non-covalent complex with GPVI[20], which could possibly explain the observation of tyrosine phosphorylation of the immunoglobulin (Ig) receptors, FcRγ and FcγRIIa, following ligand binding to GPIb-V-IX complex. These observations suggest GPIb-V-IX may utilize the same signaling pathway as GPVI and FcγRIIa [16]. In contrast, the loss of FcRγ and linker for activation of T cells (LAT) has also been shown to not affect GPIb-V-IX-mediated integrin activation and TxA_2 production [21,22].

Interestingly, vWF induction of platelet GPIb-V-IX complex has been shown to enhance intracellular cGMP levels, and subsequent cGMP-dependent protein kinase (PKG), MAPK, p38, and extracellular signal-regulated kinase (ERK) activation[23]. The cGMP signaling cascade is reported to be activated downstream of the Lyn/PI3K/Akt pathway, which leads to nitric oxide (NO) synthase production of NO. Low concentrations of NO/cGMP synthesis is demonstrated to stimulate platelet activation; whereas, high concentrations of NO and cGMP inhibit platelet reactivity[24].

There is no doubt that the main function of GPIb-V-IX is to bind to vWF and then mediate platelet adhesion at high shear rates. However, the induced signaling pathway of platelet activation remains unclear. Different aspects of receptor-induced signaling and complex interactions have been proposed and tested, but to date, the lack of consensus on the GPIb-V-IX signaling pathway still remains.

GPVI-mediated platelet activation

GPVI is a platelet-specific transmembrane type I receptor belonging to the immunoglobulin (Ig) superfamily, and estimated to express between 4000 and 6000 copies per cell. GPVI consists of

two extracellular Ig C2 loops or domains (D1 and D2) linked together by disulfide bonds, a mucin-like stalk, a transmembrane region, and a short cytoplasmic domain [25]. GPVI non-covalently associates with the Fc receptor (FcR) γ-chain, a promiscuous protein expressed on other hematopoietic cells. The FcRγ-chain is a covalently linked homodimer, whereby each chain possesses a copy of an immunoreceptor tyrosine-based activation motif (ITAM) in the intracellular domain [25,26], defined by two Yxx(L/I) sequences separated by seven amino acids. Loss of FcRγ-chain in platelets has been demonstrated to prevent GPVI from reaching to the platelet surface, resulting in abrogated collagen-induced platelet activation as well as mild bleeding diathesis. Thus, GPVI surface expression and function requires its association with the FcRγ-chain[27].

The identification of GPVI ligands (collagen type I and III, collagen-related peptides (CRPs), convulxin (snake toxin), and JAQ1 (monoclonal antibodies)) aided in elucidating the underlying mechanisms of GPVI signaling in platelet activation. It is thought that GPVI associates with its ligands, containing repeated amino acid motif sequence, glycine-proline-hydroxyproline (GPO), which induce receptor activation via clustering[28]. In resting or inactive state, GPVI is either completely or partially excluded from lipid rafts; however, upon ligand stimulation, GPVI/FcRγ complex is found to be enriched with lipid rafts[29] and some membrane-associated proteins, including Src family kinases, Lyn and Fyn, and palmitoylated adaptor protein, LAT. Lipid rafts have been reported to facilitate proximal Src kinase interaction with GPVI by which Src kinase docks on the conserved proline-rich region (PxxP) of GPVI via their Src homology 3 (SH3) domains. On the one hand, previous studies had shown that there is already a pool of active Fyn and Lyn constitutively associated with the PxxP motif of GPVI, and this interaction increases the intrinsic activity of GPVI, which places the receptor in a "ready-to-go" state[30]. Due to the

crosslinking of GPVI, Src kinases are induced to come in close proximity and engage with their substrates, the FcRγ-chain ITAM.

Following Src kinases phosphorylation of the FcRγ-chain ITAM, the tandem SH2 domain-containing tyrosine kinase, Syk, is recruited to the dually phosphorylated ITAM, undergoes autophosphorylation and phosphorylation by Src kinases, and initiates a downstream signaling cascade [31]. The initiation of the downstream signaling cascade involves the phosphorylation of LAT and concurrent assembly of a signalosome. The core of the signalosome consists of phosphorylated transmembrane adaptor LAT[32] and cytosolic adaptors, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) bound to Gads or Grb2[33,34]. These three proteins associate with a number of signaling molecules, including Bruton tyrosine kinase (Btk)[35], GTP exchange factors (Vav1 and Vav3)[36,37], small GTPase Rac, and the α and β isoforms of PI3K, which are critical for the recruitment and activation of phospholipase C (PLCγ2). For instance, the pleckstrin homology (PH) domain of PLCγ2 facilitates its recruitment to the plasma membrane through its binding of the PI3K product, phosphatidylinositol (3,4,5)-triphosphate (PIP_3). This binding plays an important role in the maximal activation of PLCγ2, which liberates second messengers 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2).

IP_3 binds to the inositol triphosphate receptor (InsP3R) to transiently release Ca^{2+} from intracellular stores, elevating cytosolic Ca^{2+} concentration from approximately .1 μM (resting) to 1-10 μM. DAG and cytosolic Ca^{2+} concurrently activate downstream effectors, which includes actin-myosin interaction, calmodulin, protein kinase C (PKC), Ca^{2+}-dependent exchange factor (CalDAG-GEF) activation, and small GTPase, Ras-related protein 1 (Rap1b) activation. Subsequently, a Rap1-GTP-interacting adaptor molecule (RIAM) enables talin to induce the

cytoplasmic tail of β_3 to undergo conformational change of the $\alpha_{IIb}\beta_3$ integrin, increasing the $\alpha_{IIb}\beta_3$ integrin affinity for fibrinogen, vWF, or fibronectin, in order to form platelet-platelet aggregates (Figure 1.2).

$\alpha_2\beta_1$-mediated platelet activation

The importance of the $\alpha_2\beta_1$ integrin in platelet function was first implicated in 1986 when a patient showed no platelet response to collagen due to the fact the individual had 15-20% of the normal level of glycoprotein Ia (GPIa) [38]. Interestingly, integrin $\alpha_2\beta_1$ had been also identified in other cells (endothelial and epithelial), serving as a receptor for both collagen and laminin. Platelet $\alpha_2\beta_1$ integrin is a receptor for collagen type I and IV, but the ligand binding is accommodated by the α_2 subunit that has a 200-amino acid inserted domain, the I domain, that is homologous to the vWF A domains[39]. Collagen interacts with the α_2-I domain via conserved sequences containing the GFOGER motif[40]. The α_2-I domain has been shown to bind to collagen in a cation-dependent manner, more specifically Mg^{2+} and Mn^{2+}, but not by Ca^{2+} [41,42]. Platelet $\alpha_2\beta_1$ integrin has primarily been recognized in providing platelets firm adhesion to the subendothelial wall following platelet translocation along the vessel wall (see section 1.2).

The role played by $\alpha_2\beta_1$ in the collagen-induced platelet activation still remains unclear. Many of the contradicting results on whether collagen binding to $\alpha_2\beta_1$ is critical for platelet activation were due to differing experimental conditions. On the one hand, one general consensus was that genetic ablation or pharmacological inhibition of $\alpha_2\beta_1$ integrin in platelets delayed platelets response to collagen without affecting the final extent of activation compared to controls. It has been demonstrated that, analogous to $\alpha_{IIb}\beta_3$ binding to fibrinogen, $\alpha_2\beta_1$ integrin undergoes a shift from low to high affinity state for collagen following stimulation[43], supporting an "inside-out" signaling mechanism. In contrast, an "outside-in" model has also been demonstrated by which

platelets spread on GFOGER motifs. Platelet spreading on $\alpha_2\beta_1$ recognition motif, GFOGER, induced activation of the Src kinases and subsequent Syk recruitment and PLCγ2 activation[44]. This observation was also supported by PLCγ2-deficient mouse platelets, which exhibited limited spreading on GFOGER coated surface. Interestingly, this study also provided insights to the underlying intracellular signaling events involved in $\alpha_2\beta_1$-mediated platelet adhesion and spreading. $\alpha_2\beta_1$-mediated spreading was demonstrated to share similar signaling pathways as the "outside-in" models of $\alpha_{IIb}\beta_3$ and FcγRIIa.

1.4 Immune receptor mediated activation of platelet

Platelets are increasingly being recognized for their functions in immunological pathological conditions due to drug exposure, bacterial and viral infections[45-49], as well as immunological host defense involving IgG-dependent platelet opsonization-destruction, activation/thrombosis, or even recruitment or modulation of leukocyte responses. One of the major mediators of immune functions is the activation of Fcγ receptors. Fcγ receptors are widely expressed throughout the human hematopoietic system, including FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, and FcγRIIIb. These receptors are distinguished by their affinity for the Fc-fragment of antibody and the inhibitory or activating signaling pathways they induce. This section will focus on the role of FcγRIIa in platelet function, since it is the only Fcγ receptor expressed by human platelets.

FcγRIIa-mediated platelet activation

The human FcγRIIa (CD32) is a 40 kDa type I transmembrane glycoprotein belonging to a subclass of FcγRs in the Ig superfamily[50]. Structurally, FcγRIIa is a single-chain receptor composed of two extracellular Ig-like domains with the second domain mediating binding to the Fc region of IgG[51] and a cytoplasmic tail containing the ITAM (see section 1.3). Although

FcγRIIa is expressed by many cell types (monocytes, neutrophils, and dendritic cells), it is the only FcγR on human platelets, ranging from 900-5000 copies [52,53], but absent in mice. FcγRIIa exhibits low affinity for single monomeric IgG molecules; however, has high avidity and affinity for multimeric IgG immune complexes[54]. In addition, a polymorphism of FcγRIIa, determined by a single-nucleotide change (G to A) resulting in the replacement of arginine with histidine at position 131, also dictates its binding efficacy to IgGs [55].

The most commonly used agonists (heat aggregated IgGs, IgG-coated beads, and anti-FγRIIa mAb IV.3 with F(ab')2 fragments of a secondary antibody) cluster FcγRIIa to activate the same ITAM signaling pathway shared by GPVI (see section 1.3.2). Upon clustering or activation, the cytoplasmic ITAM of FγRIIa is phosphorylated followed by recruitment of the proximal effector, Syk. Docked Syk is phosphorylated at tyrosine residues and subsequently activates nearby effectors in order to trigger downstream signaling transduction. A key component that is activated in this pathway is PLCγ2, which hydrolyzes PIP_2 into IP_3 and DAG, leading to calcium mobilization and PKC activation (see section 1.3). These intracellular events culminate in integrin activation as well as granule secretion (Figure 1.2).

FcγRIIa has also been demonstrated to participate in the "outside-in" signaling process by which ligand binding (fibrinogen, fibronectin, and vWF) to integrin αIIbβ3 triggers intracellular signaling events, resulting in platelet adhesion, spreading, granule secretion, stabilization of platelet aggregations, and clot retraction. A key component in the "outside-in" signaling pathway is Src family kinases activation, which has been shown to directly interact with the cytoplasmic tails of β_3 of the $\alpha_{IIb}\beta_3$ integrin and phosphorylated FcγRIIa and Syk[44,56]. For this case, FcγRIIa was demonstrated to serve as a "functional conduit" or adaptor for α_{IIb}β3-mediated outside-in signaling[57]. Platelets from FcγRIIa-positive mice showed enhanced platelet spreading, clot

retraction compared to platelets from FcγRIIa-negative mice. FcγRIIa-positive platelets also exhibited enhanced tyrosine phosphorylation of Syk and PLCγ2 upon interaction with immobilized fibrinogen.

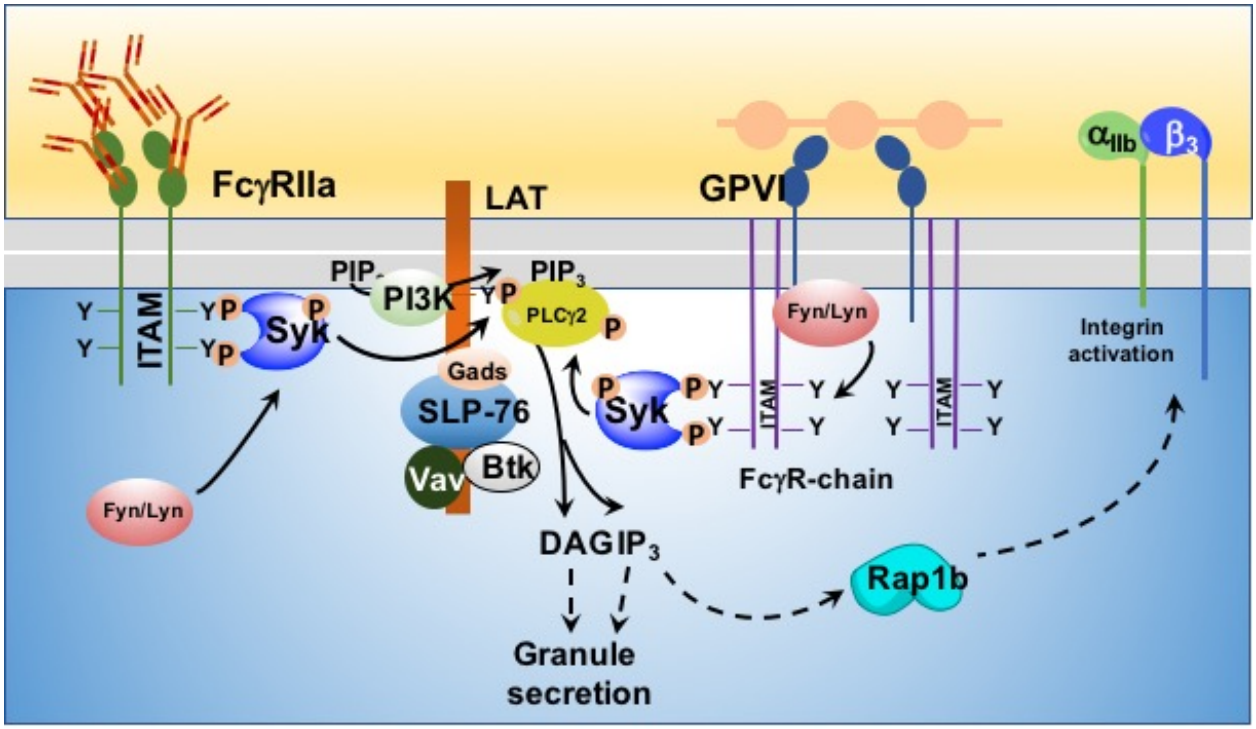


Figure 1.2 Immunoreceptor tyrosine-based activation motif (ITAM) receptors in platelets.
FcγRIIa and GPVI are immune receptors that either bears cytoplasmic ITAM or constitutively associates with the FcγR-chain that contains the ITAM, respectively. Receptor clustering initiates phosphorylation of the ITAM motif by Src family kinases (SFK), recruits spleen tyrosine kinase (Syk) and induce subsequent activation of kinases, adaptors, and effector molecules to form the signalosome. The signalosome consists of linker for activation of T cells (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), growth factor receptor-bound protein 2 (Grb2), and Grb2-related protein 2 (Gads), phosphoinositide 3-kinase (PI3K), bruton's tyrosine kinase (BTK), Vav proteins, and phospholipase C γ2 (PLCγ2). The assembly of the signalosome is critical for PLCγ2 activation, which generates second messenger signaling lipids, diacylglycerol (DAG) and inositol triphosphate (IP_3), from phosphatidylinositol 4,5-bisphosphate (PIP_2). Eventually, these intracellular signaling events culminate in the activation of platelets.

1.5 Platelet activation and signaling mediated by G-protein-coupled receptors.

A variety of soluble of platelet agonists (adenosine nucleosides, ATP and ADP, TxA_2) are released from damaged endothelial cells as well as activated platelets to enhance aggregation. Activated platelets also develop procoagulant surfaces to aid in thrombin generation in order to promote thrombus growth and fibrin formation. Unlike collagen and vWF-mediated platelet activation, this section details the mechanisms of the major agonists that exert their function

through the GPCRs. Depending on the GPCRs, agonists can induce more than one signaling mechanism. For instance, each GPCR can be associated with more than one G protein (G_s, G_i, G_q, and $G_{12/13}$) in the platelets to elicit multiple signaling cascades.

Thrombin receptors

Thrombin, a serine protease produced from coagulation factor II (FII) of the clotting cascade, not only converts soluble fibrinogen into insoluble fibrin, but also cleaves protease activated receptors (PARs) on the surface of platelets. Depending on the type of species, specific PARs are expressed. PAR1 and PAR4 are expressed on human platelets; whereas mouse platelets express PAR3 and PAR4. Thrombin cleaves the extended N-terminus of the PARs, unmasking a new N-terminus (SFLLRN) that serves as a tethered ligand[58] by binding intramolecularly to the receptor. Following ligand binding, $G\alpha_q$ and $G\alpha_{12/13}$ undergo conformational change, switching from GDP (inactive) to GTP-bound state (active), and dissociation from their $G\beta\gamma$ subunits and respective receptors. Immediately, $G\alpha_q$ and $G\alpha_{12/13}$ interact with specific effectors to activate downstream signaling cascades.

$G\alpha_q$ mainly amplifies its signal through phospholipase C (PLC) β, which hydrolyzes PIP_2 into IP_3 and DAG. These second messengers propagate downstream signaling effectors (see section 1.3.2) leading to platelet aggregation. On the other hand, the GTP-bound form of $G\alpha_{12/13}$ interacts with and activates guanine nucleotide exchange factors (GEFs), such as p115RhoGEF, which converts RhoA into active GTP-bound form, followed by its activation of Rho kinase, p160ROCK. RhoA kinase phosphorylates LIM kinase to induce cofilin activity as well as myosin light chain (MLC) phosphatase in order to induce MLC-dependent contraction. In general, $G\alpha_{12/13}$ plays a major role in mediating platelet shape change and granule secretion.

P_2Y_{12} and P_2Y_1 receptors

Platelet activation stimulated by several agonists (ie thrombin, TxA_2, collagen) results in secretion of granule contents, such as ADP, which serves as a prominent autocrine and paracrine mediator of platelet function through the purinergic receptors, P_2Y_1 and P_2Y_{12}. Optimal platelet activation by ADP requires both receptors, by which each receptor contributes uniquely through its associated G proteins. $P2Y_1$ is a GPCR coupled to $G\alpha_q$ that mediates activation of PLCβ and subsequent production of IP_3 and DAG, causing Ca^{2+} release and PKC activation (see section 1.5.1). In addition, $P2Y_1$ activation has been reported to contribute to morphological changes in platelets [59], most likely due to calcium or calmodulin-, or Rac-dependent contractile signaling[60]. On the other hand, $P2Y_{12}$ is found to be directly coupled to $G\alpha_{i2}$. $G\alpha_{i2}$ activation inhibits the ability of adenylyl cyclase to convert ATP to 3',5'-cyclic AMP (cAMP). This relieves the inhibitory effect of cAMP-dependent protein kinase on platelet activation. In parallel, the βγ subunit dissociated from the $P2Y_{12}$ coupled $G\alpha_i$ also activates a host of cellular effectors, including PI3Kγ/β, Akt/protein kinase B (PKB), Rap1b, and Src family tyrosine kinases, leading to partial platelet aggregation[61].

TPα receptor

As a consequence of agonists-mediated platelet activation, thromboxane A_2 (TxA_2) is generated from COX-1 oxidation of cytoplasmic phospholipase A_2 ($cPLA_2$) freed arachidonic acid (AA) from the lipid bilayer. Similar to the ADP released from the granules in platelets, TxA_2 behaves as a secondary mediator or potentiator of platelet activation. TxA_2 acts on the thromboxane A_2 receptor, TPα receptor, which is coupled to both $G\alpha_q$ and $G\alpha_{12/13}$ (see figure 1.3). Both $G\alpha_q$ and $G\alpha_{12/13}$ elicit unique signaling pathways, which eventually culminates or converges in integrin activation to facilitate platelet aggregation (see thrombin receptors section).

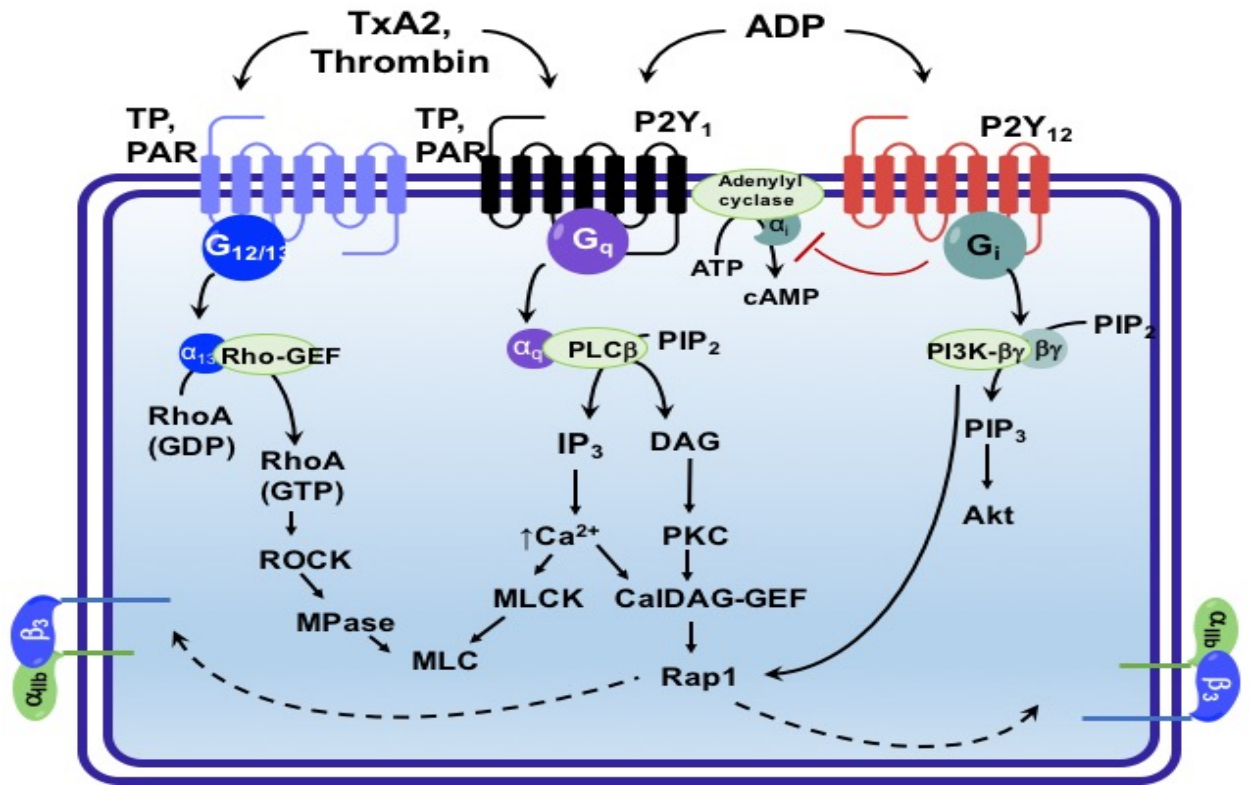


Figure 1.3 Overview of G-protein coupled receptors and signaling in platelet activation

1.6 Central hypothesis aims

The elucidation of the major signaling pathways or endpoints involved in platelet activation (described in this chapter) has facilitated in the development of a number of anti-platelet drugs to regulate platelet reactivity. While several of the current anti-platelet agents (chapter 2) had effectively reduced mortality due to thrombotic-associated cardiovascular events, morbidity and mortality still remain. However, as platelets are also essential for hemostasis, there is also the risk for bleeding as a consequence for inhibiting a number of targets in the signaling pathway involved in platelet activation. This warrants the need to identify novel or alternative targets in platelets that will effectively inhibit thrombosis, but with minimal impact on hemostasis or risk of bleeding.

Two potential therapeutic avenues for regulating platelet reactivity to prevent thrombosis were proposed in this thesis. One potential anti-platelet therapeutic avenue is targeting 12-lipoxygenase (12-LOX), a non-heme oxygenase, with the first in class selective platelet 12-LOX inhibitors, NCTT-956 and ML355. Thus, I hypothesized that selectively targeting 12-LOX with NCTT-956 or ML355, would modulate platelet reactivity through the inhibition of the predominant oxylipin, 12(S)-HETE, derived from arachidonic acid (AA). In addition, these selective inhibitors also aided in characterizing the importance of 12-LOX's role in platelet function described in chapters 5-6. The utility of targeting 12-LOX as a potential anti-platelet therapy was demonstrated in an immune-mediated thrombotic disease model, heparin-induced thrombocytopenia (HIT), described in chapter 7.

The second aim of my thesis work investigated the role of utilizing 12-LOX as an endogenous anti-platelet therapeutic approach through the modification of eicosanoid or oxylipin production. While 12-LOX predominantly generates 12(S)-HETE from AA, since it is the most abundant ω-6 polyunsaturated fatty acid (PUFA) found in the lipid bilayer of cells, 12-LOX can also oxidize a number of PUFAs to generate an assortment of other oxylipins or lipid mediators (chapter 8). Dietary supplementation or exogenous addition of plant- or fish-based oils can alter and enrich the contents of the lipid bilayer by which they shift 12-LOX substrate from AA to other PUFAs. For instance, consumption of borage or primrose oil, which is enriched in ω-6 PUFA dihomo-γ-linolenic acid (DGLA), has been shown to be cardioprotective; however. its mechanism of action had been unclear. I hypothesized that DGLA regulates platelet reactivity through its oxylipin, 12(S)-HETrE, derived from 12-LOX oxidation. The 12-LOX derived oxylipin of DGLA, 12(S)-HETrE was found to inhibit platelet activation through a Gαs-dependent manner (chapter 9).

CHAPTER 2

Anti-platelet Therapy[1]

2.1 Summary

Anti-platelet therapy remains the mainstay in preventing aberrant platelet activation in pathophysiological conditions such as myocardial infarction, ischemia, and stroke. Although there has been significant advancement in anti-platelet therapeutic approaches, aspirin still remain the gold standard treatment in the clinical setting. Limitations in safety, efficacy, and tolerability, have precluded many of the anti-platelet inhibitors from use in patients. Unforeseen incidences of increased bleeding risk and recurrent arterial thrombosis observed in patients have hampered the development of superior next generation anti-platelet therapies. The pharmacokinetic and pharmacodynamic profiles have also limited the effectiveness of a number of anti-platelet inhibitors currently in use due to variability in metabolism, time to onset, and reversibility. A focused effort in the development of newer anti-platelet therapies to address some of these shortcomings has resulted in a significant number of potential anti-platelet drugs which target enzymes (phosphodiesterase, cyclooxygenase), receptors (purinergic, prostaglandins, protease-activated receptors, thromboxane), and glycoproteins (αIIbβ3, GPVI, vWF, GPIb) in the platelet. The validation and search for newer anti-platelet therapeutic approaches proven to be superior to aspirin is still ongoing and should yield a better pharmacodynamic profile with fewer untoward side-effects to what is currently in use today.

[1] This section has been published in Journal of Blood Medicine. Yeung J, Holinstat, M. Newer agents in antiplatelet therapy: a review. ***J. Blood Med.*** 2012; 3:33-42.

[2] This section has been published in Cardiovascular & Hematological Agents in Medicinal Chemistry. Yeung J and Holinstat, M. 12-Lipoxygenase: A potential Target Novel Anti-Platelet Therapeutics. ***CHAMC*** 2011; 9(3):154-164.

2.2 Introduction

Anti-platelet drugs are the cornerstone in treatment of cardiovascular diseases. Despite the significant decrease in morbidity and mortality due to the currently approved anti-platelet drugs, recurrent ischemia, myocardial infarction, and unwanted bleeding still occur. The majority of drugs in development have focused on targeting either surface receptors or enzymes in the platelet in order to protect against unwanted clot formation following initial platelet activation. The first target for anti-platelet therapy was cyclooxygenase-1 (COX-1) by aspirin. While newer approaches for containing platelet activity have been developed, the pharmacodynamics and pharmacoeconomics suggest that aspirin will continue to be a mainstay for platelet therapy in the years to come. Currently, a combination regimen of aspirin and clopidogrel are the standard of care for prevention of platelet activation, thrombosis, and stroke. Unfortunately, many of the current anti-platelet drugs face limitations in their utility due to genetic differences in the ability to metabolize pro-drugs such as is the case with clopidogrel, acquired allergic responses such as is seen with heparin and aspirin, and resistance as has been reported with aspirin (see Table 2.1). Additional limitations observed in the application of currently approved anti-platelet drugs include a narrow therapeutic window and limited efficacy. An overview of the current federal drug administration (FDA) approved anti-platelet therapies as well as those in development will be discussed in this chapter.

2.3 P2Y Receptor Antagonists

The P2Y receptors are G-protein coupled (GPCR) purinergic receptors belonging to the P2 family. Two receptors, $P2Y_1$ and $P2Y_{12}$, are present in the platelet. $P2Y_1$ is a G_q coupled GPCR while $P2Y_{12}$ is coupled to $G\alpha_{i2}$. Activation of $P2Y_1$ signals phospholipase β, leading to DAG

formation, calcium mobilization, and eventually PKC and CalDAG-GEF activation [62]. In contrast, $P2Y_{12}$ activation inhibits adenylyl cyclase, activates phosphoinositide 3-kinase [63], the small GTPase Rap1[64], and the activation of αIIbβ3 [65].

Drug	Target	Half-life	Side effects	Bioavailability	Use
Ticlopidine (Ticlid)	P2Y12 receptor	12 hours	Bleeding, rash, neutropenia, thrombotic thrombocytopenic purpura (rare), nausea, vomiting, heartburn, indigestion	Oral	Transient ischemic attacks, patients undergoing PCI
Clopidogrel (Plavix)	P2Y12 receptor	6–8 hours	Bleeding, rash, neutropenia, thrombotic thrombocytopenic purpura (rare)	Oral	NSTEMI, STEMI, PCI, recent stroke, or established PAD
Prasugrel (Effient)	P2Y12 receptor	8 hours	Bleeding	Oral	Patients with ACS undergoing PCI
Ticagrelor (Brilinta)	P2Y12 receptor	6–12 hours	Dyspnea	Oral	STEMI, ACS
Abciximab (ReoPro)	GPIIb-IIIa	< 10–30 minutes	Bleeding, thrombocytopenia, EDTA-induced psuedothrombocytopenia	IV	PCI
Eptifibatide (Integrilin)	GPIIb-IIIa	~2.5 hours	Bleeding, thrombocytopenia, EDTA-induced psuedothrombocytopenia	IV	NSTEMI, PCI, unstable angina
Tirofiban (Aggrastat)	GPIIb-IIIa	2 hours	Bleeding, thrombocytopenia, EDTA-induced psuedothrombocytopenia	IV	NSTEMI, PCI, unstable angina
Cilostazol (Pletal)	PDE3	11–13 hours	Headache, dizziness, hypotension, flushing, nausea, vomiting, diarrhea, abdominal pain	Oral	Intermittent claudication, PAD, PCI
Dipyridamole (Aggrenox)	PDE3 and inhibition of adenosine uptake	10 hours	Bleeding, headache, diarrhea, palpitations, dizziness, rash, pancytopenia	Oral	Transient ischemic attacks

Abbreviations: ACS, acute coronary syndromes; EDTA, ethylenediaminetetraacetic acid; GP, glycoprotein; IV, intravenous; NSTEMI, non-ST elevation myocardial infarction; PAD, peripheral arterial disease; PCI, percutaneous coronary intervention; PDE, phosphodiesterase; STEMI, ST elevation myocardial infarction.

Table 2.1 Approved antiplatelet drugs.

Ticlopidine (Ticlid; Roche) is a first generation thienopyridine that requires cytochrome P450 (CYP) 1A metabolism prior to exerting its irreversible antagonistic effects on platelet reactivity via the $P2Y_{12}$ receptor [66]. Early experimental observations showed agonist-induced platelet aggregation was intermittently inhibited by ticlopidine [67,68]. Studies with ticlopidine however, exhibit off-target effects mediated by the inhibition of intracellular calcium mobilization [69]. Maximal inhibition of platelet aggregation is observed 3 to 5 days post administration of ticlopidine [70]. The delayed onset of anti-platelet effects is a consequence of metabolism of the pro-drug [67]. Clinical trials (CATS and TASS studies) have shown ticlopidine to be more effective than aspirin alone [71,72], but exhibiting significant off-target effects including minor bleeding with hemorrhagic events observed in less than 1% of subjects studied. Additionally,

ticlopidine-treated patients typically discontinue treatment due to a variety of secondary adverse events including diarrhea, skin rash, and neutropenia [71,73].

Clopidogrel (Plavix; Bristol-Myers Squibb), a second generation oral thienopyridine, also requires metabolism of a pro-drug by the CYP2C19. The active metabolite, which is a highly labile compound, irreversibly binds to and inhibits the $P2Y_{12}$ receptor through a disulfide bridge. The CURE trial has shown the clinical benefit of the dual clopidogrel-aspirin therapy compared with aspirin alone by significantly reducing mortality and nonfatal MI or stroke in patients with unstable angina; however, the dual regimen was associated with an increase in bleeding compared with placebo [74,75]. The CAPRIE trial, which evaluated the efficacy of clopidogrel monotherapy compared with dual therapy of clopidogrel plus aspirin, showed clopidogrel treatment results in a reduction of primary end points [76]. Evidence of poor metabolizers for clopidogrel has helped to explain the reduced function in patients with an altered CYP2C19 allele.[77] Poor metabolizers of clopidogrel have diminished platelet inhibition resulting in a higher rate of adverse cardiovascular events than non-carriers [78].

Prasugrel (Effient, Eli Lilly) is a third generation thienopyridine, chemically distinct from clopidogrel. *In vivo and in vitro* pharmacological studies have demonstrated that this ATP analog selectively and irreversibly inhibits ADP-induced aggregation to a greater degree than clopidogrel [79]. The irreversible binding is thought to be due to the disulfide binding between the reactive thiol group of the active metabolite and the cysteine residue of the $P2Y_{12}$ receptor [80-82]. Prasugrel is an orally available pro-drug that requires active transformation by CYP450 and esterases [83]. Activation of the pro-drug requires CYP3A4 and CYP2B6 [84]. Clinical studies have verified that inhibition of platelet aggregation is more effective with prasugrel compared to clopidogrel after a single dose in healthy subjects [84]. Furthermore, subjects who responded

poorly to clopidogrel showed greater platelet-induced inhibition in response to prasugrel [84,85]. In addition, assessment of secondary endpoints favors prasugrel due to lower incidences of cardiovascular death, nonfatal MI, and re-hospitalization due to recurrent ischemia.

Ticagrelor (Brillinta; AstraZeneca), an oral cyclopentyl-triazolo-pyrimidine (CTP) analog, unlike thienopyridines, is a direct and reversible inhibitor of the $P2Y_{12}$ receptor that is activated from its pro-drug by CYP3A [86]. Ticagrelor exerts its action via binding to the $P2Y_{12}$ receptor at a site distinct from the ADP binding site, thus making it an allosteric inhibitor [86]. As a consequence of $P2Y_{12}$ inhibition, ATP is converted to cyclic monophosphate, vasodilator-stimulated phospoprotein (VASP) is dephosphorylated, and activation of PI3-K is inhibited [87]. The PLATO trial compared ticagrelor with clopidogrel in which the primary composite endpoints, stroke, MI, cardiovascular death, and stent thrombosis, were reduced in patients with acute coronary syndromes (ACS) (with or without ST-elevation myocardial infarction (STEMI)) [88]. The benefit of ticagrelor appears to be attenuated in patients with lower body weight and those not taking lipid-lowering drugs in North American groups relative to comparative studies elsewhere [89]. There is no significant difference in major bleeding between the two agents; however, spontaneous (non-coronary artery bypass grafts) or non-procedural related bleeding is increased with ticagrelor. Additionally, off-target effects of dyspnea and asymptomatic ventricular pauses are associated with ticagrelor use [89,90]. In general, ticagrelor has so far proven superior to current treatment regimens, including a rapid onset of action, acceptable safety profile, and effectiveness in reducing the primary end points in ACS patients.

Elinogrel (PRT060128,Novartis/ Portola Pharmaceuticals) is a direct-acting reversible $P2Y_{12}$ receptor inhibitor that is currently undergoing clinical investigation (INNOVATE-PCI) for efficacy and safety in patients undergoing percutaneous coronary intervention (PCI) (see Table

2.2) [91]. Preclinical data show that intravenous or orally administered elinogrel is superior to clopidogrel and had minimal effect on bleeding times [92]. In addition, a single dose of elinogrel has been shown to overcome high platelet reactivity in patients undergoing PCI who were non-responsive to clopidogrel [92]. Elinogrel, while still in clinical development for safety and efficacy assessment in patients, shows promise as a next generation $P2Y_{12}$ antagonist.

Drug	Target	Stage of development
Elinogrel	P2Y12 receptor	Phase II
Cangrelor	P2Y12 receptor	Phase III
BX 667	P2Y12 receptor	Preclinical
Vorapaxor (SXH 530348)	PAR1	Phase III
Atopaxar (E5555)	PAR1	Phase II
S18886 (Terutroban)	TPα	Phase III
Z-335	TPα	Phase I
BM-573	TPα	Preclinical
h6B4-Fab	GPIb	Preclinical
GPGP-290	GPIbα	Preclinical
SZ2	GPIbα	Preclinical
PR-15 (Revacept)	GPVI	Phase I completed
DZ-697b	GPVI	Phase I completed
AJW200	vWF	Phase I
ARC1779	vWF	Phase II
ARC15105	vWF	Preclinical
ALX-0081	vWF	Phase II
ALX-0681	vWF	Phase II
82D6 A3	vWF	Preclinical
Z4 A5	GPIIb-IIIa	Preclinical
DG-041	PGE_2	Phase II

Table 2.2 Antiplatelet drugs under development

Cangrelor (ARC-69931MX; Medicines Company) is an intravenous non-theinopyridine and reversible $P2Y_{12}$ inhibitor. Like prasugrel and ticagrelor, cangrelor showed a more rapid onset of action and greater degree of platelet inhibition than clopidogrel. Recent evaluations of the inhibitor in the CHAMPION-PCI and CHAMPION-PLATFORM trials were stopped early due to its lack of apparent differences in the primary end point of death, myocardial infarction or

ischemia-driven revascularization 48 hours after PCI [93]. Also, the rate of major bleeding in patients undergoing PCI was higher with cangrelor compared to clopidogrel in both studies [94].

BX 667 is an orally active reversible $P2Y_{12}$ receptor antagonist that is metabolized by esterases to form the carboxylic active form, BX 048 [95]. *In vitro*, ADP-induced aggregation was potently inhibited by BX 667. Additionally, administration of BX 667 resulted in a rapid and sustained inhibition aggregation [96]. This observation is also supported by the intravenous BX 048 and oral BX 667 administration in rat arteriovenous-shunt model, which showed a similar pharmacodynamic relationship between the plasma concentration of BX 048 and thrombus inhibition [95]. This antagonist has yet to be evaluated in healthy human subjects.

2.4 Glycoprotein (GP) Antagonists

αIIbβ3 Antagonists

Glycoprotein GPIIbIIIa (αIIbβ3) is the most abundant integrin on the platelet surface [97]. αIIbβ3 is known to be involved in both inside-out or outside-in platelet signaling. The inside-out signaling in platelet activation involves the various signaling pathways that converge into a common signaling endpoint that leads to the activation of integrin αIIbβ3. Ligand binding of fibrinogen or von Willebrand factor (vWF) to αIIbβ3 mediates platelet adhesion and aggregation, triggers outside-in integrin activation and results in additional granule secretion, stabilization of platelet adhesion, aggregation, and clot retraction [98].

Abciximab (ReoPro; Eli Lilly) is an antibody developed from the murine human chimera c7E3 Fab which targets the integrin αIIbβ3 [99], preventing integrin binding to fibrinogen and vWF. Abciximab rapidly binds with high affinity and has a slow rate of dissociation from its target [100]. In addition, abciximab binds with high affinity to $\alpha_v\beta3$ (vitronectin receptor) [101] and low affinity to the leukocyte MAC-1 receptor [102]. Initial intravenous administration enables rapid onset of

platelet inhibition [103]. As abciximab has an extremely short half-life [104], platelet aggregation returns to baseline levels within 12-24 hours following discontinuation of therapy [105,106]. Interestingly, the ISAR-REACT trial demonstrated no additional benefit of abciximab over placebo in the reduction of ischemic complications or mortality [104,107]. Similarly, among diabetic patients without elevated troponin levels undergoing elective PCI, no difference was observed in primary end point events between abciximab and placebo/clopidogrel groups [108]. Conversely, in patients with elevated troponin levels, the incidence of mortality and recurrent ischemic complications was significantly reduced with abciximab [109]. Careful monitoring must be accompanied with the administration of abciximab as bleeding and thrombocytopenia have been observed [110-112].

Eptifibatide (Integrilin; Millenium Pharmaceuticals/Shering-Plough) is a cyclic heptapeptide derived from snake venom that contains a KGD (lysine-glycine-aspartic acid) sequence which selectively recognizes αIIβ3 [113]. The IMPACT-II study showed that a single loading dose followed by continuous infusion for 20 to 24 hours only resulted in 50% αIIbβ3 receptor blockade; thus limited benefits and efficacy through eptifibatide were observed [114]. The ESPRIT trial however, which utilized intravenous administration of a double bolus [115] followed by maintenance infusion, significantly reduced the 30 days incidence of death, myocardial infarction, and target vessel revascularization [116] establishing the clinical efficacy for this drug. These observations were confirmed in the PURSUIT trial, which showed an absolute reduction in the 30 day incidence of death and myocardial infarction on eptifibatide [117]. Despite the reduction in mortality, the ACUITY trial also showed an increase incidence of major bleeding in patients with ACS undergoing PCI [118,119].

Tirofiban (Aggrastat; Merck) is a tyrosine-derivative nonpeptide mimetic reversible inhibitor of αIIbβ3 that specifically and competitively binds to the receptor. Treatment with tirofiban in combination with aspirin and heparin in patients with ACS significantly reduced the 30-day post-treatment incidence of death, myocardial infarction or recurrent ischemia [120]. Further, tirofiban was superior for ACS patients recovering from invasive coronary angiography [120]. As for the use of tirofiban as an adjunct to PCI, tirofiban was shown to be inferior to abciximab in the RESTORE and TARGET trials where the incidence of composite death, non-fatal myocardial infarction, and urgent target vessel revascularization were higher with tirofiban or abciximab at 30 days [121].

Z4A5 is a novel αIIbβ3 peptide antagonist that is currently in development. This antagonist has been shown to inhibit platelet-induced aggregation and thrombi formation. Additionally, when Z4A5 was examined along with heparin and /or aspirin in the rabbit arteriovenous shunt thrombosis model, it was shown to be an effective antithrombotic agent when administered with aspirin [122]. The pharmacodynamics and pharmacokinetics in humans are currently under investigation [122].

Additional Glycoprotein Antagonists

Additional glycoprotein targets have received a fair amount of attention in the drive to develop novel approaches for anti-platelet intervention. Von Willebrand factor (vWF), a multimeric glycoprotein that acts as a bridging element between damaged endothelial sites and the glycoprotein receptors on platelets, is one such target. The A1 and A3 domains of vWF bind to collagen, while the A1 domain is bound to the GPIb-IX-V platelet receptor complex [123,124]. vWF also binds to active αIIbβ3 on the platelet surface. Interactions between αIIbβ3 and vWF contribute to the final, irreversible binding of platelets to the sub-endothelium and play a leading

role in platelet aggregation [125]. A second target receiving attention as a potential site for anti-platelet therapy is the collagen receptor glycoprotein VI (GPVI). The collagen-GPVI interaction triggers subsequent tyrosine phosphorylation of the ITAM motif of the Fc receptor γ chain, activating the Syk kinases pathway, LAT, SLP-76, and phospholipase Cγ2 [126], resulting in platelet activation or aggregation.

vWF Antagonists

AJW200 is an IgG4 humanized monoclonal antibody to vWF which has been shown to specifically inhibit high-shear-stress-induced platelet aggregation in a concentration dependent manner *in vitro* in blood from human volunteers [127].

ARC1779 (Archemix Corp) is an aptamer based antagonist. This second generation nuclease-resistant aptamer is conjugated to a 20-kDA polyethylene glycol and binds with high affinity to the active vWF A1-domain and inhibits vWF-dependent platelet aggregation [128]. A phase II trial demonstrated that continuous infusion of ARC1779 effectively increased platelet counts in critically ill thrombotic thrombocytopenic purpura patients by preventing platelet aggregation and loss of platelets. Cessation of ARC1779 infusion resulted in platelet count reduction and progression of thrombotic thrombocytopenic purpura-related organ damage [129]. This drug is currently clinical investigation.

Other vWF antagonists in clinical development or investigations include ARC15105, ALX-0081 (Ablynx), ALX-0681, and 82D6A3. *ARC15105* is a chemically advanced aptamer with assumed higher affinity to vWF, but less specific inhibitor of vWF-dependent platelet aggregation than ARC1779 based on *ex vivo* trials [130]. The preclinical and clinical trials have shown that *ALX-0081*, a bivalent humanized nanobody that recognizes the Glycoprotein Ib (GPIb) binding site of vWF, is a potent and safe inhibitor of vWF-mediate platelet aggregation over a wide range of

doses when administered in combination with aspirin, heparin, and clopidogrel. ALX-0081 is currently under investigation in PCI patients in a phase II trial. 82D6A3, a monoclonal antibody directed against amino acids Arg-963, Pro-981, Asp-1009, Arg-1016, Ser-1020, Met-1022 and His-1023 of the vWF A3 domain [131], was shown to result in complete inhibition of vWF binding to collagen during the first 3 days after stent implantation in baboons [132]. Further trials will need to follow to verify 82D6A3 efficacy, safety, and tolerability.

GPVI Receptor Antagonists

PR-15 (Revacept; ABX-CRO/Medifacts GmbH) is a soluble, dimeric glycoprotein (GPVI)-Fc that has been shown to adhere to exposed collagen in endothelial lesions preventing the binding to platelet GPVI receptors. Collagen-induced human platelet adhesion or plaque formation were significantly reduced with pre-treatment of soluble GPVI-Fc [133,134]. Similarly, infusion of GPVI-Fc was shown to virtually abolish stable arrest and aggregation of platelets following vascular injury in mice [126]. Subsequently, a phase I clinical trial demonstrated that intravenous administrated of PR-15 is safe and well tolerated by healthy volunteers [135].

DZ-697b is an orally active collagen and ristocetin inhibitor. Safety and efficacy have been assessed in a phase I trial which showed potential benefits such that bleeding time was substantially shortened compared to clopidogrel treatment [136]. DZ-697b is currently under clinical investigation.

GPIb Receptor Antagonists

Novel targets still under investigation include h6B4-Fab, GPGP-290, and SZ2. h6B4-Fab is a murine monoclonal antibody, derived from the humanized Fab fragment of 6B4 targeting GPIbα and neutralizes the binding site of the vWF A1 domain[137,138]. 6B4 had been shown to inhibit platelet adhesion by competing with vWF for binding to GPIbα under high-shear conditions.

Moreover, preliminary data show 6B4 had no effect on platelet count or bleeding times in vivo in baboons, but dose-and time-dependently inhibited ristocetin-induced platelet aggregation [139]. GPG-290 is a recombinant, chimeric antibody purified from chinese hamster ovary cell culture that contains the amino-terminal 290 amino acids of GPIbα linked to the human IgG1. GPG-290 treated dogs were shown to exhibit prolong bleeding compared to the clopidogrel-treated control, despite the prevention of coronary artery thrombosis [140]. SZ2, a monoclonal antibody developed against GPIbα, has also been shown to inhibit both ristocetin- and botrocetin-induced platelet aggregation *in vitro* [141]. Preclinical investigations are still underway to determine the *in vivo* efficacy of SZ2.

2.5 Phosphodiesterase Antagonists

Platelets express three phosphodiesterase (PDE) isoenzymes, PDE 2, 3 and 5. Phosphodiesterases regulate the levels of 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) by catalyzing the hydrolysis of cAMP and cGMP to inactive 5'-AMP and 5'-GMP, respectively.[142] Platelet activation relies on degradation of cAMP and cGMP; hence regulating these secondary messengers are fundamental in regulating platelet activation and thrombosis.

Cilostazol (Pletal, Otzuka Pharmaceutical Co.) is a type III-phosphodiesterase (PDE3) selective oral inhibitor [143]. Liu and colleagues have shown that cilostazol enhances the interstitial concentration of adenosine in several *in vitro* and *in vivo* models [144] by inhibiting adenosine uptake. This in turn stimulates A_2 receptors, which further increases cAMP levels. As a result, platelet-induced aggregation is reversibly inhibited by cilostazol [145]. Cilostazol is extensively metabolized by CYP3A4; while CYP2C19 is also shown to have a minor role in cilostazol metabolism [146]. Cilostazol is safe and effective in reducing the incidence of repeated

revascularization after PCI and risk of restenosis; however, this drug does not show superiority in reducing the primary composite end points of adverse cardiovascular events after drug-elution stent (DES) implantation [147]. Despite the functional implications of adjunctive treatment with cilostazol compared with standard aspirin and clopidogrel treatment, as shown in the OPTIMUS-2 study, the accompanied side effects (headaches, gastrointestinal symptoms, and skin rash) often lead to the discontinuation of the drug [148].

Dipyridamole (Aggrenox; Boehringer Ingelheim) is a pryimidopyrimidine derivative with both anti-platelet and vasodilator properties [149]. Similar to cilostazol, dipyridamole inhibits cyclic nucleotide phosphodiesterase and blocks adenosine uptake, which results in increased cAMP [150]. The ESPS-2 and ESPRIT trials showed that dual treatment of dipyridamole and aspirin reduced risk of stroke or death by 37% compared with aspirin alone [151,152]. Based on the ESPRIT and ESPS-2 trials, dipyridamole has been FDA approved for stroke prevention [152].

2.6 Thromboxane A_2 Receptor Antagonists

Platelets express the thromboxane receptor α (TP α), a GPCR that is coupled to G_q and $G_{12/13}$ and signals platelet activation through a number of intracellular pathways which converge to reinforce primary platelet activation through thrombin or collagen [153].

S18886 (Terutroban) is an oral reversible inhibitor of TPα. In preclinical studies, S18886 dose-dependently prolonged occlusive thrombus formation in animal models, but did not alter the size of the myocardial infarct size in the ischemia-perfusion model. S18886 and clopidogrel were effective in preventing occlusive thrombus formation with a moderate increase in bleeding time [154]. Subsequently however, in the phase III clinical trial (PERFORM), S18886 did not meeting the pre-defined criteria for non-inferiority since S18886 and aspirin had similar rates of protection without safety advantages for S18886 [155].

Z-335 ((+/-)-sodium[2-(4-chlorophenylsulfonylaminomethyl)indan-5-yl]acetate monohydrate) is an oral TPα antagonist that has previously been shown to dose-dependently inhibit the specific binding of [^{3}H]SQ-29548 (TPα inhibitor) to human and guinea pig platelet membranes [156]. In healthy male Japanese volunteers, Z-335 inhibited U46619-induced platelet aggregation within 2 hours of administration [157].

BM-573, another investigational inhibitor that targets TPα, has been shown to halt the progression of atherosclerosis in LDL receptor deficient mice [158]. Pre-clinical models have shown that arachidonic acid-induced aggregation is completely inhibited in the presence of BM-573 [159] and clinical studies on this compound are currently ongoing.

2.7 Thrombin Receptor Antagonists

Thrombin activates human platelets via two protease activated receptors (PARs), PAR1 and PAR4. PAR activation leads to a diverse range of pro-thrombotic signaling events mediated through G_q, $G_{12/13}$, and possibly G_i, resulting in phospholipase (PLC) β activation, Rho activation, and adenylyl cyclase inhibition, respectively. PAR activation requires thrombin cleavage of the amino terminus of the receptor revealing a tethered ligand. While it has been challenging to develop an inhibitor that can directly compete with the endogenous tethered ligand, development of PAR1 inhibitors as a therapeutic target to minimize uncontrolled platelet activation have recently been investigated.

SCH 530348 (Vorapaxar; Merk & Co.) is an orally active synthetic analog of himbacine [160] that competitively binds with high affinity to the PAR1. Previous *in vitro* assays show SCH 530348 inhibited thrombin- and thrombin receptor activating peptide (TRAP) – induced platelet aggregation, without affecting the aggregation induced by adenosine diphosphate (ADP), U46619, or collagen. In addition, SCH 530348 did not affect the prothrombin and activated

partial thromboplastin time, suggesting that bleeding time may not be increased. Pre-clinically, cynomolgus monkeys treated with SCH 530348 alone or in addition with aspirin and clopidogrel, showed no increase in bleeding times [161]. The TRA-PCI study verified that addition of SCH 530348 to standard anti-platelet therapy (aspirin and clopidogrel) was not associated with increases in thrombolysis in myocardial infarction (TIMI) or bleeding compared with the control group [162]. The phase III clinical trials TRA-CER and TRA 2ºP-TIMI, which sought to assess the impact of vorapaxar on cardiovascular death, MI, stroke, and recurrent vascular events in patients with established coronary, cerebral, or peripheral atherosclerosis [163] failed due to unforeseen intracranial bleeding [163].

E5555 (Atopaxar; Eisai Limited) Pre-clinical trials showed that oral administration of the PAR1 antagonist, *E5555*, significantly prolonged bleeding times in guinea pigs [164]. Further, PECAM-1, active αIIbβ3, GPIb, thrombospondin, and vitronectin expression, were significantly reduced by E5555 in whole blood flow cytometry. Clinical studies have shown that E5555 attenuated thrombin-induced but not ADP-induced platelet aggregation [165,166].

Additional PAR-1 antagonists: SCH 205831 and SCH 602539 are still under investigation. Preliminary data show SCH 205831 derived from himbacine, inhibited platelet deposition in baboons with arteriovenous-shunt thrombosis. Similarly, SCH 602539 inhibited thrombosis in a dose-dependent manner in the Folts model of thrombosis in anesthetized cynomolgus monkeys [167]. These compounds continue to be developed in pre-clinical models.

2.8 Conclusion

Significant progress has been made in advancing our understanding of how platelet activation directly regulates thrombus formation in the vessel leading to occlusive thrombi and stroke. However, a continued need for the development of new anti-platelet therapies exists as the risk

for myocardial infarction, stroke, and death, remain a persistent problem for individuals suffering from cardiovascular disease. Further, while aspirin continues to be the first line of pharmacological intervention in anti-platelet therapy, the risk of bleeding is significantly exacerbated by its irreversible action coupled to the additional regimen of dual therapy often employed to minimize thrombotic events. In hopes of reducing prolonged bleeding or myocardial infarct events, newer compounds continue to be developed to target alternative sites in the platelet. The successful implementation of these strategies may significantly reduce the morbidity and mortality in cardiovascular disease due to unwanted platelet activation as well as excessive bleeding due to traditional approaches. Even with the newer anti-platelet drugs entering the market in the near future, we are faced with the realization that activation of the platelet involves an increasingly complex signaling network. Hence, new frontiers will need to be explored which will take advantage of this signaling to reveal novel therapeutic targets with diminished off-target effects.

CHAPTER 3

12-Lipoxygenase: A Novel Target for Anti-Platelet Therapeutics[2]

3.1 Summary

Platelets play an essential role in the regulation of hemostasis and thrombosis and controlling their level of activation is central to prevention of occlusive clot formation and stroke. Although a number of anti-platelet targets have been identified to address this issue including COX-1, the $P2Y_{12}$ receptor, the integrin αIIbβ3, and more recently the protease-activated receptor-1, each of these targets has resulted in a significant increase in bleeding which in many cases may be more deleterious than the clot itself. Therefore, alternative approaches to treat uncontrolled platelet activation are warranted. Platelet-type 12-lipoxygenase is an enzyme which oxidizes the free fatty acid in the platelet resulting in the production of the stable metabolite 12-hydroxyeicosatetraenoic acid (12-HETE). The role of 12-HETE in the platelet has been controversial with reports associating its function as being both anti- and pro-thrombotic. In this review, the role of 12-lipoxygenase and its bioactive metabolites in regulation of platelet reactivity, clot formation, and hemostasis is described. Understanding the mechanisms by which 12-lipoxygenase and its metabolites modulate platelet function may lead to the development of a novel class of anti-platelet therapies targeting the enzyme in order to attenuate injury-induced clot formation, vessel occlusion and pathophysiological shifts in hemostasis.

[2] This section has been published in Cardiovascular & Hematological Agents in Medicinal Chemistry. Yeung J and Holinstat, M. 12-Lipoxygenase: A potential Target Novel Anti-Platelet Therapeutics. ***CHAMC*** 2011; 9(3):154-164.

3.2 Platelet Therapy in Cardiovascular Disease

Incidence of CVD. Recently, the American Heart Association reported that cardiovascular diseases (CVD) accounted for 33.6% of all deaths in the United States based on the 2007 data [168], and is the single leading cause of death in the nation and worldwide [169] occurring before age 65. Furthermore, more than 67.3% of adults over the age of 20 are reported to be obese, which is one of the major contributing factors to developing CVD along with smoking. Surgical procedures for cardiovascular complications have also increased by 27% from 1997 to 2007 [168]. Despite these alarming statistics, the mortality rate resulting from CVD complications has slightly decreased, although the burden of the disease still remains high. This is due, in part, to the successful development and widespread use in the clinic of a number of anti-platelet drugs.

Current anti-platelet drugs. One of the earliest targets for anti-platelet therapy was cyclooxygenase-1 (COX-1), responsible for the formation of prostanoids, which are involved in inflammation [170]. A number of pharmacological approaches have been developed over the years to inhibit COX activation in platelets including aspirin, indomethacin, and nonsteroidal anti-inflammatory drugs (NSAIDS) [171] The efficacy in treating and preventing vascular-occulsive events with COX-1 inhibitors has been limiting in a significant proportion of the population [172,173]. One study for example, reported that greater than 33% of the patients with cardiovascular diseases (CVD) exhibited aspirin resistance [170,174]. Another target on the platelet which has successfully been inhibited in order to limit platelet activation is the ADP receptor, $P2Y_{12}$. Targeting $P2Y_{12}$ with clopidogrel, a pro-drug that binds to and inhibits receptor activation, has been shown to be effective in further reducing platelet activation when given as dual anti-platelet therapy with aspirin [175]. However, due to the nature of this pro-drug and the genetic variability of the P450 enzyme required to convert clopidogrel to its active form its utility is limited as is

evident from the development of next generation $P2Y_{12}$ inhibitors ticangralor and presagrel [176,177]. Integrin IIb/IIIa inhibitors (abciximab, eptifibatide, tirofiban), although a breakthrough in antibody-based anti-platelet therapy [177-179], have resulted in an increase in hemorrhagic complications following PCI. Furthermore, some patients developed frequent myocardial infarction and refractory ischemia post-tibrofiban administration [180].

Adverse side effects such as bleeding are of primary concern for current treatment against platelet activation in CVD therapy, especially prior to and during surgical procedures [181]. Thus, alternative strategies which would inhibit platelet activation while minimizing the bleeding side effect are warranted. A novel target for anti-platelet therapy in the treatment of patients with cardiovascular related diseases may be human 12-lipoxygenase (12-LOX). In order to develop potential 12-LOX inhibitors however, we must have a comprehensive understanding of its pathophysiological and biochemical implications and the role 12-LOX metabolites play in the vascular system. Our current knowledge of this enzyme and its oxidized products in platelets and other tissues is still limited, but the preliminary evidence shows the potential advantages of inhibiting 12-LOX on the platelet to treat human diseases.

History of lipoxygenases. Lipoxygenases (LOXs) are a family of nonheme iron dioxygenases, originally known as lipoxidases that catalyzes the oxidation of polyunsaturated fatty-acids such as linoleic acid or arachidonic acid (AA), containing the *cis*-methylene interrupted diene structure and esters yielding conjugated hydroperoxides [182]. The earliest lipoxygenase studies showed 15-lipoxygenase purified from soybean oxidized unsaturated moieties of fatty acids [183] at a specific carbon site of the acyl chain; that eventually allowed lipoxygenase isoforms to be identified according to their site of carbon oxidation and stereochemistry. The transformation of AA to 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) was first demonstrated in

human and bovine platelets in the mid-1970s [184]. These metabolites, produced by 5-, 8 -, 12-, and 15- LO, have been reported to potentially form signaling lipid mediators that exert their effects through the G protein –coupled receptor (GPCR) [185]. Lipoxygenases and their metabolites have been demonstrated experimentally and clinically to be restricted to specific cells or tissues and show significant species specificity. To date, three isoforms of 12(S)-lipoxygenases have been identified accordingly to their cell type: epidermis, leukocyte, and platelet [186]. 12-LOX activation has been reported to play a role in a number of diseases including psoriasis[187] and ulcerative colitis [188,189]. Despite intensive research in this field, the majority of the discoveries of 12-lipoxygenase functions and their implications have been established using animal models. Thus, our current understanding of the biological significance of these enzymes and products in humans is still limited. 12-LOX isoforms and their products will be the focus of this review showing their involvement in platelet reactivity contributing to hemostasis and thrombosis and the overall scheme in developing alternative targets to prevent vascular occlusion, myocardial infarction, and stroke.

3.3 12-LOX in the Human Platelet

Activation of platelets through a number of receptors is known to result in activation of cytosolic phospholipase A_2 ($cPLA_2$), a lipid lipase that generates free fatty acid such as AA from the phospholipid membrane by cleaving the sn-2 position [184]. Once AA is formed, it is available for oxidation by either COX-1 or 12-LOX and can produce a number of bioactive lipid metabolites [184]. In leukocytes, oxidized AA will result in the production of PGE_2 and LTB_4 [190,191] as pro-inflammatory effectors or thromboxane (TxA_2) and 12-HETE in platelets. Recently, we have shown that COX-1 and 12-LOX-mediated signaling may rely on different pools of AA based on the kinetics of TxA_2 and 12-HETE formation and their differential reliance on $cPLA_2$ at the

surface of the platelet [192]. Unpublished data further indicates that 12-LOX activation is required for dense granule secretion in platelets as well as normal platelet aggregation and adhesion**.** This is not surprising considering blocking 12-LOX attenuates aggregation and integrin activation in the presence of thromboxane, collagen, thrombin, and protease-activated receptors (PARs) [193-195]. Additionally, 12-LOX has also been implicated to play a role in regulating calcium mobilization. A role for 12-LOX in the platelet using one of the classical 12-LOX inhibitors, baicalein, was first described in the mid-1990s, where stimulation with AA in the absence of 12-LOX resulted in a significant attenuation of thrombin-induced calcium $[Ca^{2+}]_i$ transients and aggregation [196]. In addition to oxidation, 12-LOX in platelets has also been reported to have lipoxin synthase activity. Lipoxins, such as LXA_4 and LXB_4, are tetraene-containing eicosanoids generated from exogeneous leukotriene A_4 (LTA_4) that induces vasoconstriction of the smooth muscles and regulates neutrophil function via binding at specific recognition sites [197-200].The molecular observations above confirm an important role for 12-LOX in human platelet reactivity and a renewed interest in this field attests to the therapeutic potential inherent with regulation of 12-LOX. Finally, although human studies to date are limited to platelet reactivity and thrombosis, a number of animal models have added crucial information as to the potential role of 12-LOX in hemostasis including studies with 12/15-LO knockout mice, canines, porcine, and rabbits, show varying and sometimes unrelated physiological effects compared with humans [201] (Table 3.1).

Species	Cell Type	Function
Human	Adrenal glomerulosa cells	LO activation participates in aldosterone's stimulatory effects of angiotensin II (ANG) [37]
	Epithelial lens cells (HLECs)	Growth in response to EGF and insulin [38]
	platelet	12-HETE and other metabolites formation [26, 39]; platelet activation [2, 40]
Mouse	Pancreatic islets and adipose tissue	Cytokine production in adipocytes and macrophages shown to impair insulin signaling such as tumor necrosis factor-alpha (TNF-α), interleukin 6(IL-6), IL-12p40, and monocyte chemoattractant protein (MCP-1) [35]
	Melanoma cells	Cell proliferation [39, 41]
	Platelets	12-HETE and other metabolites formation shown to modulate platelet reactivity [42]
Porcine	Anterior pituitary	Possible gonadotroph regulation [43]
Rabbit	Corneal epithelial cells	Regulation of epithelial cell proliferation and the rate of corneal re-epithelialization following an injury [44]
Rat	Mesangial cells in renal	Indirectly involved in the expression of P-cadherins [45-47]
	Endothelium	Vasconstriction [48]
Canine	Gingival tissue, hepatocytes [49], Kupffer cells [50] and platelets [51]	Involved in neutrophil activity [50] and vasoconstriction and thrombosis [51]

Table 3.1 12-LOX expression and function different species

3.4 12-LOX Substrates

Polyunsaturated fatty acids (PUFA) as regulators of 12-LOX-mediated platelet activity. A possible alternative approach to anti-platelet therapy may lie in the dietary intake of certain essential fatty acids (EFA). Large prospective studies have shown that there is a positive relationship between increased dietary intake of ω-3 fatty acids and reduced CVD. For instance, Eskimos from the West Coast of Greenland with greater whale, fish, and seal intake correlated with lower incidence of myocardial infarction, stroke, and mild tendency to bruise [202]. Another study showed rats receiving Thomas-Hartroft thrombogenic diet were subsequently fed with unsaturated fatty acid supplements, which comparatively showed reduced thromboplastin generation and mortality [203]. In addition, the epidemiological studies of high eicosapentaenoic acid (EPA) to AA consumption in Japan had shown that the incidence of mortality due to ischemic heart disease and cerebrovascular disease between 1950 and 1982 tended to be lower in the fishing residents than the farming villages [204-206]. Hence 12-LOX may be the key to regulating platelet function through oxidation of various free fatty acid substrates. While the addition of polyunsaturated fatty acids as dietary supplement may be beneficial, the amount of fatty acids added to the diet needs to be carefully monitored in patients with existing cardiovascular risk. Previous studies for example, have suggested that docosahexaenoic acid (DHA) has an adverse effect on diabetics and elderly who are already suffering from a lower antioxidant potential. In one study, platelets from an elderly subject with low DHA were given low DHA supplements and exhibited lower lipid peroxidation activity, whereas incubated with high DHA concentration induced higher 12-HETE formation [207,208]. As for EPA, 30mg and 200mg/kg/day of the fatty acid supplement given to patients resulted in attenuated platelet

aggregation. TxB_2 production had also been observed to be blocked in platelets pre-treated with EPA[207].

12-LOX substrate availability. Dietary intake of certain essential fatty acids (EFAs), ω-3 or ω-6, have been shown to impact the type of metabolic products generated by 12-LOX. For instance, AA, a ω-6 series fatty acid derived from cis-linoleic acid (LA), can be found mostly in peanut oil. As for ω-3, fish oil, flaxeed, and algal oil, are the common sources for α-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) [209]. Platelet membranes containing DHA have been reported to be catalyzed by 12-LOX to various hydroxyl docosahexaenoic acid (HDoHE) isoforms, but largely forming 14-HDoHE in an agonist and calcium-dependent manner [184]. A number of metabolic products are formed following 12-LOX oxidation of another fatty acid, EPA, which include AA, TxB_3, 12(S)-hydroxyeicosapentanoic acid (12-HEPE), and hydroxy-5,8,10-14-heptadecatetraenoic acid (HhTE) [210,211]. One study showed that EPA conversion to TxB3 depended on the presence of hydroxy-5,8,10, 14-eicosatetraenoic acid (12-HpETE), whereas, pre-incubation with 12-HETE did not induce EPA metabolism. In another case, pre-incubation with 12(S)-HpETE has been shown to increase the amount of nonesterified AA in collagen stimulated platelets, significantly enhancing platelet aggregation and the formation of TXB_2[212].. The eicosanoid metabolites, once formed, can have a number of regulatory functions in the platelet probably through both autocrine as well as paracrine signaling schemes. The physiological and cellular effects due to oxidized products are further discussed in detail below.

Direct 12-LOX regulation of eicosanoid metabolites. Eicosanoid metabolites are able to be further oxidized by 12-LOX. 12-LOX can catalyze 5-HETE to generate 5(S), 12-dihydroxyeicosatetraenoic acid (12(S)-DHETE) and 15-HETE to 14,15-DHETE in platelets

under conditions of prolonged exposure to the enzyme [213]. Similarly, exogenous AA and 5-HETE showed reduced production of 5(S), 12(S)-DHETE. 11,12-DHETE had also been reported to be generated from 12-LOX[214].

3.5 The Biological Role of Metabolites

Metabolite profiling in the blood. Profiling of lipid metabolites and mediators in whole blood in the presence of the calcium ionophore A23187, demonstrated that 12-LOX contributes a large proportion of the total products formed [215] compared with the other lipoxygenases. The predominant products were 12-HETE, 12-HEPE, and 12- hydroxydocosahexaenoic acid (12-HDHA). A significant number of 5-LOX products, including leukotriene B4 (LTB_4), LTB_5, and prostaglandin E_2 (PGE_2), were also found in circulation under these conditions. Shifting ω-3 or ω-6 content in the lipid bilayer of the cells has been shown to contribute to differences in their respective lipid mediators and metabolites and thus regulate biochemical and physiological events in the cells of the individuals [210,216].

12-LOX metabolite regulation of various tissues. 12-LOX oxidation of various fatty acids in the platelet can result in the formation of a number of unique eicosanoid metabolites. Many, if not all, of these eicosanoids can play a regulatory role both within the platelet itself as well as at distal tissues and organ systems. Previous studies support a pro-inflammatory role for eicosanoid formed through 12-LOX oxidation of ω-3 fatty acids and AA in a number of animal models including the mouse and rabbit [217] Further, work in cell lines confirms a role for these eicosanoids in a number of distant tissue beds as well. For example, mouse pre-adipocytes 3T3-L1 cells pre-treated with 12(S)-HETE and 12(S)-HpETE induce upregulation of proinflammatory cytokine genes, such as tumor necrosis factor-alpha (TNF-α), interleukin 6(IL-6), IL-12p40, and monocyte chemoattractant protein (MCP-1) [201]. In the human epidermis,

12(R)-HETE, which is sterochemically different from platelet-derived 12(S)-HETE, has been reported to increase DNA synthesis and plays a significant role in psoriasis [218,219].

12-LOX metabolite regulation of platelets. As for platelets, the role of the AA oxidized metabolite, 12-HETE, is not well understood. Furthermore, work in the field is controversial as to whether 12-HETE is pro-thrombotic, anti-thrombotic, or inert towards platelet activity. One study indicated that 12-HETE had no effect on either basal or thrombin-induced $[Ca^{2+}]_i$ levels or aggregation[220] Conversely, other reports showed a clear potentiation of thrombin-induced aggregation in platelets in the presence of 12-HETE [221].. 12-HpETE has also been shown to stimulate 12-LOX but inhibit COX-1 in lysed platelets[222] and one report indicated that 12(S)-HETE acts as an inhibitor of platelet and neutrophil PLA_2 [223]. Additionally, the eicosanoid, 15-HETE, which is primarily produced in leukocytes, has been reported to act as an inhibitor of 12-LOX[224]. Other eicosanoid products, such as 12-HPEPE and 12-HEPE originating from 12-LOX oxidation of EPA, are thought to elicit an inhibitory effect on platelet aggregation [204,205]. In addition to their effects on aggregation, 12-HPEPE and 12-HEPE have been shown to attenuate serotonin (5-HT) release mediated by AA and collagen in a dose dependent manner[225]. The level of fatty acid substrate available to 12-LOX may also play a role in its physiological regulation of platelet function. On the one hand, incorporation of DHA into one's diet lowers lipid peroxidation, which lends to attenuating platelet reactivity at high concentrations by inhibiting TXA_2 induced aggregation[226], whereas higher DHA concentrations result in potentiation of platelet reactivity. This paradoxical phenomenon has also been observed for the 12-LOX metabolite, 12-HEPE.

12-LOX metabolite regulation of CVD. Patients with essential hypertension, a significant risk factor for vascular occlusion [227], have shown an increase in the basal level of 12(S)-HETE in the

platelets and in urinary excretion of 12(S)-HETE compared to healthy subjects [228]. Further, the protein levels of 12-LOX in the cytosolic fraction from hypertensive patients was much higher than in normotensive subjects. Thrombin-mediated 12(S)-HETE generation however, did not differ between the groups implying a potential role for genetic variation in the levels of 12-HETE formed [228]. SNP analysis has uncovered an association in the coding polymorphism of the 12-LOX gene with essential hypertension and urinary production of 12(S)-HETE [229].

Age is known to be directly correlated to an increased risk for cardiovascular disease[230,231]. Similarly, platelet reactivity is also increased in older patients due, in part, to increasing levels of AA and TxA_2 formation [85-87] Although 12-LOX product formation has not been studied in young versus older subjects, it is likely that 12-HETE is also increased in this segment of the population. Additionally, diabetics have been shown to exhibit an increase in PLA_2 activity linked to an increase in TxA2 formation[232]. 12-LOX has been reported to have a link to platelet function in diabetes as well. 12-HETE levels in platelets from a group of Japanese with type 2 diabetes were observed to be decreased compared with healthy subjects [233], whereas noninsulin dependent patients in the US showed an increase in 12-HETE formation in the urine samples compared to healthy subjects [234]. These controversial observations, although perplexing, support a link between the levels of 12-LOX activity in the platelet and diabetes.

3.6 12-LOX Inhibitors

The studies described above show that regulating amount of essential fatty acids and their metabolites via 12-LOX is essential in understanding both the pathophysiological processes of the platelets and CVD. Various groups have screened for potential natural and small molecule drugs targeting 12-LOX, however, many of these screens have failed due to problems with efficacy, off-target effects, and adverse events, both in animals and human platelets (Table 3.2).

One of the earliest drugs tested on arachidonate 12-LOX was an acetylenic acid, 4,6-10-13-eicosatetrayonic acid (4,7,10,13-ETYA) [235]. This approach however, also targeted human peripheral neutrophil 5-LOX with an ID_{50} of 2-3uM and other lipoxygenases from different sources and was therefore not developed further. Esculetin, also known as curcumin, was shown to inhibit 12-HETE production in both human and rat platelets [236], but did not inhibit formations of TxB2 and HHT [237]. Besides curcumin, baicalein (5,6,7-tihydroxyflavone), a compound extracted from Scutellariae roots [238], was first reported to selectively inhibit 12-LOX in human platelets in the 1980s [239] without affecting cyclooxygenase activity [240]. In addition, platelet activation and ATP secretion stimulated by *Chlyamydia pnemoniae* was markedly reduced by this inhibitor [241]. More recent data suggests that baicalein inhibits $cPLA_2$ in human platelets and that some of its effects may be due to a lower level of AA formation following initial platelet activation. Baicalein has also been reported to be an inhibitor of CYP2C9, an enzyme involved in drug metabolism [242] as well as other human LOs and COXs [243]. In addition to its off target effects, baicalein in rats showed that the amount of 12-HETE produced in the presence of the inhibitor and thrombin stimulation did not correlate with the potentiation of contractile responses in the artery [244].

Other potential inhibitors which have shown little efficacy toward 12-LOX include 1) Dicranin (acetylenic fatty acid: 9,12,15-octadecatrien-6-ynoic), extracted from Dicranum Scoparium, which weakly inhibited COX-1, but resulted in an increase in 12-HETE [245], 2) Knipholone, which is isolated from the roots of Kniphofa foliosa and shown to inhibit leukotriene synthesis, but only weakly inhibit 12-HETE production [246], and 3) OPC-29030 which inhibits thrombin-mediated 12(S)-HETE production [247]. Additionally, Hinokitiol, extracted from

Japanese wood, was shown to be a selective 12-LOX inhibitor. Unfortunately, Hinokitiol has also been reported to be cytotoxic and teratogeneic on living tissues [248,249].

Recently, there has been an increased interest in developing a highly selective small molecule inhibitor targeting 12-LOX. These compounds structurally exhibit greater selectivity than the previous natural inhibitors described above due to their selectivity in distinguishing and LO paralogs in species specific tissues/cells [250].These small molecule inhibitors may possibly reduce off-target effects in the system due to their greater selectivity and aid in clarifying the role of 12-LOX in the pathophysiology of thrombosis in the human.

3.7 Conclusions

Cardiovascular disease remains the leading cause of death in the world and is a growing problem both globally as well as within the United States. Research spanning over three decades has convincingly established a central role for platelet activation in the pathophysiology of cardiovascular disorders and acute coronary syndrome. Current pharmacological therapy for treatment of diseases caused by blood clots, such as heart disease and stroke, often involves the use of drugs that do not reflect current scientific understanding of these pathologies. Although targeting of enzymes such as COX-1 or surface receptors including $P2Y_{12}$, PAR1, and integrin receptor αIIbβ3, has been extremely useful in decreasing morbidity due to MI, these therapies have failed to shift the incidence of mortality in these patients. This may be due, in part, to the fact that these anti-platelet drugs do not fully attenuate platelet activation, can have delayed onset and long durations of action, and may result in significant morbidity due to bleeding complications [76,251]. New therapeutic approaches targeting the level of platelet activation necessary to inhibit vessel occlusion and stroke without significantly increasing bleeding are needed. 12-LOX may be a viable target for anti-platelet therapy. Studies have shown that a link

exists between the level of 12-LOX and cardiovascular risks such as type 2 diabetes and hypertension. Furthermore, 12-LOX metabolites such as 12-HETE, have been shown to potentiate platelet activation, thrombin generation, and calcium mobilization. Recent unpublished work using small molecule inhibitors now supports a pro-thrombotic role for 12-LOX in the human platelet. Thus, ,targeting this enzyme may allow for attenuation of the platelet clotting cycle without a significant increased risk of bleeding.

Although 12-LOX was identified in the early 1970s by Hamberg and Samuelsson [252], identifying the regulatory role of 12-LOX and its metabolites in platelet function has been difficult, in no small part due the poor selectivity of naturally occurring lipoxygenase inhibitors (see table 3.2). Recently however, several research groups have revisited this enzyme and are developing a number of natural and synthetic molecule approaches in order to identify highly selective inhibitors against platelet-type 12-lipoxygenase in the human. The first generation of these inhibitors is now being tested in human platelets and early results support targeting this enzyme for future use as an anti-platelet therapy. Platelet-type 12-LOX is not the only potential target in development, however, its relatively selective expression in megakaryocytes and platelets, and its pro-thrombotic activity in human platelets supports further development of this target for anti-platelet therapeutics.

Inhibitor	Origin	Target(s)
CDC		12-LOX in human platelets; 5-LO in rats [91-93], 5-LO in human polymorphonuclear leukocytes and monocytes [93, 94]
Baicalein	Scutellariae roots	human reticulocyte 15-LOX and 12-LOX in platelets [27, 95, 96] , prolyl-4 hydroxylases (PHDs) in human hepatoma cells and mouse fibroblasts 3T3-L1 [97]; Raf-1 mediated phosphorylation of MEK-1 in rat glioma cells [98]; phosphatidylinositol 3-kinase (PI3K) inhibitor in human cancer cell lines e.g. colorectal, lung, gastric, ovarian, renal, melanoma, breast, glioma, prostate [99]
(NDGA)	Creosote bush	5-LOX, 12-LOX prostaglandin [100], inhibits HER2 and IGF-1 receptor tyrosine kinases in human breast cancer cells, [101, 102]; neutrophil phospholipase A2 [103]
BW755C	Phenidone analogue	dual inhibitor 12-LOX and COX-1 in platelets [104]; 5-LOX in leukocytes [105]
AA-861		5-LO in human and guinea pig peritoneal leukocytes [106]; 12-LO in mouse epidermis [107, 108]; tyrosine phosphatase of the receptor tyrosine kinase signaling pathway in mouse embryonic fibroblasts [109]
Timegadine		COX and LO in rabbit, rat, bovine, horse platelets [110-112] [110]
ETYA		12-LOX [113]; COX-1 [114-116], cytochrome P-450 ensymes in rat thyroid FRTLA-5 cells [117]; neutrophil phospholipase A2 [103]; Δ-6 desaturase in mouse hepatic cells [118]
Esculetin		12-LOX in human platelets [61, 119]; 5-LO [120],
BHPP	Hydroxamic acids	12/15-LO in rats and mice [121]
Panaxynol	a polyacetylene compound from Ginseng radix, Fang-Feng and Panx ginseng	COX, leukocyte 12-LO and platelet 12-LO in humans and porcine. 15-LO in rabbit [122]; cholesterol acyltransferase in rat liver [123]; 15-hydroxyprostaglandin dehydro-genase (PGDH) in rabbit gastric mucosa [124]
Falcarindiol	Panaxynol analog	leukocyte 12-LO and platelet 12-LO in human, and 15-LO [122]in rabbit reticulocyte; GABA degradative enzymes GABA transaminase (GABA-T) [125]
BW A4C	acetohydroaxmic acid	12- and 15-LO in bovine leukocyte, and 15-LO human leukocyte [126, 127]; secretory type II phospholipase A2 (sPLA2-II) in guinea-pig alveolar macrophages [128]
Hinokitiol	Derived from tropolone, a constituent of the wood *Chymacyparis taiwanesis*	12-LOX in human platelet [129, 130]; HIF-specific prolyl-4-hydroxylases (PHDs) [131] in human HepG2 hepatoma and HeLa cervical epithelial cells; metalloprotease in injects [132]
KY11449	Derived from *Streptoverticillium hadanonense*	Bovine 12-LO, rat basophilic leukemia cells (RBL-1) 12-LO [133]
Gossypol	Polyphenolic derived compound from cotton plant	5-LO and 12-LO [134] in human neutrophils and platelets, and rat basophilic leukemia cells (RBL-1) [135]; 11β-hydroxysteroid dehydrogenase (11β-HSD) activity in human and rat kidney [136]; Bcl-2 in human ovarian cancer cell lines OV433 and TOV112D [137]
Catechins	Green tea leaves	soybean lipoxygenases and 12-LO in rabbit and human [130]; tumor-associated NOX (tNOX) in human mammary adenocarcinoma cells (BT-20) and HeLa [138]

Table 3.2 12-LOX inhibitors and targets

Inhibitor	Origin	Target(s)
Quercetin		LOs in rat mast cells, 12-LOX, and 5-LO in RBL-1[139]; neutrophil phospholipase A2 [103]; P-glycoprotein-mediated efflux transport and CYP3A4 enzyme [140], human glycolate oxidase [141], sPLA2 in human platelets [142]
MK 866		12-LOX in human platelets [127] , 5-LO in rats [143, 144]
12-LOX small molecule inhibitors	Small molecule screen	selectively targets 12-LOX in human platelets [145]

CHAPTER 4

Oxygenases and Their Derived Oxylipins Regulation of Platelet Function[3]

4.1 Summary

In mammals, three major oxygenases, cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP450), generate an assortment of unique lipid mediators (oxylipins) from polyunsaturated fatty acids (PUFAs) which exhibit pro- or anti-thrombotic activity. Over the years, novel oxylipins generated from the interplay of the oxygenase activity in various cells, such as the specialized pro-resolving mediators (SPMs), have been identified and investigated in inflammatory disease models. Although platelets have been implicated in inflammation, the role and mechanism of these SPMs produced from immune cells on platelet function are still unclear. This review highlights the burgeoning classes of oxylipins that have been found to regulate platelet function; however, their mechanism of action still remains to be elucidated.

4.2 Introduction

Cardiovascular disease remains the leading the cause of mortality globally accounting for nearly 1 in 3 deaths annually[253]. Platelet activation leading to clot formation and thrombosis is an essential component of both the hemostatic and thrombotic responses in the blood following physiological and pathophysiological disturbance of the endothelium lining the vessel wall [254]. The inability to properly regulate platelet reactivity often leads to atherothrombotic events, including myocardial infarction and stroke. Recent work in the field has uncovered a number of

[3] This section has been published in Journal of Molecular Medicine. Yeung J, Hawley, M, and Holinstat, M. The expansive role of oxylipins on platelet biology. ***J. Mol. Med.*** 2017; 95(6):575-588.

lipid products, eicosanoids, derived from ω-3 or -6 polyunsaturated fatty acids (PUFAs) that significantly regulate and alter platelet function. The PUFAs include: arachidonic acid (AA), linoleic acid (LA), eicospentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), and dihomo-γ-linolenic acid (DGLA). Understanding how these newly identified lipids fit into the overall regulation of platelets in the vessel will aid in our understanding of lipid-platelet interactions, often resulting from altered diet or fatty acid supplementation, that play key roles in the ability of the platelet to form a hemostatic "plug" following vascular injury or alternatively form an occlusive thrombus following pathophysiologic insult to the vessel. Finally, understanding how these lipids and lipid products are generated and regulate platelet reactivity should reveal novel targets for therapeutic intervention to prevent thrombosis while limiting the risk for bleeding following vessel injury. Thus, this chapter will be limited to describing the various lipids and bioactive lipid products shown to regulate platelet function and modulate hemostasis and thrombosis in the vessel.

PUFAs are generally inert and depend on oxygenase activity to generate a wide array of structurally distinct bioactive fatty acids metabolites. The formation of lipid products is typically initiated by stimulation of the cell that results in an increase in intracellular calcium. This calcium flux results in translocation of cytosolic phospholipase A2 ($cPLA_2$) to the lipid membrane where it cleaves the fatty acid from the sn-2 position of the phospholipids to release free fatty acids for oxidation in the cell. Once cleaved from the lipid membrane, the freed fatty acids can be metabolized by cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 (CYP450) to form oxidized lipids (oxylipins). Oxylipins have been thought to predominantly function by regulating cellular properties and signaling through one of three pathways. The first involves binding to G protein-coupled receptors (GPCRs) to further propagate intercellular

signaling. Secondly, fatty acids or their oxylipins can directly interact with peroxisome proliferator-activated receptors (PPARs) within the cell. While fatty acids are thought to be weak activators of PPARs, when they accumulate in the vicinity of the PPAR, reports have shown their affinity for activating PPAR signaling is significantly increased [255]. The third regulatory mechanism utilized by fatty acids and oxylipins in the platelet is direct inhibition of oxylipin-producing enzymatic pathways or further metabolic transformation of lipids within the cell (Figure 4.1). The chapter will cover the oxygenase pathways, classes of structurally distinct oxylipins, and their biological effects on the platelets.

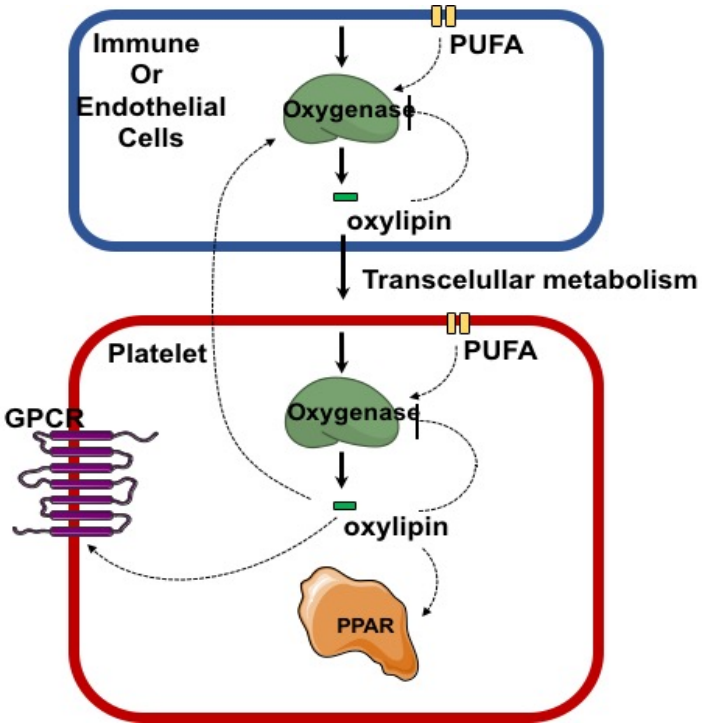


Figure 4.1 Mechanisms of oxylipin regulation of platelet function.
Polyunsaturated fatty acids (PUFAs) are released from the embedded phospholipid bilayer membrane, which are then converted by intracellular oxygenases (COX, LOX, or CYP450) to generate wide array of oxylipins that can diffuse across the cellular membrane to be further converted by oxygenases, act on intracellular signaling component, PPAR, or act on receptor to regulate platelet function. This illustration was generated by Jennifer Yeung and Megan Hawley

4.3 Cyclooxygenase

Cyclooxygenase (COX) exists in two isoforms, COX-1 and COX-2 in the body; however, the platelet expresses primarily COX-1, and its inhibition is thought to be a primary target for

reduction of platelet reactivity in the patients with cardiovascular risk. COX activation primarily results in the generation of prostanoids (prostaglandins (PGs) and thromboxanes (TXs)) derived from PUFAs that are responsible for maintaining either physiological or pathophysiologic states, such as inflammation and tumorigenesis [256]. This section describes the select prostanoid lipids generated from the PUFAs through the COX pathway that regulate platelet function.

COX derived metabolites and their regulatory roles on platelet function

COX transforms AA to series 2 PGs (PGE_2, PGD_2, PGI_2) and thromboxanes (TX) A_2 that can exhibit either pro-thrombotic or anti-thrombotic modulation of platelet function (Table 4.1) [257,258]. In terms of thrombosis, TXA_2, when formed, is released and acts through the thromboxane receptor (TPα), which is coupled to G α_q and G α_{13} and functions to amplify platelet activation leading to enhanced aggregation and thrombosis [259]. In contrast, PGD_2 derived mainly from mast cells, leukocytes and some platelets[260], had been shown to dampen platelet activation [261-263] through its binding to the DP_1 receptor and subsequent elevation of cAMP [264-266]; however, there are evidence that PGD_2 can also directly activate PPARs [267]. PGD_2 can be further dehydrated to PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2, and inhibit platelet activation through a number of signaling pathways including activation of PPARs [268,269]. While PGD_2 derivatives are known PPAR ligands, the role of PPARs in platelet activation has not been fully elucidated. Similar to PGD_2, PGI_2 (prostacyclin), a well-characterized vasodilator [270], has been shown to activate adenylate cyclase in the platelet via the prostacyclin (IP) receptor and in turn antagonizes platelet aggregation at sites of injury [258,271]. Although PGI_2 has been shown to exert antiplatelet effects *in vivo* and is also clinically available to treat cardiovascular related diseases, a major concern of the use is the increased occurrence of hypotension. Moreover, PGE_2 exhibits pleiotropic effects by which it can induce both pro- and anti-platelet responses depending on the concentration

[272,273] through the binding of one of more of its prostaglandin receptors: EP_1, EP_2, EP_3 and EP_4 [274].

DGLA is an omega-6 (ω-6) PUFA that can be acquired through supplementation of γ-linolenic acid (GLA) in the diet. COX converts DGLA to series 1 prostaglandins (PGD_1, PGE_1) and TXA_1 [275,276], which inhibit platelet function *in vitro* and *in vivo* [277]. These DGLA-derived COX prostanoids exerted their antiplatelet action by activating the $G\alpha_s$-coupled GPCRs, prostaglandin receptors (EP_2 and EP_4) or IP receptor.

Similar to DGLA, COX acts on EPA, an ω-3 PUFA, to generate anti-inflammatory [278] lipid mediators, known as series 3 PGs and TXs [279,280]. EPA-derived metabolites of COX (PGE_3, PGD_3, PGI_3) inhibit platelet aggregation [281-284] and P-Selectin expression induced by platelet activating factor (PAF) as well as inhibiting platelet-rich plasma (PRP) [259]. Evidence for the series 3 PGs receptors is scant. PGE_3 had been suggested to be a partial agonist of the EP receptors in human kidney cells with varying degree of affinity potencies[281] and secondary messenger actions. PGE_3 mediated $G\alpha_q$ activation and intracellular calcium release through the EP_1 receptor. While $G\alpha_s$ and enhanced cAMP formation were observed in EP_2 and EP_4 overexpressed cells treated with PGE_3, EP_3 ligand binding resulted in reduced cAMP generation and augmented IP formation coupled to $G\alpha_i$ activation[281]. Platelets treated with EP_3, EP_4, IP and receptor antagonists (DG-41, ONO-AE3-208, and CAY10441 respectively) demonstrated that PGE_3 acted as an antagonist to the EP_3 to further inhibit platelet function, but with no effect mediated by the IP receptor on platelet reactivity. In contrast, CAY10441 reversed the ability of PGE_3 to inhibit platelet function [282]. These studies indicate that PGE_3 is also capable of producing multiple and simultaneous effects, resulting in either pro- or anti-thrombotic outcome.

Oxygenase	PUFA	Oxylipin	Actions
COX	AA	PGD_2	Inhibits platelet activation via DP receptor and possibly PPAR
	AA	PGE_2	Exhibits both anti-platelet and pro-platelet activation, depending on concentrations and binding to receptors EP_1-EP_4
	AA	PGI_2	Inhibits platelet function via IP receptor and PPAR
	AA	TXA_2	Activates platelet function via TPα receptor
	DGLA	PGD_1	Inhibits platelet function via EP_2, EP_4 and IP receptors
	DGLA	PGE_1	Inhibits platelet function via EP_2, EP_4 and IP receptors
	DGLA	TXA_1	Inhibits platelet function via EP_2, EP_4 and IP receptors
	EPA	PGD_3	Inhibits platelet function via DP receptor and PPAR
	EPA	PGE_3	Inhibits platelet function via EP_2 and P_4 receptors
	EPA	PGI_3	Inhibits platelet function via IP receptor and PPAR
	EPA	TXA_3	Inhibits platelet function via EP_2 and EP_4 receptors
5-LOX	AA	5-HETE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	AA	LTA_4	Unknown function
	AA	LTB_4	Unknown function
	AA	LTC_4	Activates platelet function via $CysLT_2R$
	AA	LTD_4	Potentiation of platelet activation; unknown mechanism of action
	AA	LTE_4	Potentiation of platelet activation; unknown mechanism of action
	AA	5-oxo-ETE	Unknown function
12-LOX	AA	12-HETE	Exhibits both anti-platelet and pro-platelet activation possibly through 12-HETER
	DGLA	12-HETrE	Inhibits platelet through $G\alpha_s$
	DHA	11-HDHA	Inhibits platelet activation; unknown mechanism of action
	DHA	14-HDHA	Inhibits platelet activation; unknown mechanism of action
	DPA	11-HDPA	Inhibits platelet activation possibly through antagonism of COX-1
	DPA	14-HDPA	Inhibits platelet activation possibly through antagonism of COX-1
	EPA	12-HEPE	Inhibits platelet activation; unknown mechanism of action
15-LOX	AA	15-HETE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	DGLA	15-HETrE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	DHA	17-DHDA	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
	DPA	13-HODE	Exhibits both anti-platelet and pro-platelet activation; unknown mechanism of action
CYP450	AA	5,6-EET	Inhibits platelet activation; unknown mechanism of action
	AA	8,9-EET	Inhibits platelet activation; unknown mechanism of action
	AA	11,12-EET	Inhibits platelet activation; unknown mechanism of action
	AA	14,15-EET	Inhibits platelet activation; unknown mechanism of action
	AA	19-HETE	Inhibits platelet function via IP receptor
	AA	20-HETE	Inhibits human platelet activation; antagonism of PGH_2/TXA_2 receptor or further oxidation to inactive metabolites
	DHA	7,8-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	10,11-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	13,14-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	16,17-EDP	Inhibits platelet activation; unknown mechanism of action
	DHA	19,20-EDP	Inhibits platelet activation; unknown mechanism of action
	EPA	8,9-EEQ	Inhibits platelet activation; unknown mechanism of action
	EPA	11,12-EEQ	Inhibits platelet activation; unknown mechanism of action
	EPA	14,15-EEQ	Inhibits platelet activation; unknown mechanism of action
	EPA	17,18-EEQ	Inhibits platelet activation; unknown mechanism of action
5-LOX, 15-LOX	DHA	Resolvin D_1	Potentiation of platelet activation possibly through GPR32
12-LOX	DHA	MaR1	Exhbits both anti-platelet and pro-platelet activation; unknown mechanism of action
15-LOX, 5-LOX	DHA	Protectin DX	Inhibits platelet activation; unknown mechanism of action

Table 4.1 Oxylipin regulation of platelets

Oxygenase	PUFA	Oxylipin	Actions
COX-2, 5-LOX, CYP450	EPA	Resolvin E_1	Inhibits platelet activation possibly through ChemR23
15-LOX, 5-LOX, 12-LOX	AA	LXA_4	Modulate neutrophil-platelet aggregation through LXA_4 receptor; however unknown direct effect on platelet function and mechanism of action
	AA	LXB_4	Unknown function

Both PGD_3 and PGI_3 are deemed to behave similarly to PGD_2 and PGI_2 agonists as well as exerting through the same cognate receptors to increase cAMP. PGD_3 is also shown to be further dehydrated PGJ_3, Δ^{12}-PGJ_3, and 15-deoxy-$\Delta^{12,14}$-PGJ_3 [285] and act on DP receptor or possibly function through PPARs [286]. PGI_3 is an unstable analog of PGI_2 that exerts its action on the IP receptor, but has been observed to activate PPAR [287]. TXA_3 is presumed to act on EP_2 and EP_4 receptors to to enhance cAMP [283] to inhibit platelet function similar to TXA_1, but further cell line and pharmacological models are required to verify this assumption.

4.3 Lipoxygenases

Mammalian lipoxygenases (LOXs) constitute the following heterogeneous group of lipid-peroxidizing enzymes that are categorized accordingly to their positional specificity of AA oxygenation: 5-LOX, 12-LOX, and 15-LOX. While these enzymes are expressed in a number of cells, they each produce oxylipins that function in part to regulate platelet activity, hemostasis, and thrombosis.

5-Lipoxygenase (5-LOX)

5-LOX is best known for its ability to produce leukotrienes (LTs) [280]. LTs are synthesized in myeloid cells (eosinophils, neutrophils, mast cells, macrophages, monocytes, dendritic cells, basophils, and B-lymphocytes) that are involved in inflammatory, immune, and allergy responses. 5-LOX also produces non-LT products (5-hydroxyeicosatetraenoic acid (5-HETE)) and two structural forms of LTs, which consist of cysteinyl-free (LTA, LTB) and cysteinyl-LTs (LTC, LTD, LTE, LTF)[288] (Figure 4.2, Table 4.1). The type of PUFA substrate being oxidized

dictates the class of LTs formed to modulate inflammatory response and vascular tone. While platelets lack 5-LOX, there are numerous studies suggesting the interplay between leukocytes and platelets through their eicosanoid production [289] by which platelets can modulate immunological response or vice versa [290,291].

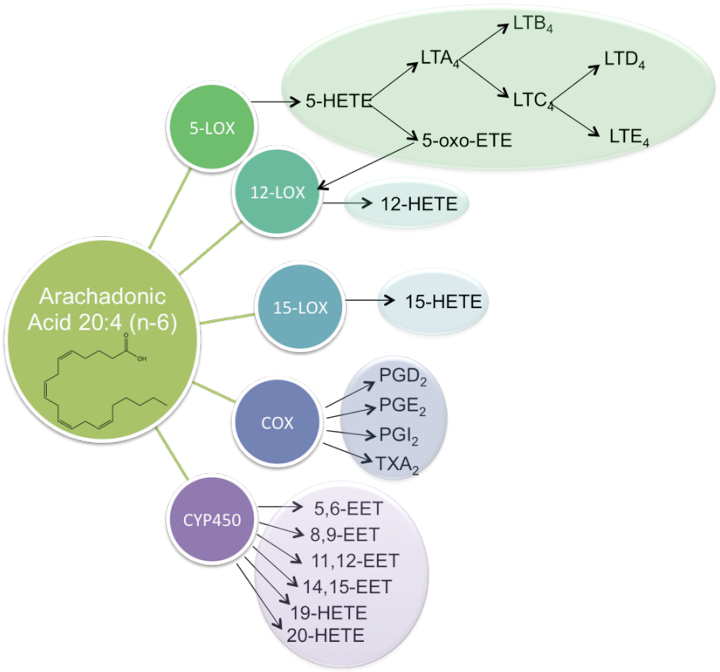


Figure 4.2 Oxidation of arachidonic acid into their respective products by oxygenases.
Arachidonic acid (AA) is oxidized by 5-LOX, 12-LOX, 15-LOX, COX, and CYP450 into their respective classes of oxylipins: 1) non-leukotrienes (LTs) (5-HETE, 5-oxo-ETE) or cysteinyl-free LTs (LTA_4, LTB_4) and cysteinyl-LTs (LTC_4, LTD_4, LTE_4); 2) 12-HETE; 3) series 2 prostaglandins (PGD_2, PGE_2, PGI_2, and TXA_2); 4) epoxyeicosatrienoic acids (EETs) (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET) and hydroxylates (19-HETE, 20-HETE). This illustration was generated by Jennifer Yeung and Megan Hawley

5-LOX derived metabolites and their regulation of platelet function

Cellular induction by various stimuli (chemotactic agents, immune complexes, bacterial peptides) leads to translocation of 5-LOX to the membrane [292-294] to convert AA to 5-HETE. 5-HETE can further be converted by 5-hydroxyeicosanoid dehydrogenase to form 5-oxo-6,8,11,14-eicatetraenoic acid (5-oxo-ETE) (Figure 4.2), or dehydrated to a member of series 4 epoxide intermediate leukotriene, LTA_4 [295].

Previous studies have demonstrated a platelet role in the transcellular metabolism of LTs released by neutrophils, enhancing platelet-neutrophil interactions. The interplay between platelet-adherent leukocyte interactions has been implicated in chronic inflammatory diseases in patients, including aspirin-exacerbated respiratory disease (AERD). While neutrophils lack LTC_4 synthase to convert LTs, platelets have been shown to possess abundant LTC_4 synthase [296-299] to convert unmetabolitzed LTA_4 secreted from neutrophils or monocytes to LTC_4 requiring P-selectin-dependent interaction [300-302]. Through this interaction, transcellular metabolism of LTC_4 is increased, resulting in exacerbated inflammatory response [303-305].

Although the secreted LTs function principally to activate and recruit additional neutrophils to propagate inflammatory responses in allergy or asthma, platelets have also been shown to respond to LTs. Human PRP pretreated with exogenous LTs (LTE_4, LTD_4, LTC_4) for example, showed potentiation in aggregation and TXB_2 production following sub-threshold stimulation with thrombin and epinephrine [306]. The effects of LTs potentiation of human PRP aggregation and TXB_2 generation are presumed to be mediated through the platelet cys-LT receptors, type 1 and type 2 ($CysLT_1R$ and $CysLT_2R$) [307], which are involved in chemokine, RANTES, release in inflammation [307-311]. The functional importance of $CysLT_2R$ in platelet function was demonstrated in transgenic mice deficient in $CysLT_2R$ (*Cysltr2*$^{-/-}$) where it was shown in *Cysltr2*$^{-/-}$ that $CysLT_2R$ expression was required for low nanomolar LTC_4 induction of P-selectin, ADP, and TXB_2 release from platelets [312]. On the other hand, LTE_4 and LTD_4 did not augment P-selectin expression from wild-type, *Cysltr1*$^{-/-}$ or *Cysltr2*$^{-/-}$ platelets suggesting metabolite specificity for these biochemical regulatory steps. Interestingly, LTC_4 induction of P-selectin expression was also observed to be markedly impaired in purinergic receptor $P2Y_{12}$ knockout mouse platelets. Together, these studies suggest that $P2Y_{12}$-targed thienopyridine drugs

used for the management of cardiovascular ischemic events may also interfere with the LTC_4/ $CysLT_2R$-dependent pathway of platelet activation.

The biological activity of 5-HETE on platelet function has been controversial. *In vitro* studies showed 5-HETE inhibits the binding of the radiolabeled thromboxane mimetic, [^{125}I]BOP to the PGH_2/TXA_2 receptor in washed human platelets with IC_{50} values greater than 25 μM. This observation suggests that 5-HETE directly inhibits platelet activation through direct competition with PGH_2/TXA_2 [313]. Conversely, thrombin-induced platelet aggregation and ADP release was shown to be potentiated with 30 μM of 5-HETE [221]. In light of clinical studies being conducted with the use of 5-HETE inhibitors, it will be of high importance to delineate the potential effect of these inhibitory strategies targeting 5-HETE production on platelet reactivity and hemostasis.

12-Lipoxygenase (12-LOX)

Both 12*S*-LOX and 1*2R*-LOX isoenzymes, which generate distinct chiral metabolites from PUFAs, are expressed in selective mammalian tissues and cells. 12-LOX is further classified as platelet, leukocyte, or epithelial-type. Platelet-type 12-LOX is expressed in all mammalian species, whereas the leukocyte-type 12-LOX is found in murine, porcine, and bovine, but not in humans or rabbits [314-316]. Conventionally, 12-LOX is characterized for its ability to convert AA to 12-hyderoperoxyeicosatetraenoic acid (12-HpETE), which is rapidly reduced to 12-hydroxyeicosatetraenic acid (12-HETE) (Figure 4.2). To date, majority of the platelet related studies have focused on 12*S*-LOX products, since no 12*R*-LOX products have been found to regulate platelet function. Thus, for the purpose of this review only the *S* configuration metabolites of 12-LOX will be discussed.

Regulation of platelet function by 12-LOX derived metabolites

The major metabolite of 12-LOX, 12-HETE, has been described to have both anti-thrombotic and pro-thrombotic effects. The anti-thrombotic effect of 12-HETE was first implicated by its direct inhibition of neutrophil PLA_2 activity by which the availability of AA was reduced *in vitro* [317]. In support, exogenous 12-HETE suppressed collagen-induced liberation of AA in bovine platelets [318]. Platelets from 12-LOX deficient mice were hyper-responsive to aggregation induced by ADP, and this phenomenon was reversed by 12-HETE treatment [319]. 12-HETE and 12-HpETE were also reported to inhibit PGH_2- and collagen- induced platelet aggregation [320-322] as well as to prevent binding of PGH_2 and TxA_2 to their cognate receptors [319].

In stark contrast, 12-HpETE and 12-HETE have been demonstrated to potentiate platelet activation and aggregation. Exogenous 12-HpETE, at nanomolar concentrations, activated platelet p38 mitogen-activate protein kinase involved in platelet activation, as well as phosphorylation of cytosolic PLA_2, increased TxB_2 and dense granule secretion [322-325]. 12-HETE was also shown to potentiate bovine platelet aggregation induced by thrombin as well as inhibiting PGE_1-induced elevation of cAMP. Pharmacological inhibition and genetic ablation of 12-LOX have demonstrated the importance of 12-HETE in potentiation of platelet activation [325-327].

The pro-thrombotic effect of 12-HETE is thought to be mediated through its esterification into the lipid membrane following formation in the platelet, which results in enhanced tissue factor-dependent thrombin generation in the vessel [328]. It is also possible that 12-HETE could be mediating its effect on platelet function through high affinity binding to an orphan GPCR, GPR31 (12-HETER) [329], which was originally discovered in cancer cells to promote survival and metastasis as well as neuronal cells that modulate voltage-sensitive calcium channels [330]. Alternatively, 12(S)-HETE had also been shown to enhance and activate peroxisome

proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor family of ligand-dependent transcription factors [331]. To date, the expression of 12-HETER in platelets has not been confirmed.

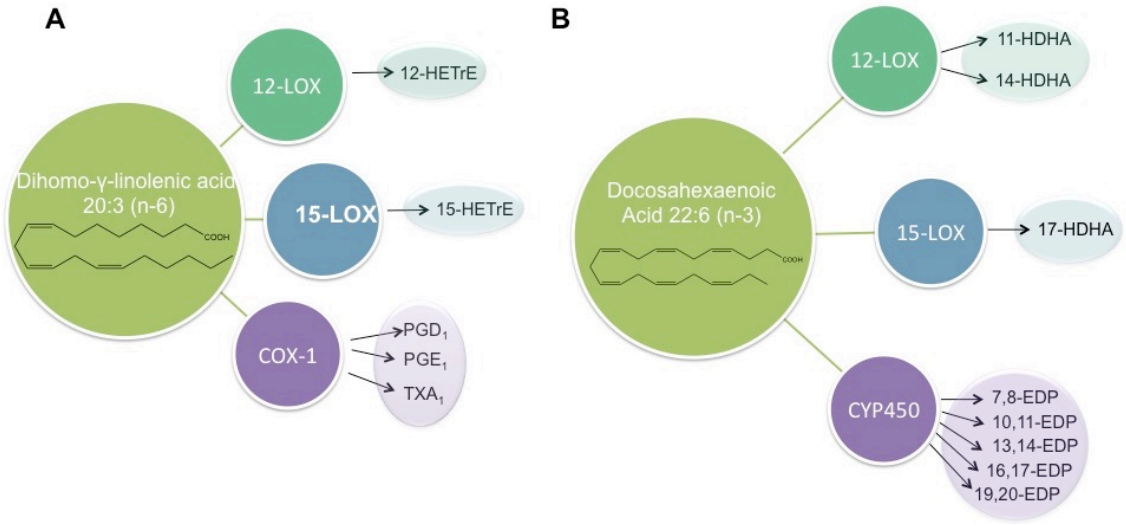


Figure 4.3 PUFA oxidation by oxygenase.
a) Dihomo-γ-linolenic acid (DGLA) is oxidized by 12-LOX, 15-LOX, and COX into the corresponding metabolites: 12-HETrE, 15-HETrE, and series 1 prostaglandins (PGD1, PGE1, TXA2). b Docosahexaenoic acid (DHA) is also metabolized by the oxygenases into the following: 11- or 14-HDHA by 12-LOX, 17-HDHA by 15-LOX, and epoxydocosapentaenoic acids (EDPs) (7,8-EDP, 10,11-EDP, 13,14-EDP, 16,17-EDP, 19,20-EDP) by CYP 450 isoforms. This illustration was generated by Jennifer Yeung and Megan Hawley.

While the predominant metabolite, 12-HETE, has been shown to have contradicting roles, other 12-LOX derived metabolites from EPA, DHA, DPA, and DGLA (12-HEPE, 11/14-hydroxydocosahexaenoic acid (11/14-HDHA), 11/14-hydroxydocosapentaenoic acid (11/14-HDPA), and 12-hydroxyeicosatrienoic acid (12-HETrE)) [321,327,332] have been shown to exert anti-platelet or anti-thrombotic *in vivo* effects. These metabolites vary in potency and ability to be synthesized. For instance, only trace amounts of 11/14-HDHA (Figure 4.3) were detected in EPA or DHA pretreatment compared to 12-HEPE and 12-HETE following thrombin-stimulation in platelets, suggesting that higher concentrations of DHA are needed for platelet inhibition [333]. Additionally, DPA is observed to exert its anti-platelet effect [334] through inhibition of COX-1 activity by 11/14-HDPA[335] (Figure 4.4).

Though previous studies have implicated the 12-LOX-derived metabolites in cardioprotection through the dampening of platelet activation, there was no direct *in vivo* evidence to support those claims. More recently, the role of platelet 12-HETrE on thrombosis and underlying mechanisms were investigated *in vivo* and *ex vivo*. Mice intravenously administered 6 mg/kg of 12-HETrE or 50 mg/kg DGLA were protected from thrombus accumulation at the site of arteriole vessel injury [336]. The antiplatelet effects of DGLA *in vivo* were also shown to be dependent on the presence of functional platelet 12-LOX in mouse platelets. For instance, even though mice lacking 12-LOX (*ALOX12*$^{-/-}$) had attenuated thrombus formation within the vessel following laser injury, DGLA treatment did not further prevent thrombus growth in the *ALOX12*$^{-/-}$ mice compared to the wild-type. This demonstrated that 12-LOX was required for DGLA-mediated inhibition of platelet activation and thrombus formation. Finally, the anti-platelet effect of 12-HETrE was shown to be mediated through a $G\alpha_s$-linked GPCR, which activates adenylyl cyclase and subsequent downstream effectors to inhibit platelet activation.

15-Lipoxygenase (15-LOX)

Two forms of 15-LOX isoforms exist in mammalian tissues, leukocyte-type 15*S*-LOX (15-LOX-1) and epidermis-type 15-LOX type B (15-LOX-2) [337-339]. Tissue distribution of 15-LOX-2 is limited when compared to that of 15-LOX-1. 15-LOX-1 is expressed in eosinophils, leukocytes, reticulocytes, macrophages, dendritic, epithelial cells (bronchial, corneal, and mammary)[340,341], whereas, 15-LOX-2 is predominantly found in skin, prostate, lung, and cornea [342].

15-LOX derived metabolites and regulation of platelet function

While the existence of 15-LOX-1 and -2 in platelets is questionable, platelets have demonstrated the ability to generate the 15-LOX oxylipin products, 15-hydroxyeicosatetraenoic acid (15-

HETE), 8,15-dihydroxyeicosatetraenoic acid (8,15-diHETE), and 14,15-dihydroxyeicosatetraenoic acid (14,15-diHETE) from AA (Figure 4.2), [338] and 17-hydroxydocosahexaenoic acid (17-HDHA) [343] from DHA (Figure 4.3, Table 4.1). Both 15-HETE and 8,15-diHETE were shown to inhibit platelet aggregation induced by collagen, ADP, epinephrine, AA, or prostaglandin H2 analog [344-346]. Conversely, 15-HETE and 15-HpETE were also demonstrated to enhance whole blood aggregation and thrombin generation in the presence of macrophages [347]. 15-HETE (between 1 and 100 nM) enhanced thrombin-stimulated platelet aggregation, ADP release, and secondary messengers (inositol-1,4,5-triphosphate, diacylglycerol, and intracellular calcium) production[221]. Similarly, 17-HDHA was shown to potentiate ADP-induced platelet aggregation and spreading, but inhibited alpha granule secretion [348]. The latter data suggests that 15-LOX products can function as pro-coagulant mediators.

Both 13-hydoxyoctadecadienoic acid (13-HODE) and 15-hydroxyeicosatrienoic acid (15-HETrE), major metabolites of 15-LOX derived from linolenic acid (LA) (Figure 4.4) and DGLA (Figure 4.3), respectively, were shown to inhibit rabbit [349] and human platelet aggregation [350]. Additionally, 13-HODE was demonstrated to inhibit thrombin-induced TxB2 and 12-HETE production in platelets as well as platelet adherence to endothelial cells *in vitro* [350,351]. Interestingly, 15-HETrE exhibited biphasic effects on platelet aggregation in which low concentrations potentiated and higher concentrations inhibited platelet aggregation [352].

4.4 Cytochrome P450

Cytochrome P450 enzymes (CYP450s) belong to a large group of oxygenases, with at least 57 putatively functional subfamilies in humans and upwards of 102 in mice [353,354]. CYP450s are expressed primarily in the liver, with some detection in the heart, lung, vasculature, kidney and gastrointestinal tract. Traditionally, these membrane-bound and heme-containing oxygenases are

recognized for their xenobiotic metabolism and detoxification of drugs, however multifaceted functions have been investigated. These enzymes are also involved in the metabolism of eicosanoids from fatty acids, vitamin D3 synthesis, biosynthesis of cholesterol and bile acids, and synthesis and metabolism of steroids [355].

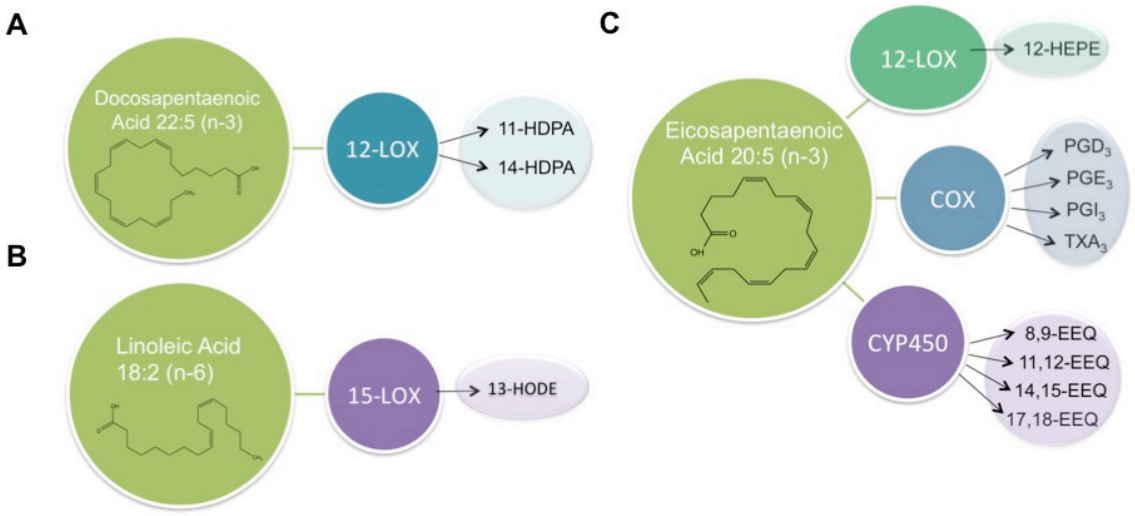


Figure 4.4 PUFA oxidation by oxygenases.
a 12-LOX acts on docosapentaenoic acid (DPA) to convert to 11- or 14-HDPA. **b** Linoleic acid (LA) is metabolitzed by 15-LOX to generate 13-HODE. **c** Eicosapentaenoic acid (EPA) is oxidized by 12-LOX, COX, CYP 450 to 12-HEPE, series 3 prostaglandins (PGD_3, PGE_3, PGI_3, and TXA_3), and epoxyeicosatetraenoic acids (EEQs) (8,9-EEQ, 11,12-EEQ, 14,15-EEQ, and 17,18-EEQ). This illustration was generated by Jennifer Yeung and Megan Hawley.

Regulation of platelet function by CYP450 epoxygenase and hydroxylase derived metabolites

AA can be synthesized by endothelial epoxygenase into a number of epoxyeicosatrienoic acids (5,6-epoxyeicosatrienoic acid (5,6-EET), 8,9-epoxyeicosatrienoic acid (8,9-EET), 11,12-epoxyeicosatrienoic acid (11,12-EET), and 14,15-epoxyeicosatrienoic acid (14,15-EET), which are further catalyzed to dihydroxyeicosatrienoic acids (5,6-diHETrE, 8,9-diHETrE, 11,12-diHETrE, 14,15-diHETrE) by soluble expoxide hydrolase (sEH), and the CYP450 ω-

hydroxylase to generate 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE) that had been demonstrated to maintain vascular tone and hemostasis (Figure 4.2). Early studies demonstrated that many epoxygenase isomers ranging from 1 to 10 μM, regardless of their regiochemical, geometric, and steriochemical structures, were effective at inhibiting human platelet [356-358] or PRP [359] aggregation independent of TxB_2 and cAMP formation [358]. However, in contrast to earlier reports, more recent reports suggest that 11,12-EET (ranging from 1 to 10 μM) do not inhibit platelet aggregation stimulated with collagen, ADP or a thrombin-receptor activating peptide [360]. The conflicting observations from several research groups will require further study to determine if this class of EETs is pro-thrombotic, anti-thrombotic, or is bi-functional depending on platelet conditions (PRP or isolated platelets) and agonist stimulation used.

EETs (5,6-EET, 11,12-EET, 8,9-EET, and 14,15-EET) have additionally been demonstrated to hyperpolarize platelets through the activation of calcium potassium channels resulting in decreased ADP-induced P-selectin expression on platelet surface as well as platelet adhesion to cultured endothelial cells under physiological shear stress [361]. To further support CYP450-derived products from endothelial cells regulate platelet function, supernatant releasate from bradykinin-stimulated cultured endothelial cells overexpressing CYP2C9 were shown to inhibit platelet adhesion. Finally, *in vivo* anti-thrombotic effects of CYP2C9 derived metabolites were demonstrated in the arteriolar wall of hamster. Hamsters administered with CYP2CP inhibitor, sulfaphenazole, at doses known to block endothelium-derived hyperpolarizing factor-dependent dilations, significantly enhanced platelet-vessel wall interactions. The firm adhesion of platelets to vessel wall was reversed when superfused with 10 μM of 11,12-EET [362].

The CYP450 ω-hydroxylase product of AA, 19-HETE, was found to be an orthosteric prostacyclin receptor agonist that inhibited mouse platelet aggregation. To verify that 19(S)-HETE, and not its regioisomer 19(R)-HETE, was responsible for binding to the prostacyclin receptor and inhibiting platelet activity, a megakaryocyte cell line, MEG-01 [363], with intact Gαs expression was shown to enhance cAMP formation following dose-dependent 19(S)-HETE treatment. Additionally, COX-1/2 inhibition of COS-1 human IP receptor expressing cells did not interfere with the ability of 19(S)-HETE to directly induce cAMP formation in MEG-01. Blocking the IP receptor with the selective prostacyclin receptor inhibitor Cay104401 prevented 19(S)-HETE stimulation of cAMP generation in MEG-01 cells. Similarly, 19(S)-HETE was able to displace ^{3}H-iloprost in COS-1 cells expressing IP receptor demonstrated that 19(S)-HETE behaved as a competitive agonist binding to the same domain of the IP receptor as Iliprost (and likely PGI_2). These observations were Confirmed when Pretreatment of IP deficient mouse (*Ptgir*$^{-/-}$)_platelets with 3 μM 19(S)-HETE failed to block thrombin-induced platelet aggregation.

Another eicosanoid found to have potent inhibitory properties against the platelet is 20-HETE. This eicosanoid was found to have a potent effect on inhibiting human platelet aggregation and TxB_2 formation induced by AA, calcium ionohophore, A23187, and the TxB_2 mimetic without affecting thrombin-induced aggregation [364]. The proposed inhibitory effect of 20-HETE on platelet activation was also presumed to be its antagonism of the PGH_2/TxA_2 receptors [364,365]. However, aside from receptors antagonism, 20-HETE was also demonstrated to be further metabolized by COX-1 and 12-LOX to the inactive 11,12-dihydroxyeicosatetraenoic acid (11,12-diHETE) and 12,20-dihydroxyeicosatetraenic acid (12,20-diHETE), respectively, in human platelets [364]. Thus, it is possible that at least some of the observed anti-platelet effect could be attributed to its metabolic transformation to the diHETEs.

Upon dietary supplementation of ω-3 or -6 PUFAs, AA-derived products of CYP450 epoxygenase are partially replaced by EPA and DHA-derived epoxyeicosatetraenoic acids (EEQs) and (epoxydocosapentaenoic acids (EDPs), respectively [366]. In the case of CYP450 epoxygenase derived metabolites of EPA (8,9-EEQ, 11,12-EEQ, 14,15-EEQ, 17,18-EEQ) (Figure 4.4) and DHA (7,8-EDP, 10,11-EDP, 13,14-EDP, 16,17-EDP, and 19,20-EDP) (Figure 4.3), all metabolites were shown to inhibit AA-induced platelet aggregation [367] (Table 4.1). Even though all the diols produced by sEH conversion of the EEQs (8,9-DiHETE, 11,12- DiHETE, 14,15- DiHETE, 17,18- DiHETE) and EDPs (7,8-DiHDPA, 10,11-DiHDPA, 13,14-DiHDPA, 16,17-DiHDPA, 19,20-HiDPA) inhibited platelet aggregation; they were less potent at inhibiting platelet aggregation than the parent epoxides.

Although several of these studies have demonstrated EET and hydroxylate metabolites are derived from cells with intact CYP450, preformed expoxides and 20-HETE of AA have been found as integral components of human platelet membrane [357]. Thus, it is possible that circulating EETs and their diol products, DHETs, and hydroxylates are avidly taken up by platelets and endothelial cells [368,369]. These products can be released during receptor-mediated hydrolysis of platelet phospholipids [357] or further metabolized by COX [370] and LOX [364]. For instance, once stimulated, EETs are de-esterified in platelets and released to influence the migration pattern of nearby neutrophils [357,371]. In contrast, CYP450 ω-hydroxylase inhibitor, HET0016, blocked angiotensin and endothelin-stimulation of 20-HETE secretion from platelets, suggesting that CYP450 isoforms exist in the platelet [366]. Based on study discrepancy, the endogenous expression of CYP450 in the platelet has not been confirmed and will need to be definitively determined before platelet generation of expoxide or hydroxylate products can be

assigned to the platelet itself or alternatively if these products are presented to the platelet from other blood cells including the endothelium and neutrophils.

4.5 The interplay of oxygenases and generation of specialized pro-resolving lipid mediators (SPMs)

Over the past decade, studies have focused on the role of specialized pro-resolving lipid mediators (SPMs) on preventing excessive inflammation, infection, and wound repair, through their ability to attenuate or dissipate chemotactic and pro-inflammatory signals. SPMs, which include lipoxins (LX), D and E series resolvins (Rv), (neuro)protectins (PD), and maresins (MaR), are synthesized by the sequential action of LOXs on PUFAs to resolve and restrain inflammation [351] (Figure 4.5). Despite the prevalence of platelet involvement in the inflammatory process, little is known on how and whether SPMs play a direct role on regulation of platelet function.

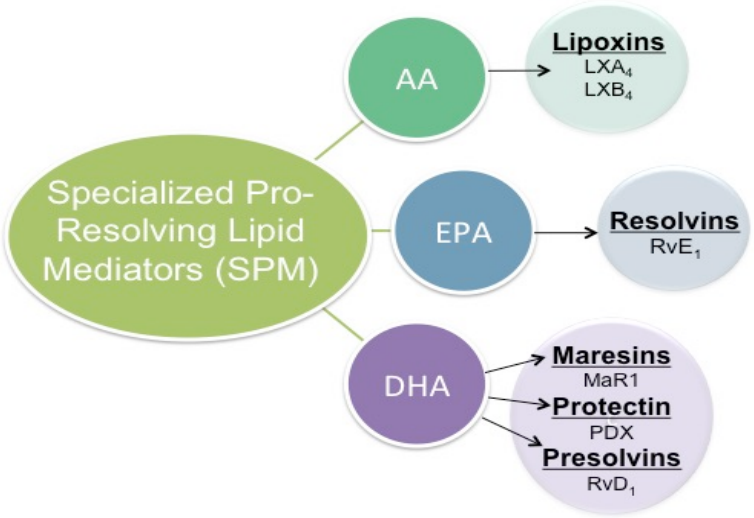


Figure 4.5 Specialized pro-resolving lipid mediators (SPMs) constitute a wide array of lipids classes derived from the interplay of oxygenase activity on AA, EPA, and DHA.
Lipoxins, (LXA_4, LXB_4) and E series resolvin (RvE_1) are derived from AA and EPA, respectively. DHA can be indirectly converted by the interaction of the oxygenases into MaR1, PDX, or D series resolvin, RvD_1.). This illustration was generated by Jennifer Yeung and Megan Hawley.

Lipoxins A (LXA_4) and B (LXB_4) were one of the first SPMs to be identified from the combination of 5- and 15-LOX in human leukocytes [43] as well as neutrophil-derived 5-LOX and platelet 12-LOX [372-375] from AA. Although platelets express LXA_4 receptor (ALX) [376], LXA_4 does not directly inhibit platelet aggregation induced by ADP [349] and bacterial infection [349]. Alternatively, aspirin-triggered lipoxin (ATL), 15(R)-epi-lipoxin A_4 (15(R)-epi-LXA_4)) [377], is indirectly derived from the acetylated COX-2 metabolism of AA. Both LXA_4 and 15(R)-epi-LXA_4 had been demonstrated to modulate neutrophil-platelet aggregation through ALX; however, it remains unclear whether these lipoxins can directly regulate platelet function based on limited studies.

Resolvin E_1 (RvE_1), synthesized by acetylated COX-2 or sequential CYP450 and 5-LOX activity of EPA, was demonstrated to inhibit human PRP aggregation stimulated by ADP and TxB_2, but not collagen [378]. RvE_1 was also shown to inhibit P-selectin expression on activated platelets and platelet actin polymerization, without affecting calcium mobilization. The observed anti-platelet effects of RvE_1 were shown to act through the ChemR23 receptor on the surface of platelets [379]. In contrast, resolvin D_1 (RvD_1) and its intermediary precursor 17-HDHA, derived from 15-LOX and 5-LOX synthesis of DHA, potentiated ADP-mediated platelet aggregation and platelet spreading on fibrinogen. ADP-mediated release of alpha granules in platelets were not affected by RvD_1 and 17-HDHA; however, thrombin stimulation of alpha granules was significantly attenuated by these SPMs [380]. RvD_1 is presumed to exert its effect on its cognate receptor, GPR32, on the platelet surface. Thus far, while data support a role for platelets in the generation of inflammatory markers, the mechanism by which resolvins regulate platelets remains unclear.

Maresin 1 (MaR1) is derived from the biosynthesis of DHA by both neutrophil 15-LOX and platelet 12-LOX [349]. Early studies demonstrated MaR1 anti-inflammatory and pro-resolving properties in lung catabasis. While MaR1 was shown to potentiate platelet aggregation and spreading, it also dampened pro-inflammatory and pro-thrombotic granules, suggesting MaR1 differentially regulates platelet function through a mechanism that has not been fully elucidated to date.

Protectin DX (PDX) belongs to a group of di-oxygenated derivatives of PUFAs, called poxytrins [381]. PDX is an isomer of neuroprotein D1 (PD1) [382], which was originally discovered to attenuate brain ischemia-reperfusion [383,384]. The PDX isomer was demonstrated to be biologically less potent than PD1 in the resolution of inflammation; however, effective in inhibiting collagen-, AA-, and thromboxane -induced platelet aggregation through inhibition of COX-1, at nanomolar concentrations [385].

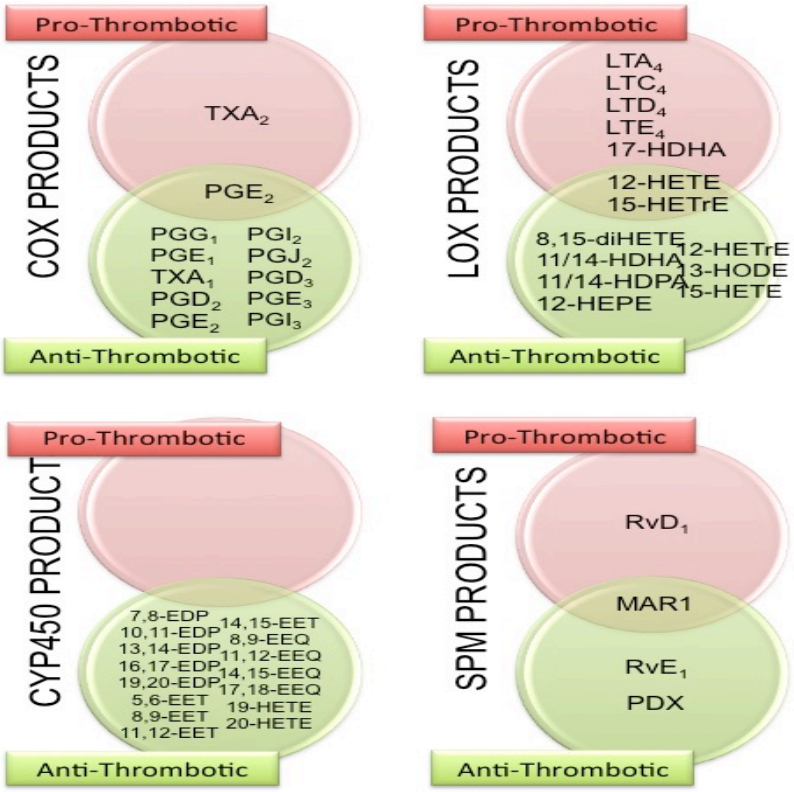


Figure 4.6 LOX, COX, CYP 450 derived lipid mediators and SPMs can be divided into either pro- or anti-thrombotic classes based on their effects on platelet function.). This illustration was generated by Megan Hawley.

4.6 Discussion and Future Implications

Regulation of platelet function is a key step in both physiological and pathological hemostatic processes. While inhibition of platelet activation remains a first line approach for prevention of myocardial infarction and stroke, morbidity and mortality due to cardiovascular diseases and stroke remain the top causes of death globally. Hence a greater understanding of the regulators of platelet function *in vivo* will significantly aid in the development of novel treatments to prevent unwanted clotting and occlusive thrombosis. Lipids and their oxylipins have long been known to regulate platelet function; however, until recently, the breadth of regulators and functions they control in the platelet have not been fully appreciated.

In this chapter, we have highlighted some of the major breakthroughs in identifying oxylipins we now know have direct effects on the platelet. The use of genetic manipulation and

pharmacological tools in both mouse and cellular models to determine the oxygenases and their lipid contributions to vascular and platelet functions has progressed considerably over the last two decades. These tools have greatly enhanced our understanding of the varying roles of oxylipins in platelet biology; however, these studies are still limited to another layer of complexity. The difficulty in targeting the precise pathway or oxylipins associated with pathophysiological disease states stems from the source of oxylipins generated by the involvement of multiple oxygenase enzymes localized in different organs and cell types. Even with the use of pharmacological tools to determine the contributions for each oxygenase metabolites to platelet function, selectivity of drug target still remains a major setback. For instance, chemical compounds designed to inhibit CYP epoxygenase enzymes also antagonized CYP hydroxylase enzymes activity. Therefore, interpretation of results with pharmacological inhibitors should be taken with caution. Future work will thus focus on further delineating the full breadth and diversity of oxylipins regulating platelet function *ex vivo* and *in vivo* and determining the mechanism(s) by which they exert their regulatory function on the human platelet to develop newer pharmacological approaches for targeting pathways involved in the regulation of platelet to address pathological conditions whereby normal regulation of hemostasis and thrombosis has become dysfunctional.

CHAPTER 5

Protein Kinase Regulation of 12-Lipoxygenase-Mediated Human Platelet Activation[4*]

5.1 Summary

Platelet activation is important in the regulation of hemostasis and thrombosis. Uncontrolled activation of platelets may lead to arterial thrombosis which is a major cause of myocardial infarction and stroke. Following activation, metabolism of arachidonic acid (AA) by 12-lipoxygenase (12-LOX) may play a significant role in regulating the degree and stability of platelet activation as inhibition of 12-LOX significantly attenuates platelet aggregation in response to various agonists. Protein kinase C (PKC) activation is also known to be an important regulator of platelet activity. Using a newly developed selective inhibitor for 12-LOX and a pan–PKC inhibitor, we investigated the role of PKC in 12-LOX-mediated regulation of agonist signaling in the platelet. To determine the role of PKC within the 12-LOX pathway, a number of biochemical endpoints were measured including platelet aggregation, calcium mobilization, and integrin activation. Inhibition of 12-LOX or PKC resulted in inhibition of dense granule secretion and attenuation of both aggregation and $\alpha IIb\beta_3$ activation. However, activation of PKC downstream of 12-LOX inhibition rescued agonist-induced aggregation and integrin activation. Furthermore, inhibition of 12-LOX had no effect on PKC-mediated aggregation indicating that 12-LOX is upstream of PKC. These studies support an essential role for PKC downstream of 12-

[4]This section has been published in Molecular Pharmacology. Yeung J, Apopa PL, Vesci J, Kenyon V, Rai G, Jadhav A, Simeonov A, Holman TR, Maloney DJ, Boutaud O, Holinstat M. Protein kinase C regulation of 12-lipoxygenase-mediated human platelet activation. Molecular pharmacology. 2012 Mar 1;81(3):420-30.

LOX activation in human platelets and suggest 12-LOX as a possible target for anti-platelet therapy.

5.2 Introduction

Platelet activation plays a significant role in hemostasis and thrombosis and a central role in the pathophysiology of cardiovascular disease. Platelet activation can be initiated through a number of different receptor pathways including thrombin and collagen. Reinforcement of the initial activation signal is known to be regulated in part by secondary signaling events mediated by arachidonic acid (AA) released from the phospholipid membrane. Although active metabolites formed by the oxidation of AA by cyclooxygenase-1 (COX-1) are known to regulate platelet reactivity [386], the role of metabolites produced by the oxidation of AA by platelet-type 12-lipoxygenase (12-LOX) is controversial. Some reports have shown that metabolic products of 12-LOX attenuate AA-induced aggregation [387] and also inhibit AA release from membrane phospholipids by blocking PLA_2 [318], while other studies suggest 12-LOX activation is pro-thrombotic and is linked to calcium mobilization [196], regulation of tissue factor activation, and thrombin generation in the platelet [329]. The mechanistic basis for these physiological changes in platelet activity through the 12-LOX pathway is not clear. In particular, the events that occur both upstream and downstream of 12-LOX upon agonist stimulation have not been well characterized.

Protein kinase C (PKC), which is known to play an important role in a number of biochemical activation steps in the platelet [388,389], has also been suggested to play a role in 12-HETE regulation in tumor cells (Szekeres et al., 2000). In platelets, similarly to 12-LOX, PKC has been shown to regulate aggregation and play an important role in granule secretion and integrin activation [390]. Further, protease-activated receptor-1 (PAR1) and PAR4 signaling in the platelet

have been shown to result in Ca^{2+} mobilization and PKC-mediated aggregation and secretion [391]. However, the underlying mechanism by which PKC regulates platelet activity is controversial. Kim et al [392] reported that PKC inhibition by the pan-PKC inhibitor, Ro 31-8220, potentiated epinephrine induced platelet aggregation and Unsworth et al [393] showed that PKC inhibition potentiates platelets secretion in the presence of Ca^{2+}. Other reports have shown that PKC inhibition attenuates platelet aggregation [394].

In this study, we investigated the coupling between the activation of 12-LOX and PKC in regulating platelet aggregation and integrin activation. We sought to determine if PKC acted downstream of 12-LOX upon agonist stimulation. Agonist-mediated platelet aggregation was significantly decreased in the presence of either a 12-LOX or PKC inhibitor. Inhibition of 12-LOX activity by selective small molecule inhibitors [395] which leads to attenuation of aggregation was overcome when the PKC activator, PMA, was added together with agonist to the platelets. Furthermore, inhibition of 12-LOX had no effect on PMA-mediated platelet aggregation. Finally, $\alpha IIb\beta_3$ attenuation in the absence of 12-LOX was rescued by addition of PMA. Hence, this is the first report to show that PKC activity occurs downstream of 12-LOX in human platelets and begins to elucidate how this essential pathway mediates normal platelet activation through a number of GPCR and non-GPCR receptors.

5.3 Materials and Methods

Materials: 12-LOX inhibitor (NCTT-956) was synthesized at the NIH Chemical Genomics Center (Rockville, MD) and provided by David Maloney. Baicalein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK and total-ERK antibodies were purchased from Cell Signaling Technology (Boston, MA), PAR1-AP (SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai, China). Fluo-4 AM was from Invitrogen (Eugene, OR). Human α-thrombin was purchased from Enzyme Research Labs (South Bend, IN). Convulxin was purchased from Center Chem (Norwalk, CT). Fluorescein isothiocyanate (FITC)-conjugated PAC1 antibody was purchased from BD Biosciences (San Jose, CA). C6 flow cytometer was from Accuri (Ann Arbor, MI). Aggregometer, collagen, chronolume reagent, and other aggregation supplies were purchased from Chrono-Log Corp. (Havertown, PA).

Human Platelets: Human platelets were obtained from healthy volunteers within the Thomas Jefferson University community and the Philadelphia area. These studies were approved by the Thomas Jefferson University Institutional Review Board and informed consent was obtained from all donors before blood draw. Blood was centrifuged at 200 *g* for 13 minutes at room temperature. Platelet-rich plasma was transferred into a conical tube containing a 10% acid citrate dextrose solution (39 mM citric acid, 75 mM sodium citrate, and 135 mM glucose, pH 7.4) and centrifuged at 2000 *g* for 15 minutes at room temperature. Platelets were resuspended in Tyrode's buffer (12 mM $NaHCO_3$, 127 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES), and the final platelet concentration was adjusted to 3 X 10^8 platelets/ml after counting with a ZI Coulter particle counter (Beckman Coulter, Fullerton, CA). Reported results are the data obtained using platelets from at least three different subjects. Agonists and inhibitors were used at concentrations indicated in the figures and figure legends.

GC/MS analysis of [$^2H_8$] Thromboxane synthesis in platelets: 2 ng of [$^2H_4$] TxB_2 was added to the samples as internal standards. Briefly, the sample prepurified with a C18 SepPak column. [$^2H_8$] TxB_2 was eluted with 10 ml heptane/ethyl acetate (1:1), dried, dissolved in acetonitrile and TxB_2 was converted to pentafluorobenzyl esters. TxB_2 was then purified and the sample was converted to O-trimethylsilyl ether derivatives and analyzed by gas chromatography/electron capture negative chemical ionization mass spectrometry, using an SPB-1 column (15 meters), with a temperature gradient from 190°C to 300°C at 20°C/min. The ion corresponding to the derivatized TxB_2 was monitored by selected ion monitoring (SIM). The signal for TxB_2 is *m/z* = 622. The signal for the internal standard [$^2H_4$] TxB_2 is *m/z* = 618.

Measurement of 12-HETE: Secretion of 12-HETE was measured from platelet supernatants by liquid chromatography LC/APCI/MS/MS following addition of an internal standard (2 ng of [$^2H_8$] 12-HETE) as described previously [396]. The concentration of 12-HETE was determined by isotopic dilution.

$cPLA_2$ activation assay: The effect of the different inhibitors on $cPLA_2$ was tested with recombinant human $cPLA_2$ using the enzymatic activity assay described previously [397] with the following differences. The inhibitors were added at a final concentration of 50 μM in DMSO right before the recombinant enzyme was added to initiate the reaction. After 5 minutes of incubation, the products of the reaction were analyzed.

Platelet Aggregation: Washed platelets were adjusted to a final concentration of 3 x 10^8 platelets/ml. Where indicated, platelets were pretreated with 12-LOX inhibitors for 10 minutes or PKC inhibitor for 1minute. The aggregation response to PAR1-AP, PAR4-AP or collagen was measured using an aggregometer with stirring at 1100 rpm at 37°C.

Dense-Granule Secretion: ATP release was assayed as an indication of dense granule secretion. For ATP studies, washed platelets adjusted to a final concentration of 3 x 10^8 platelets/ml were pretreated with 12-LOX inhibitors for 10 minutes or PKC inhibitor for 1 minute. ATP release in response to agonist was measured using a Lumi-aggregometer at 37°C with stirring at 1100 rpm.

Flow Cytometry: Integrin αIIbβ3 activation on the surface of the platelet was measured by flow cytometry using FITC-conjugated PAC1 (an antibody which only recognizes the active form of αIIbβ3). For these experiments, 40 µl aliquots of washed platelets adjusted to a final concentration of 2.5 x 10^7 platelets/ml were pre-treated with inhibitors for 10 minutes. After addition of 1 µl of PAC1, platelets were stimulated with agonist for 10 minutes and then diluted to a final volume of 500 µl using Tyrode's buffer. The fluorescence intensity of platelets was immediately measured using an Accuri flow cytometer.

Western Blotting: Washed platelets adjusted to 1 x 10^9 platelets/ml were stimulated with indicated agonists and lysed with 3x Laemmli buffer was then added to the samples, boiled for 5 minutes, and subjected to Western blot analysis.

Calcium Mobilization: Platelets were re-calcified to a final concentration of 1 mM followed by pre-incubation with Fluo-4 AM for 10 minutes. The platelets were then treated with a 12-LOX inhibitor for 10 minutes before stimulation with indicated agonist. Calcium mobilization was measured using Accuri C6 flow cytometer.

Statistical Analysis: Comparison between experimental groups was made using appropriate statistical analyses (paired *t* test program or ANOVA with post-test analysis) using Prism software. Differences in mean values (measured as standard error of the mean) were considered significant at $p < 0.05$.

5.4 Results

Specificity of 12-LOX inhibitors. To determine the role of the lipoxygenase pathway in human platelet reactivity, the recently identified and highly selective 12-LOX small molecule inhibitor NCTT-956 [395] and the less selective commercially available 12-LOX inhibitor (baicalein) were studied. To assess for possible off-target effects on the related cyclooxygenase-1 pathway which leads to formation of thromboxane A_2 (Figure 5.1), platelets were treated with deuterated-AA in the presence or absence of NCTT-956 or baicalein and the deuterated-TxB_2 product was measured (Figure 1a). Values are expressed as the % of control to which no inhibitor was added. Deuterated-TxB_2 was not inhibited by NCTT-956 or Ro 31-8220, indicating that COX-1 and thromboxane synthase are not directly inhibited by these pharmacological agents. Treatment with baicalein, however, resulted in a significant decrease in the production of [$^2H_8$] TxB_2 suggesting this drug may have a direct inhibitory effect on COX-1 or thromboxane synthase (Figure 5.1a). As [$^2H_8$] TxB_2 was increased in the presence of the PKC inhibitor, the level of 12-HETE formation was also measured to determine if Ro 31-2880 directly inhibited 12-LOX. No decrease in 12-HETE formation was observed in the presence of Ro 31-2880 (data not shown). To confirm that NCTT-956, baicalein, and Ro 31-8220 did not inhibit the release of arachidonic acid, $cPLA_2$ activity was measured in the absence or presence of each inhibitor (Figure 5.1b). Neither 50 μM NCTT-956 nor 10 μM Ro 31-8220 inhibited $cPLA_2$ activity. 50 μM baicalein, however, resulted in almost a 50% decrease in $cPLA_2$ activity ($P<0.001$) confirming the higher level of selectivity of NCTT-956 toward 12-LOX.

PKC regulation of platelet aggregation and dense granule secretion. Since PKC plays an important role in granule secretion and integrin activation [390], we investigated PKC involvement in the transduction of 12-LOX signaling in the platelet. First, we confirmed the role

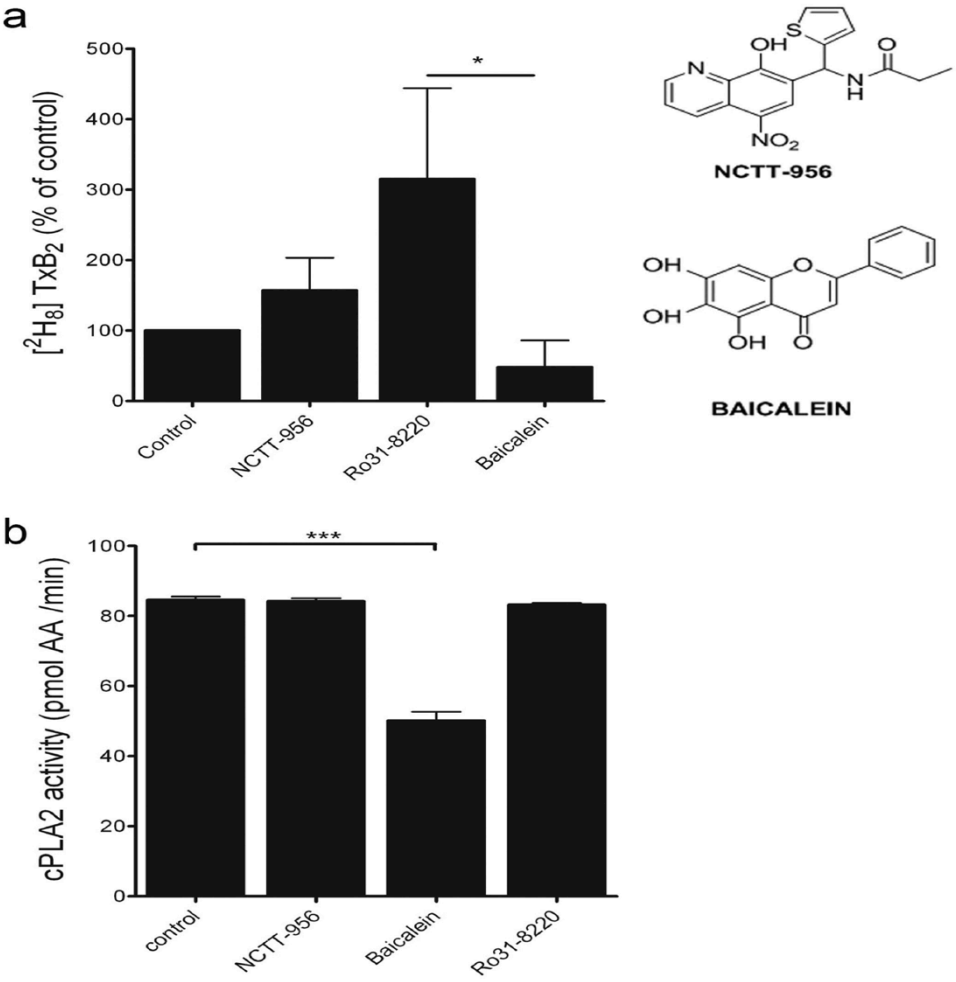


Figure 5.1 Inhibitor selectivity.
a) Washed platelets were stimulated with [$^2H_8$] arachidonic acid and the level of [$^2H_8$] TxB_2 relative to no inhibitor treatment (control) was measured (N=3). No inhibition of [$^2H_8$] TxB_2 was observed in the presence of 50 μM NCTT-956 or 10 μM Ro 31-2880 relative to control. 50 μM baicalein induced an inhibition of [$^2H_8$] TxB_2 compared to control levels and [$^2H_8$] TxB_2 formation in the presence of baicalein was significantly inhibited compared to [$^2H_8$] TxB_2 in the presence of Ro 31-2880. N=3. b) Human recombinant $cPLA_2$ activity in the absence or presence of 50 μM baicalein, 50 μM NCTT-956, or 10 μM Ro 31-2880 (N=3). AA: arachidonic acid released from vesicles. *, $P<0.05$; ***, $P<0.001$. This figure was provided our collaborator Dr. Olivier Boutaud.

of PKC in platelet activation by measuring platelet aggregation in the presence of increasing concentrations of the pan-PKC inhibitor, Ro 31-8220, following stimulation with 20 μM PAR1-AP, 200 μM PAR4-AP, or 10 μg/ml collagen (Figure 5.2). Our data show that inhibiting

activation of PKC resulted in a dose-dependent attenuation of platelet aggregation, suggesting that PKC is important for normal platelet aggregation to occur.

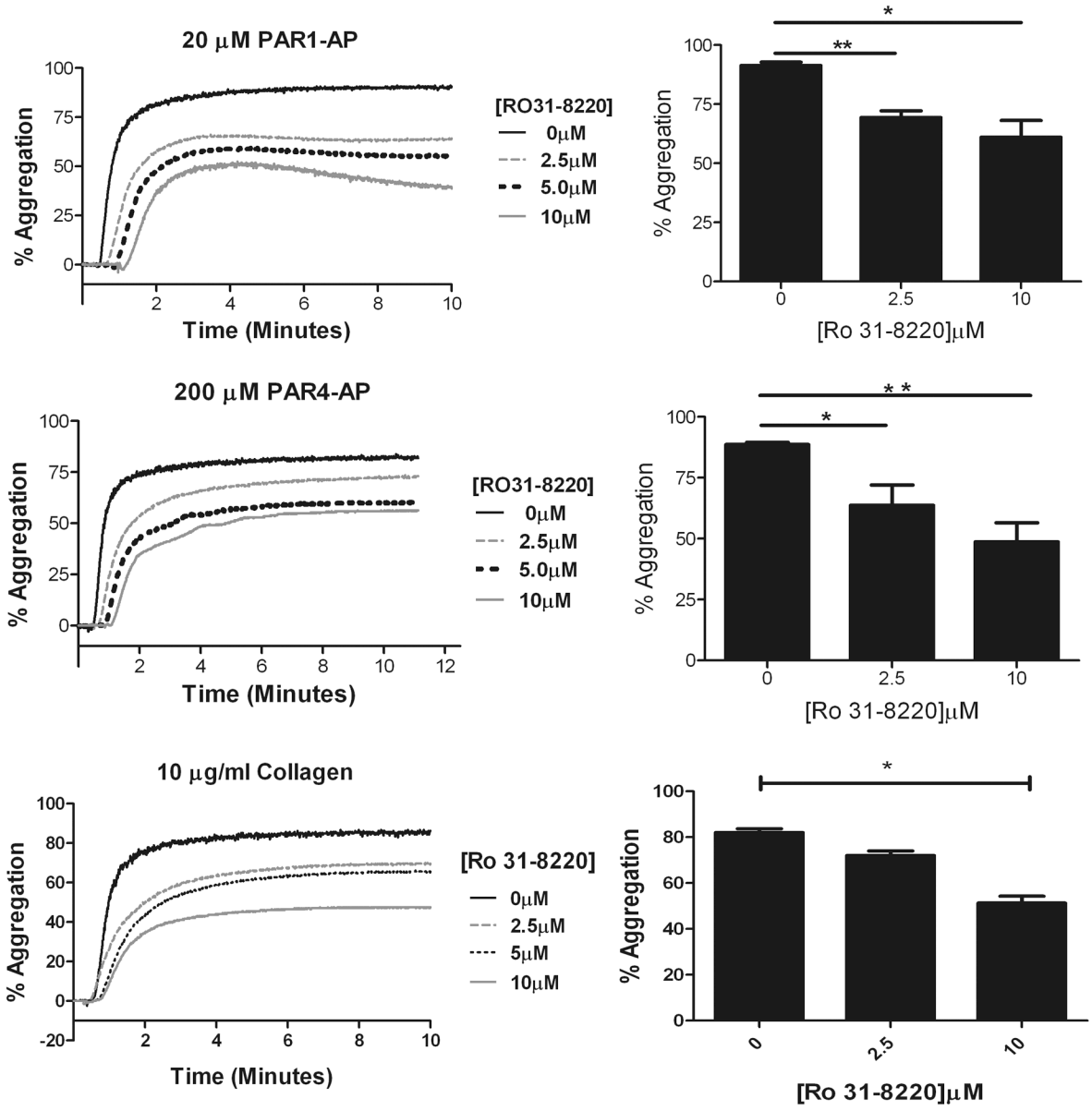


Figure 5.2 PKC regulation of platelet aggregation.
Platelets were pre-treated with increasing concentrations of pan-PKC inhibitor, Ro 31-8220 from 0 to 10 μM for 1 minute followed by stimulation with 20μM PAR1-AP, 200 μM PAR4-AP, or 10 μg/ml collagen and platelets aggregation measured. Pre-treatment with increasing concentration of Ro 31-8220 attenuated agonist-mediated platelet aggregation in a dose dependent manner. The bars represent mean±SEM (**P*<0.05; **P<0.001) for aggregation, n=3. This figure was provided by Dr. Patrick Apopa.

To identify if PKC regulates dense granule secretion, washed platelets were stimulated with 20 μM PAR1-AP, 200 μM PAR4-AP, 10 μg/ml collagen or the PKC activator, PMA (Figure 5.3).

ATP, which is secreted from the dense granule, was measured by luminescence in the absence or presence of increasing concentrations of Ro- 31 8220. As the concentration of the Ro 31-8220

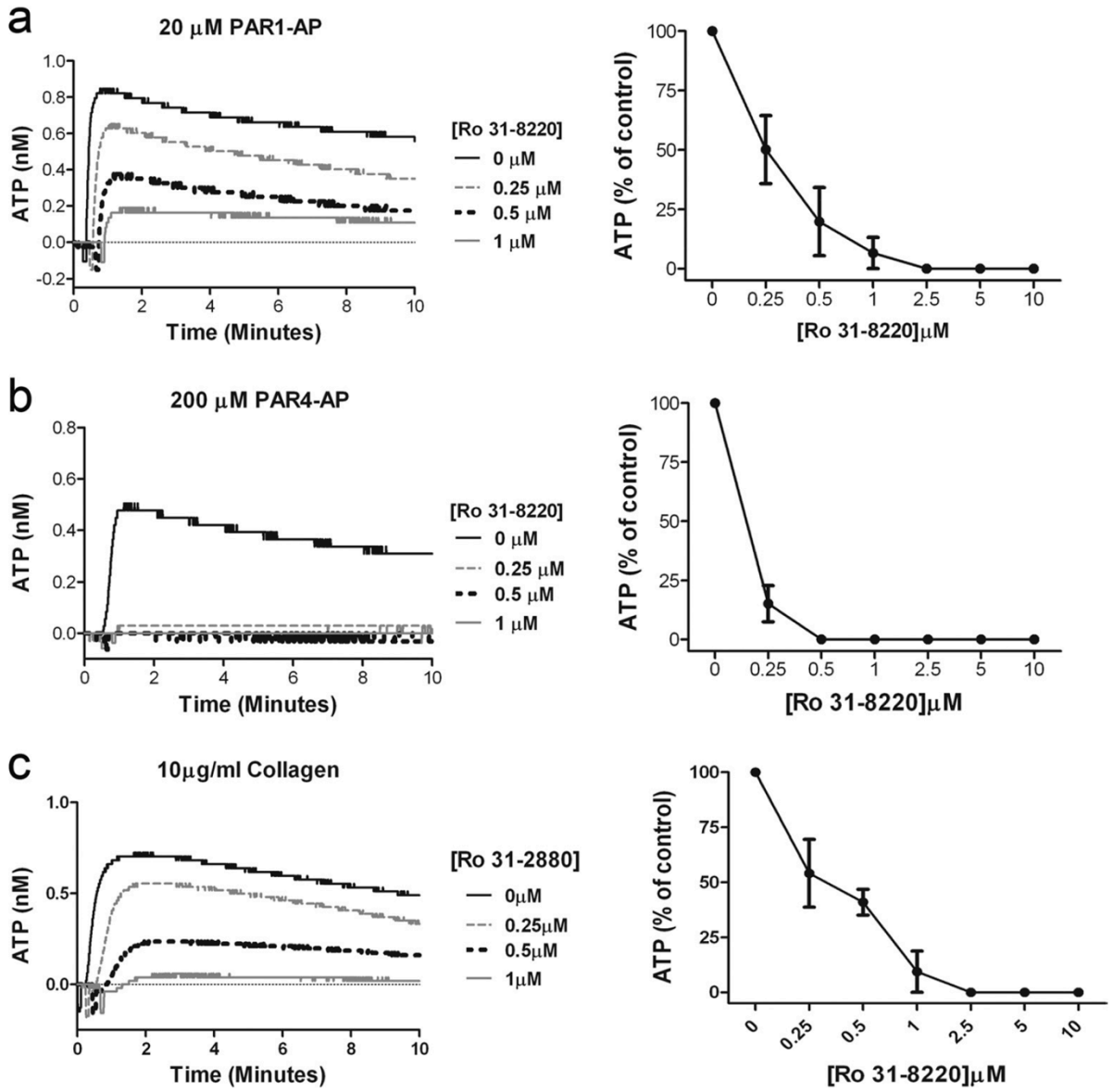


Figure 5.3 PKC is an important determinant of platelet ATP secretion.
Washed platelets were treated with or without increasing concentrations of pan-PKC inhibitor, Ro 31-8220 for 1 minute and platelet ATP secretion was measured following stimulation with (a) 20 μM PAR1-AP, (b) 200 μM PAR4-AP, or (c) 10 μg/ml collagen. The right panel graph represents normalized ATP secretion (n=3). This figure was provided by Dr. Patrick Apopa.

was increased, the level of ATP secreted from the dense granule was decreased with full inhibition observed at 1 μM Ro- 31 8220 (Figure 5.3a-c).

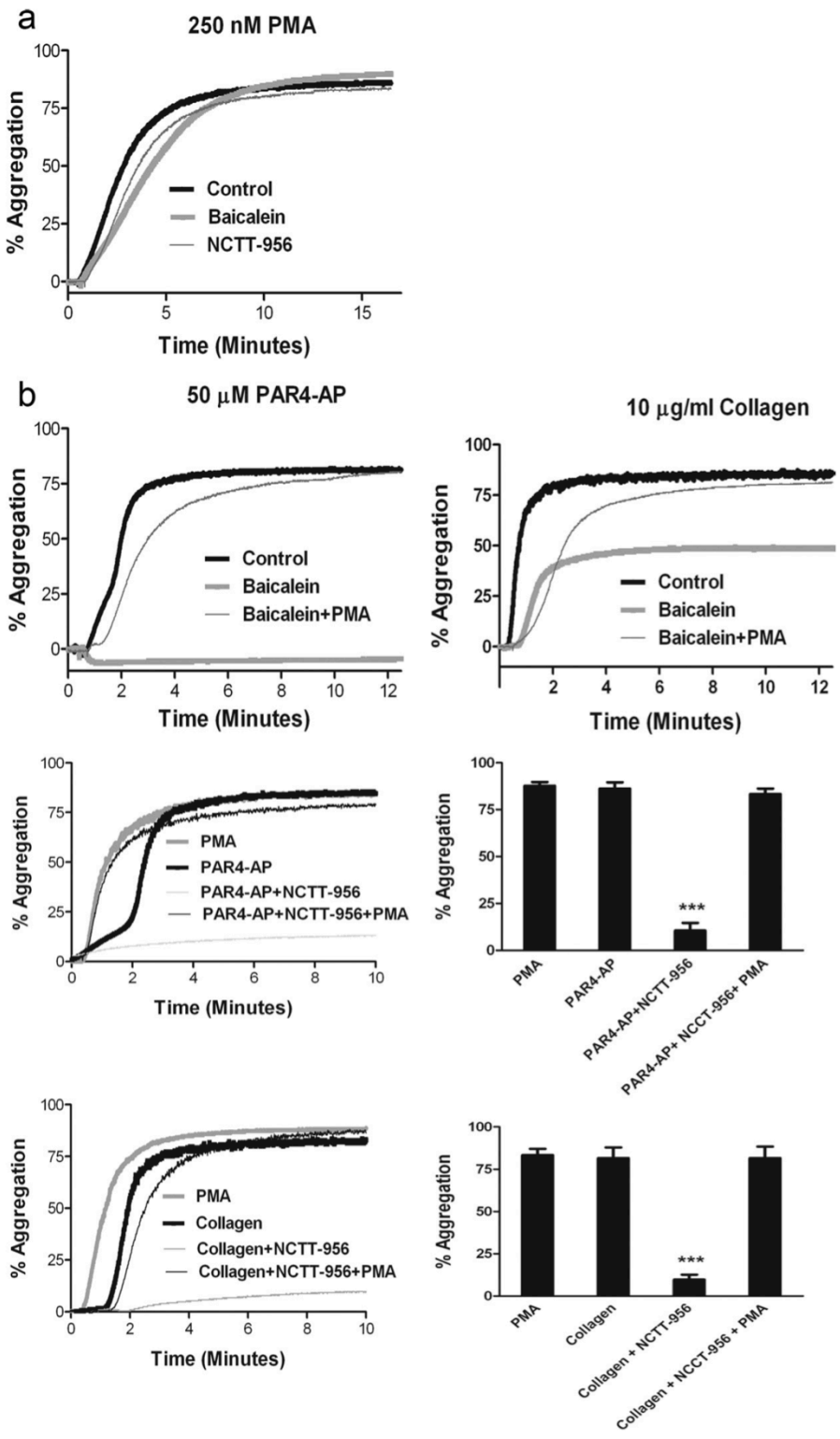


Figure 5.4 PKC activation rescues platelet aggregation downstream of 12-LOX.
a) Washed human platelets were treated with or without 25 μM NCTT-956 or 50 μM baicalein followed by 250 nM PMA stimulation. Platelet aggregation was then measured for 16 minutes. b) Washed platelets were treated with or without baicalein, followed by receptor agonist alone, 50 μM PAR4-AP (left panel) or 10 μg/ml collagen (right panel), or receptor agonist plus PMA and platelet aggregation measured for 12 minutes. c) Washed platelets were treated with or without, NCTT-956, followed

by stimulation with PMA, PAR4-AP, collagen, or a combination of PMA and PAR4-AP (or collagen) and platelet aggregation measured. (***P<0.001) for aggregation, n=3. This figure was co-provided by Jennifer Yeung and Dr. Patrick Apopa.

To determine if PKC activation can rescue platelet activation in the absence of 12-LOX activation, washed platelets were stimulated with the diacylglycerol mimetic (PMA) in the absence or presence of the 12-LOX inhibitors baicalein or NCTT-956 and platelet aggregation was measured for 15 minutes (Figure 5.4). Stimulation of washed platelets with 250 nM PMA resulted in full platelet aggregation which was sustained over time. PMA-mediated aggregation was not affected by either 12-LOX inhibitor, indicating that 12-LOX activation is not required for PMA-mediated platelet aggregation (Figure 5.4a).

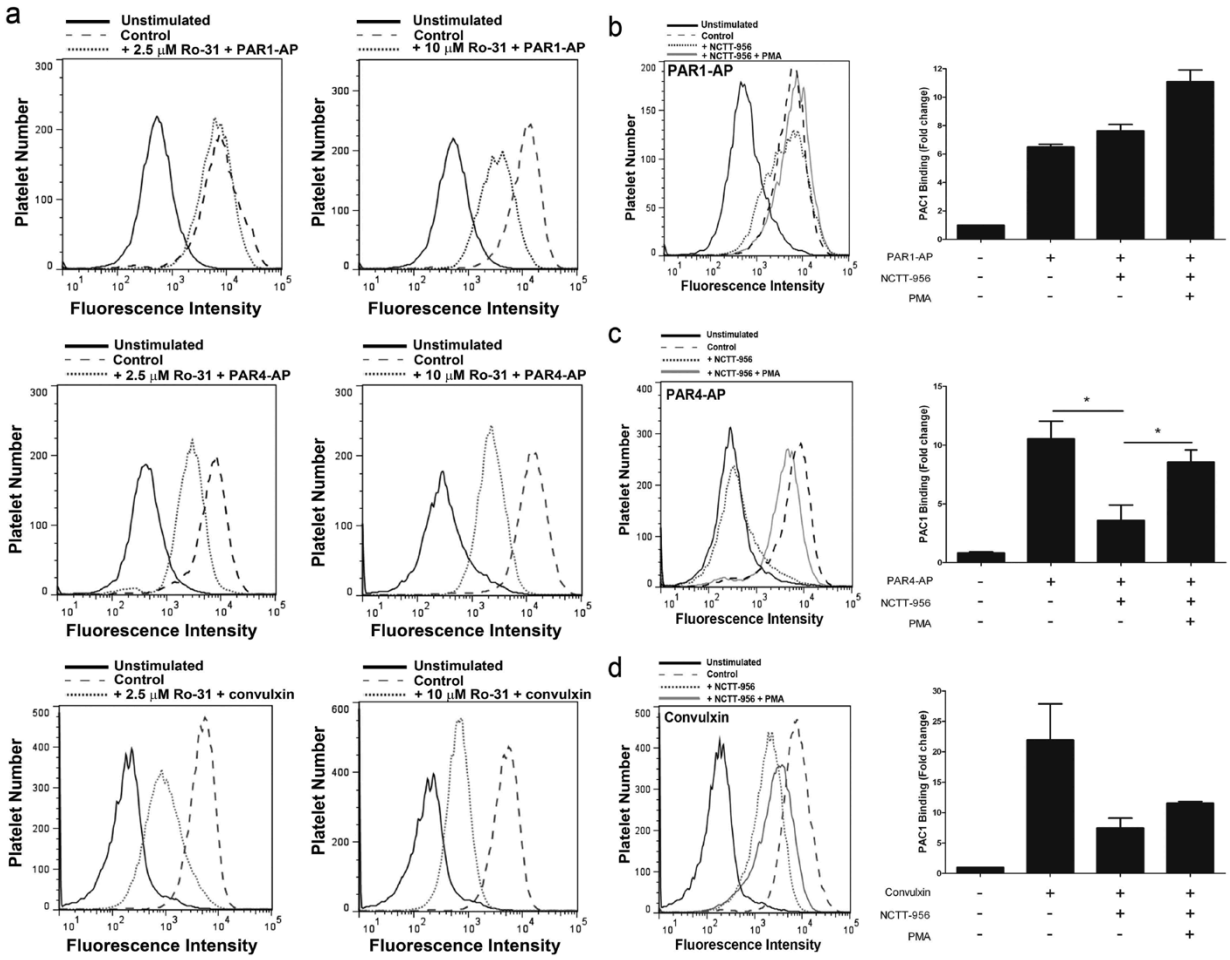


Figure 5.5 Role of PKC in PAR-mediated integrin αIIbβ3 activation.
a) Washed platelets were pre-incubated with PAC1 antibody and treated with 2.5 μM or 10 μM pan-PKC inhibitor, Ro 31-8220, for 1minute. αIIbβ3 activation by flow cytometry was measured following stimulation with 5 μM PAR1-AP, 25 μM PAR4-AP,

or 0.1 μg/ml convulxin for 10 minutes. The histograms shown are representative of three different experiments. b) Washed platelets were pre-incubated with PAC1 antibody and treated with the 25 μM NCTT-956 for 10 minutes followed by stimulation with PAR1-AP, c) PAR4-AP, or d) convulxin in the presence or absence of 1 μM PMA. αIIbβ3 activation was measured by flow cytometry with FITC-PAC1. The bars represent mean±SEM for fluorescence, n=3. *,P<0.05.

Although stimulation with PMA results in aggregation of washed platelets, we had previously shown that PMA does not induce calcium mobilization and neither thromboxane nor 12-hydroxyeicosatetraenoic acid were produced, giving evidence that PKC activation alone does not liberate arachidonic acid from the plasma membrane of the platelet [398]. To determine if PKC activation acts downstream of 12-LOX, we assessed whether PMA might rescue the aggregation defect observed in the presence of the 12-LOX inhibitors. Washed platelets were stimulated with either 50 μM PAR4-activating peptide (PAR4-AP) or 10 μg/ml collagen in the absence or presence of 12-LOX inhibitors (Figure 5.4b-c). Treatment with either baicalein or NCTT-956 significantly inhibited PAR4-AP and collagen-induced platelet aggregation. However, addition of PMA fully rescued agonist-mediated platelet aggregation in the presence of 12-LOX inhibitors suggesting that PKC activation may play a role in 12-LOX-mediated platelet aggregation through a number of signaling pathways including PAR4 and collagen.

Role of PKC in PAR-mediated integrin αIIbβ3 activation. The primary adhesive receptor mediating platelet aggregation is the integrin αIIbβ3. Since there is a direct correlation between activation levels of αIIbβ3 and platelet aggregation, the role of PKC in this pathway was investigated (Figure 5.5). Washed platelets were stimulated with PAR1-AP, PAR4-AP, or convulxin (a snake venom known to activate the collagen GPVI receptor) in the absence or presence of two concentrations of the PKC inhibitor and active αIIbβ3 was assessed. For all agonists, αIIbβ3 activation was partially blocked in the absence of PKC activity (Figure 5.5a). To confirm that PKC could rescue agonist-mediated αIIbβ3 activation in the absence of 12-LOX activity, platelets were pre-treated with or without 25 μM NCTT-956 and stimulated with PAR1-

AP, PAR4-AP, or convulxin in the presence or absence of PMA (Figures 5.5b-d). NCTT-956

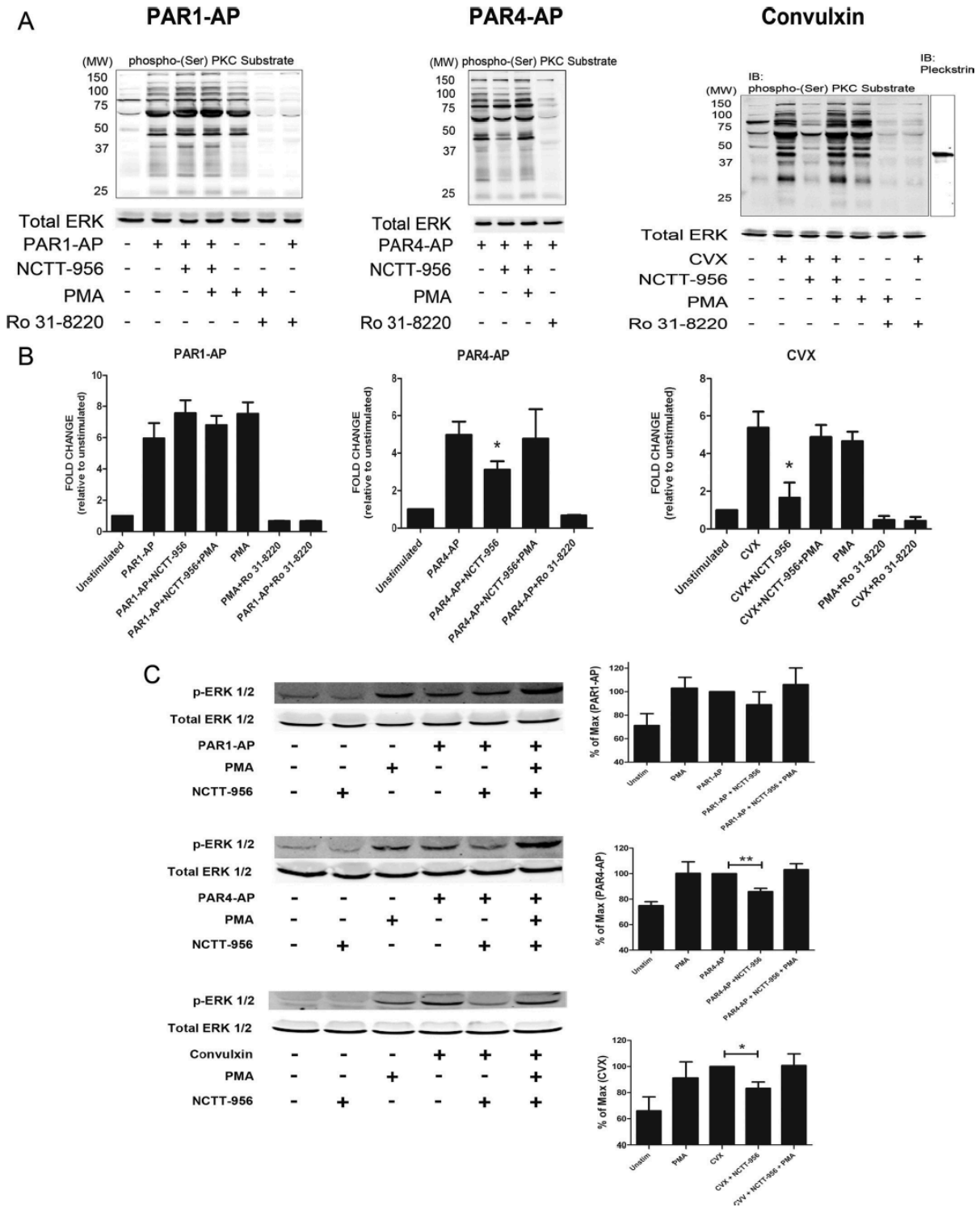


Figure 5.6 12-LOX regulates agonist-mediated ERK phosphorylation.
Washed Platelets were treated with 25 μM NCTT-956 for 10 minutes followed by stimulation with 5 μM PAR1-AP, 25 μM PAR4-AP, or 0.1 μg/ml convulxin in the presence or absence of 1 μM PMA for 3 minutes under stirring conditions. The

cytosolic fraction was assessed by western blot for phosphorylation of ERK (N=2). Total ERK antibody was used as a loading control. This figure was co-provided by Jennifer Yeung and Joanne Vesci.

inhibited more than 50% of PAR4-AP-mediated αIIbβ3 activation (P=0.02), and this inhibition was significantly overcome in the presence of PMA (P=0.04), further supporting a role for PKC following 12-LOX activation in regulating agonist-mediated platelet activation. By contrast, inhibition of 12-LOX by NCTT-956 did not affect PAR1-AP-induced αIIbβ3 activation either alone or in combination with PMA, suggesting another pathway of transduction through PAR1 requiring PKC activation but not 12-LOX (Figure 5.5a). Interestingly, the effect of NCTT-956 on αIIbβ3 activation in platelets stimulated with convulxin was not rescued by PMA (Figure 5.5d). These results clearly indicate a very complex agonist-dependent mechanism of regulation of integrin activation in platelets.

12-LOX regulation of p-ERK. To confirm that 12-LOX is an upstream regulator of PKC activation, washed platelets were stimulated with PAR1-AP or PAR4-AP in the absence or presence of NCTT-956 with or without PMA. Following platelet activation, ERK phosphorylation was measured, as ERK has been shown to be partially regulated by PKC in the platelet (Figure 5.6). Stimulation with PAR-AP, or PMA resulted in a significant phosphorylation of ERK. In the presence of NCTT-956, PAR4-AP-mediated ERK phosphorylation was significantly reduced and was partially rescued with the addition of PMA. Interestingly, PAR1-AP-mediated ERK phosphorylation was not significantly affected by treatment with NCTT-956. To determine if ERK phosphorylation was regulated downstream of 12-LOX solely through the PAR-4 pathway, platelets were treated with convulxin, the snake venom known to specifically activate the collagen receptor. Convulxin alone induced phosphorylation of ERK and this phosphorylation event was significantly attenuated in the presence of NCTT-956. However, similar to PAR4-AP, the presence of PMA fully rescued

NCTT-956-induced inhibition of convulxin-mediated ERK phosphorylation, supporting a proximal role for 12-LOX in regulating platelet activation upstream of PKC and ERK.

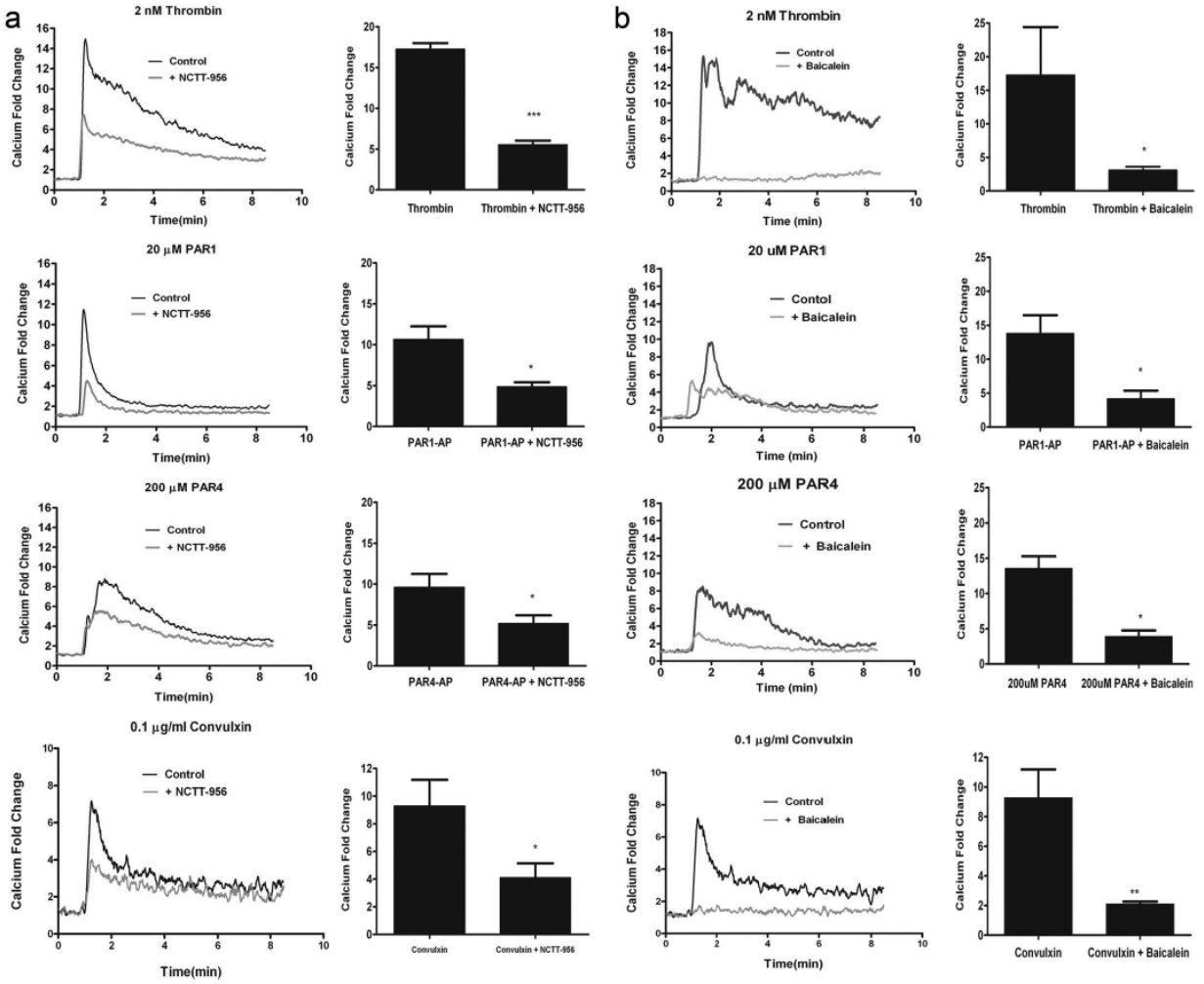


Figure 5.7 12-LOX inhibition attenuates calcium mobilization in human platelets.
Calcium mobilization was measured in re-calcified washed platelets in the presence or absence of 12-LOX inhibitors following stimulation with 2 nM thrombin, 20 μM PAR1-AP, 200 μM PAR4-AP, or 0.1 μg/ml convulxin. a) Platelets loaded with Fluo-4 AM for 10 minutes were incubated with NCTT-956 for an additional 10 minutes followed by stimulation with thrombin, PAR1-AP, PAR4-AP, or convulxin and calcium mobilization was monitored for 8 minutes post-stimulation. Representative curves on the left show the fold change in free calcium relative to the unstimulated condition over 8 minutes. The bar graphs on the right indicate the maximal increase in calcium mobilization (N=3). b) Platelets incubated with 50 μM baicalein were stimulated with thrombin, PAR1-AP, PAR4-AP, or convulxin and calcium mobilization was monitored for 8 minutes post-stimulation. Representative curves on the left show the fold change in free calcium relative to the unstimulated condition over 8 minutes. The bar graphs on the right indicate the maximal increase in calcium mobilization (N=3). Composite bar graphs are calculated as mean ± SEM; *, P<0.05; **, P<0.01; ***, P<0.001. This figure was co-provided by Jennifer Yeung and Joanne Vesci.

12-LOX inhibition attenuates calcium mobilization in human platelets. It has been reported that inhibition of 12-LOX attenuates calcium entry into platelets [196]. As calcium mobilization also plays a role in regulation of eicosanoid production and platelet activation, calcium levels were

monitored following stimulation with thrombin, PAR1-AP, PAR4-AP, or convulxin in the absence or presence of 12-LOX inhibitors (Figure 5.7). Agonist stimulation induced a significant and transient increase in free calcium in the platelet. In the presence of NCTT-956, free calcium in the platelet was significantly diminished following stimulation with thrombin, PARs, or convulxin (Figure 5.7a). Inhibition with baicalein more severely attenuated platelet mobilization compared to NCTT-956 which may be due to the higher level of selectivity toward 12-LOX exhibited with NCTT-956 relative to baicalein (Figure 7b) [395].

5.5 Discussion

Platelet reactivity plays a critical role in hemostasis and thrombosis. Much attention has been given to limiting unwanted platelet activation and vessel occlusion through inhibition of the ADP receptor ($P2Y_{12}$) and cyclooxygenase-1[399,400] . These therapies, while successful in decreasing the morbidity due to myocardial infarction and stroke [401,402], have significant shortcomings including genetic variability [403] and aspirin resistance [404].

Furthermore, all of these approaches result in a significant increase in bleeding which can be more deleterious than the clot itself. Therefore, alternative approaches with fewer side effects are warranted. Targeting 12-LOX, which metabolizes AA in a stereo-specific manner to generate 12(S)-HpETE, may be one such target [405]. 12-LOX and its metabolites have been shown to promote cancer progression and metastasis through a MAPK-dependent pathway [406,407]. Inhibition of 12-LOX in tumor cells was shown to induce apoptosis and was blocked by either over expression of 12-LOX or addition of 12-HETE [407,408]. In platelets however, the underlying signaling mechanisms regulating 12-LOX-mediated platelet reactivity have not been well characterized. Our data demonstrates that inhibition of 12-LOX significantly attenuates agonist-mediated platelet aggregation. This is in line with the pro-thrombotic actions attributed to

production of 12(S)-HETE in the platelet [329]. Furthermore, we showed for the first time that 12-LOX-mediated regulation of platelet activity is controlled, at least in part, through activation of PKC.

PKC has been shown to regulate a number of biochemical pathways in platelets, affecting platelet physiology by modulating aggregation and dense granule secretion [389,409]. While some studies have indicated that arachidonic acid may activate PKC directly through 12-HETE [410,411], others have proposed an indirect mechanism for eicosanoid regulation of PKC activation [412,413]. Our present work showed that inhibition of 12-LOX resulted in attenuation of platelet aggregation. Similarly, we found that PKC inhibition attenuated PAR-induced platelet aggregation. Therefore, we hypothesized that 12-LOX regulation of platelet reactivity may in some way be coupled to that of PKC. Through a number of approaches, PKC was determined to be downstream of 12-LOX activation, as activation with PMA was able to rescue 12-LOX-mediated inhibition of platelet aggregation, αIIbβ3 activation, and ERK phosphorylation. Together with our earlier work which showed PMA activation of platelet aggregation did not result in calcium mobilization or formation of 12-HETE [398], this data is suggestive of a signaling cascade in which PKC is downstream of 12-LOX activation.

Platelet aggregation requires activation of the integrin αIIbβ3. 12(S)-HETE has been linked to regulation of integrin activation in other cells [414] and we observed partial inhibition of αIIbβ3 activation in platelets in the absence of PKC activity. Similar attenuation of PAR4-induced integrin activation was observed in the absence of 12-LOX activity, attenuation that was also rescued by addition of PMA. These results support a role for 12-LOX-dependent PKC regulation of integrin activity following activation of platelets by PAR4-AP. Interestingly, we found that the integrin activity induced by PAR1 and by convulxin was not significantly affected

by the 12-LOX inhibitor. Together with our observation that the PKC inhibitor attenuates αIIbβ3 activation induced by these two agonists, our results indicate that, contrary to PAR4, activation of the integrin by PAR1 and GPVI agonists is mediated by a 12-LOX-independent, but PKC-dependent mechanism.

Although several MAPKs have been identified in platelets including ERK1/2, p38MAPK and JNK [415-417], their role in mediating platelet function downstream of 12-LOX activation is unclear. 12-HETE has been reported to induce ERK activation in human epidermal carcinoma cells and this activation could be inhibited by pertusis toxin suggesting the potential involvement of a G protein coupled receptor [407]. ERK has also been shown to be regulated following PKA activation [418,419]. Our data supports a role for 12-LOX regulation of ERK in human platelets as well, since inhibiting 12-LOX activation resulted in a partially attenuated activation of ERK by PAR4-AP or convulxin. The mechanism by which 12-LOX regulates ERK, whether it be through 12-HpETE, 12-HETE, or some other bioactive metabolite, is under current investigation and understanding its regulation will significantly aid our understanding of 12-LOX metabolite regulation of platelet function. The observation that attenuation of ERK phosphorylation in the absence of 12-LOX activation was partially rescued by PMA (Figure 5.6), lends strong support for PKC regulation of platelet function downstream of 12-LOX.

Several isoforms of PKC are activated by calcium in the human platelet [420,421] and previous reports have indicated that calcium may be partially regulated by 12-LOX [196]. However, this study was conducted with the less selective 12-LOX inhibitor, baicalein, which has been shown to inhibit a number of enzymes in addition to 12-LOX [243]. The current study is the first to show that baicalein inhibits a number of enzymes in the bioactive lipid pathways in the platelet including $cPLA_2$, COX-1, and perhaps thromboxane synthase, while NCTT-956 was shown not

to directly affect any of these off-target enzymes (Figure 5.1). To determine if calcium mobilization is specifically regulated by 12-LOX, platelets were treated with the highly selective 12-LOX inhibitor, NCTT-956, and agonist-induced calcium mobilization was measured (Figure 5.6). Calcium mobilization was significantly attenuated in the presence of NCTT-956 and baicalein, supporting the earlier reports attributing 12-LOX activation to this biochemical step [196]. Since calcium can activate PKC directly, these results give evidence for agonist-mediated activation of calcium downstream of 12-LOX activation and upstream of PKC. Interestingly, calcium mobilization was not completely inhibited by NCTT-956 following platelet activation by either PAR1 or PAR4-AP, suggesting a 12-LOX-independent component of calcium signaling. By contrast, after activation by convulxin, calcium mobilization is completely abrogated by the 12-LOX specific inhibitor. These data suggest a differential regulation of ERK phosphorylation that involves both 12-LOX-dependent and 12-LOX-independent mechanisms contingent upon the agonist used to activate the platelets.

Taken together, our results clearly show that 12-LOX plays an important role in platelet reactivity. This is the first report to show that 12-LOX activity occurs upstream of PKC and that integrin αIIbβ3 activation occurs downstream of both 12-LOX and PKC in human platelets. Importantly, this report also demonstrates the selectivity of 12-LOX sensitivity towards the PAR4 activation pathway. Finally, this study identifies PKC as an important biochemical intermediate in both 12-LOX-dependent and independent regulation of platelet activation. Future investigations will focus on identifying the feedback mechanisms by which 12-LOX regulates platelet function, presumably through an eicosanoid-dependent pathway, resulting in PKC-dependent activation of the human platelet.

CHAPTER 6

12-Lipoxygenase Activity Plays an Important Role in PAR4 and GPVI-mediated Platelet Reactivity[5]

6.1 Summary

Following initial platelet activation, arachidonic acid is metabolized by cyclooxygenase-1 and 12-lipoxygenase (12-LOX). While the role of 12-LOX in the platelet is not well defined, recent evidence suggests that it may be important for regulation of platelet activity and is agonist-specific in the manner in which it regulates platelet function. Using small molecule inhibitors selective for 12-LOX and 12-LOX-deficient mice, the role of 12-LOX in regulation of human platelet activation and thrombosis was investigated. Pharmacologically inhibiting 12-LOX resulted in attenuation of platelet aggregation, selective inhibition of dense versus alpha granule secretion, and inhibition of platelet adhesion under flow for PAR4 and collagen. Additionally, 12-LOX-deficient mice showed attenuated integrin activity to PAR4-AP and convulxin compared to wild-type mice. Finally, platelet activation by PARs was shown to be differentially dependent on COX-1 and 12-LOX with PAR1 relying on COX-1 oxidation of arachidonic acid while PAR4 being more dependent on 12-LOX for normal platelet function. These studies demonstrate an important role for 12-LOX in regulating platelet activation and thrombosis. Furthermore, the data presented here provide a basis for potentially targeting 12-LOX as a means to attenuate unwanted platelet activation and clot formation.

[5] This section has been published in Thrombosis and Hemostasis. Yeung J, Apopa PL, Vesci J, Stolla M, Rai G, Simeonov A, Jadhav A, Fernandez-Perez P, Maloney DJ, Boutaud O, Holman TR. 12-lipoxygenase activity plays an important role in PAR4 and GPVI-mediated platelet reactivity. Thrombosis and haemostasis. 2013 Sep;110(3):569.

6.2 Introduction

Platelet activation plays a crucial role in hemostasis and thrombosis [422,423] and a central role in the pathophysiology of cardiovascular disorders such as acute coronary syndrome [424-426]. Activation of human platelets is initiated through primary signaling by thrombin or collagen and reinforced by secondary signaling events initiated by arachidonic acid (AA) release from the phospholipids, formation of bioactive metabolites, and granule secretion. The clinical importance of this positive feedback to platelet activation is evident by the widespread use of aspirin, which specifically blocks AA metabolism to thromboxane A_2 [427]. An equally important metabolic pathway which utilizes AA as its substrate is platelet-type 12-Lipoxygenase (12-LOX), resulting in the formation of 12-(*S*)-hydroperoxyeicosatetraenoic acid (12-HpETE) [428,429]. 12-LOX is primarily expressed in the platelet and megakaryocyte and its metabolic products have been shown to play a role in regulating vascular and non-vascular processes such as integrin activation, vascular hypertension, and progression of certain types of cancer [196,315,322,405,430-432]. While we have recently shown an important role for 12-LOX in platelet activation [326], the extent to which 12-LOX activation directly regulates platelet activity and its agonist-specific role in regulating thrombosis is unclear. We therefore sought to determine if pharmacological inhibition or genetic ablation of 12-LOX activity plays a role in PAR and collagen-mediated platelet activation and clot formation.

12-LOX utilizes AA as a substrate to form 12-(*S)*-hydroperoxyeicosatetraenoic acid (12-HpETE) [428] which is further metabolized by glutathione peroxidase to form 12-HETE [433]. Once formed, the majority of 12-HETE is released to the extracellular space and is postulated to signal through a number of potential mechanisms including GPCRs [330,434], cytosolic signaling complexes, or alternatively incorporation into the plasma membrane [412,435,436]. The biological

function of 12-LOX activation in platelets has been complicated by the observation that its metabolites are reported to mediate both an increase as well as decrease in platelet reactivity [196,320,326,335,437,438]. In support of 12-LOX as a mediator of platelet activation, 12-LOX activity has been linked to alpha granule secretion [430] and surface integrin expression and activation in both the platelet and tumor cells [326,414,439,440]. Additionally, although 12-LOX has been postulated to play a role in dense granule secretion in platelets [320], specific inhibitors which would enable direct investigation of 12-LOX regulation of platelet reactivity, adhesion, and clot formation have been lacking [250,423,441]. The recent development, however, of selective platelet 12-LOX inhibitors has allowed for a more detailed investigation of how 12-LOX selectively regulates platelet reactivity, clot formation, and dense granule secretion in the human platelet [326,395,441]. While these inhibitors selectively block 12-LOX activity in human platelets while not affecting activity of other enzymes in this pathway (5-LOX, 15-LOX, COX-1, thromboxane synthase, $cPLA_2$, or PKC) [326,442], their effectiveness in inhibiting the 12-LOX homolog expressed in murine platelets (12/15-LOX) is unclear limiting the use of these selective inhibitors in the murine system in vivo.

Here we show that inhibition of 12-LOX activity inhibits platelet aggregation, which is coupled to a significant shift in the ability of the platelet to induce normal integrin activation, dense granule secretion, and platelet adhesion under venous shear. Additionally, αIIbβ3 activation in the 12-LOX$^{-/-}$ mouse is attenuated providing strong evidence for the involvement of 12-LOX in formation and long-term stability of the platelet clot. Finally, we show that while COX-1, but not 12-LOX activation, is essential for normal platelet activation through PAR1 [398], 12-LOX activation plays an important role in normal platelet function through PAR4 [326]. Thus, these studies provide the first reported evidence supporting an important physiological role for

12-LOX in adhesion of platelets under shear which is coupled to platelet aggregation, integrin activation, and secretion and support further investigation of 12-LOX as a potential target for regulation of platelet activation and uncontrolled thrombus formation.

6.3 Materials and Methods

Materials: 12-LOX compounds (NCTT-956 and -694) were synthesized at the NIH Chemical Genomics Center (Rockville, MD). For a list of all materials, see supplemental methods.

Human platelets: Human platelets were obtained from healthy volunteers from within the Thomas Jefferson University community and the Philadelphia area. These studies were approved by the Thomas Jefferson University Institutional Review Board and informed consent was obtained from all donors before blood draw. Experiments were performed on both whole blood and washed platelets as previously described [443,444]. Unless otherwise noted, all experiments were conducted with a platelet concentration of 3 X 10^8 platelets/ml.

Mice: 12-LOX$^{-/-}$ mice and strain-matched controls (B6129SF2/J) were obtained from Jackson Labs. Experimental procedures were approved by the Animal Care and Use Committee of Thomas Jefferson University.

Platelet Aggregation and dense granule secretion: Washed platelets were pretreated with inhibitors for 10 min. The aggregation response to agonist was measured using a lumi-aggregometer (Model 700D, Chronolog Corp) with stirring at 1100 rpm at 37°C. ATP luminescence was used to detect dense granule secretion. Aggregation in whole-blood was measured with a lumi-aggregometer following manufacturers recommendations.

Platelet adhesion under flow: *In vitro* platelet adhesion under venous and arterial shear rates was performed in a microfluidic apparatus [445]. Whole blood was incubated with NCTT-956 or NCTT-694 for 15 minutes followed by incubation with calcein green fluorophore and infused at

venous (400^{-S}) or arterial (2000^{-S}) wall shear rates for 5 minutes. Adhesion of platelets was monitored continuously with a Nikon Ti-U inverted microscope equipped with a Retiga EXL monochrome camera. Images were analyzed using Nikon NIS Elements software.

Flow Cytometry: P-selectin expression and integrin αIIbβ3 activation on the surface of the platelet were measured by flow cytometry (Accuri C6) as a marker of α-granule secretion and integrin activation, respectively [326,333].

Tail-bleeding assay: Tail bleeding of $ALOX12^{-/-}$ and wild-type mice was performed on an anesthetized mouse as previously described [446]. Time in seconds required for cessation of blood flow was recorded.

Statistical Analysis: Comparison between experimental groups was made using appropriate statistical analyses (paired *t* test program or ANOVA with post-test analysis) using Prism software. Differences in mean values (measured as standard error of the mean) were considered significant at $p < 0.05$.

6.4 Results

12-LOX inhibition attenuates platelet aggregation

Platelet aggregation is essential for agonist-mediated clot formation. The role of 12-LOX in agonist-induced platelet aggregation was examined by measuring the dose-response for PAR1-AP, PAR4-AP, collagen, and ADP-mediated aggregation in the absence or presence of NCTT 956 or baicalein (12-LOX inhibitors).

In the absence of 12-LOX inhibitors, all agonists were able to induce full and stable aggregation. Interestingly, in contrast to PAR1 sensitivity to COX-1 for normal aggregation [398], PAR1-mediated platelet aggregation was insensitive to 12-LOX inhibition. A significant shift in the dose-response for aggregation was observed with all other agonists tested. ADP, which normally

induces full aggregation at 5 μM, was not able to overcome the inhibition of 12-LOX and full aggregation was not reached even at an ADP concentration of 40 μM. These data support an important role for 12-LOX in regulating agonist-induced platelet aggregation through a number of receptor pathways.

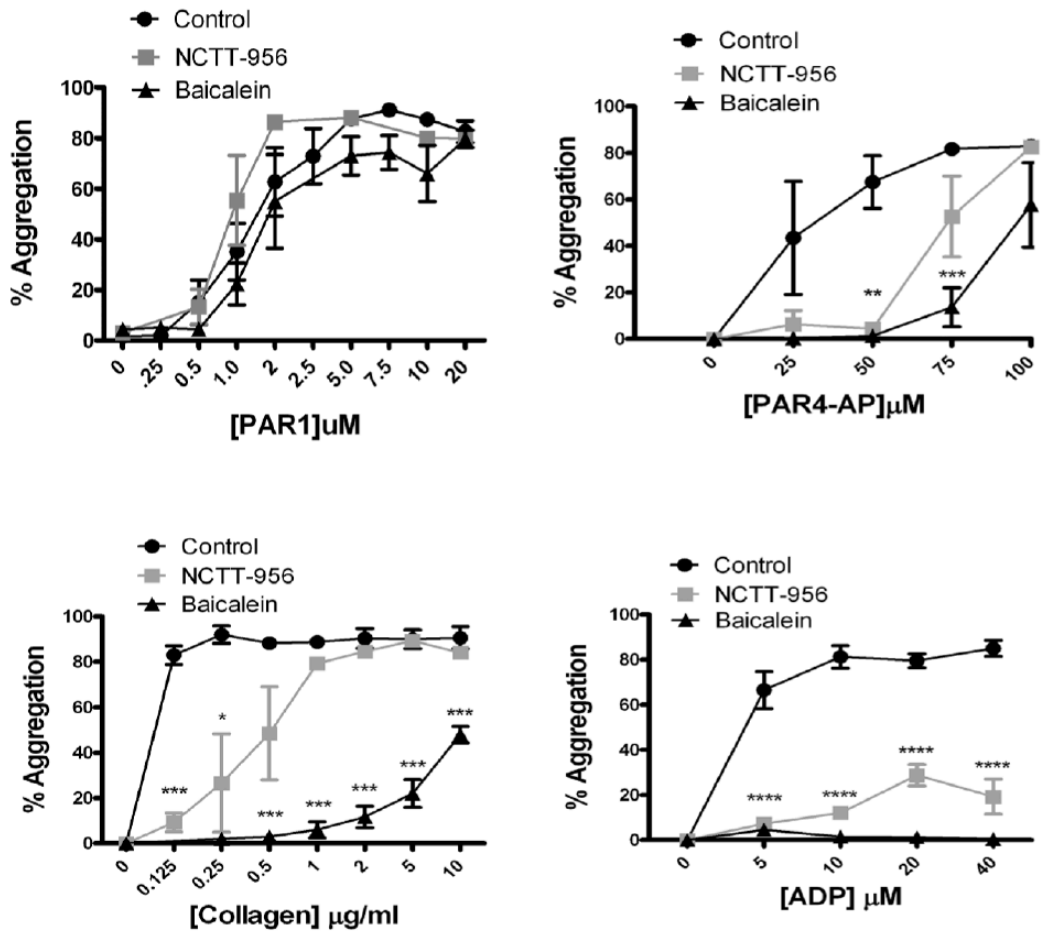
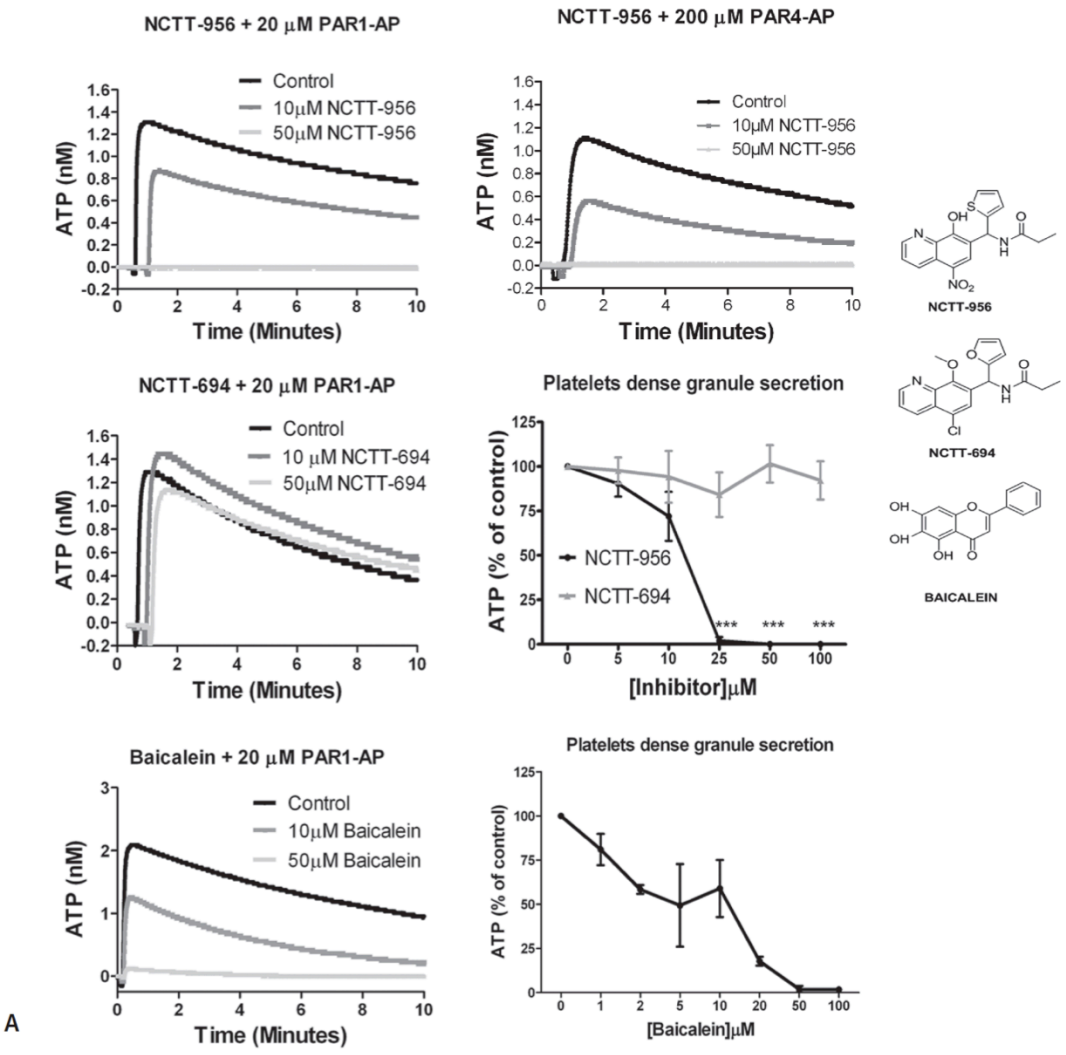


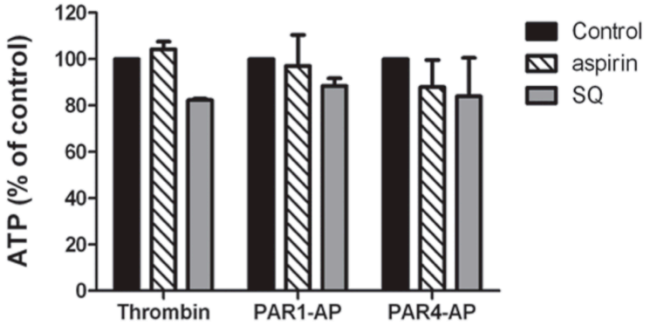
Figure 6.1 12-LOX is an important determinant of platelet aggregation.
Washed platelets were treated with or without 50 μM baicalein or 25 μM NCTT-956 and platelet aggregation was measured following stimulation with increasing concentrations of collagen, ADP, PAR4-AP, or U46619. All agonists tested showed a shift to the right in the EC_{50} for platelet aggregation (N=3). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. This figure was co-provided by Jennifer Yeung, Joanne Vesci and Dr. Patrick Apopa

12-LOX regulation of dense granule secretion. As granule secretion plays an important role in the regulation of platelet clot growth and stability, secretion of dense granules was measured in platelets treated with or without 12-LOX inhibitors (Figure 6.2). Treatment with the active 12-LOX inhibitor (NCTT-956) resulted in complete inhibition of PAR1-AP and PAR4-AP-induced

ATP secretion, while treatment with the inactive analog (NCTT-694) had no effect on dense granule secretion at concentrations as high as 100 μM NCTT-694 (Figure 6.2A). The effects of NCTT-956 were also compared to the 12-LOX inhibitor baicalein. Similar to NCTT-956, baicalein induced a dose-dependent inhibition of PAR1-AP-mediated dense granule secretion. To ascertain that the analogs did not interfere directly with the luciferase-based assay of ATP release, control reactions were performed where a known concentration of ATP was measured in presence of analogs or vehicle. The results showed that neither NCTT-956 nor NCTT-694 inhibit the luciferase assay (Supplemental Figure 1). This was a surprising result based on the observation in figure 6.1 that inhibition of 12-LOX attenuated PAR4, but not PAR1-mediated platelet aggregation. However, this is in line with the observation that PAR4 is more sensitive to positive feedback through secretion and ADP compared to PAR1 [444].


NCTT-956 + 20 μM PAR1-AP
Control
10μM NCTT-956
50μM NCTT-956
ATP (nM)
Time (Minutes)
NCTT-956 + 200 μM PAR4-AP
Control
10μM NCTT-956
50μM NCTT-956
ATP (nM)
Time (Minutes)
NCTT-956
NCTT-694 + 20 μM PAR1-AP
Control
10 μM NCTT-694
50μM NCTT-694
ATP (nM)
Time (Minutes)
Platelets dense granule secretion
ATP (% of control)
NCTT-956
NCTT-694
***
[Inhibitor]μM
NCTT-694
BAICALEIN
Baicalein + 20 μM PAR1-AP
Control
10μM Baicalein
50μM Baicalein
ATP (nM)
Time (Minutes)
Platelets dense granule secretion
ATP (% of control)
[Baicalein]μM

A


ATP (% of control)
Control
aspirin
SQ
Thrombin
PAR1-AP
PAR4-AP

B

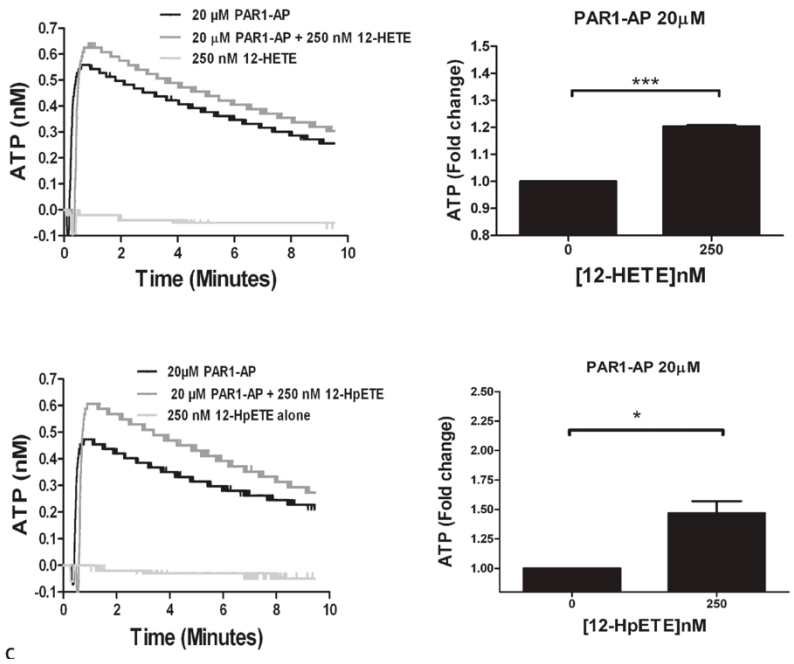


Figure 6.2 12-LOX activation is required for dense granule secretion in platelets.
Washed platelets were treated with active or inactive 12-LOX inhibitors and the level of dense granule secretion was monitored by measuring ATP release following stimulation with different agonists. A) ATP release induced by 20 μM PAR1-AP with increasing concentration of NCTT-956 (N=5) or NCTT-694 (N=3). Composite analysis of dose-response for dense granule secretion with PAR1-AP in the presence of increasing concentrations of NCTT-956 (N=5) and NCTT-694 (N=3). ***, P<0.001. C) Representative and composite for dense granule secretion following PAR1-AP in the presence of increasing concentrations of baicalein (N=3). Structure of NCTT-956, NCTT-694, and baicalein are shown for comparison. B) Composite analysis of ATP secretion in the presence of the 100 μM aspirin or 100 nM SQ29548 followed by stimulation with 10 nM thrombin, 20 μM PAR1-AP, or 200 μM PAR4-AP for 10 minutes. C) Dense granule secretion was measured in absence or presence of 12-LOX metabolites to determine their role in the regulation of agonist-mediated secretion. Washed platelets were treated with either 250 nM 12(*S*)-HETE (N=3) or 12(*S*)-HpETE (N=3). Dense granule secretion was measured following activation with PAR1-AP. ***, P<0.001; *, P<0.05. This figure was co- provided by Dr. Patrick Apopa and Joanne Vesci

To determine if 12-LOX regulation of dense granule secretion was agonist specific, the same experiments were repeated with a number of agonists known to induce dense granule secretion (Supplemental Figure 2). Inhibition of 12-LOX completely eliminated dense granule secretion induced by thrombin, PAR4-AP, collagen, U46619 (an agonist for the TPα receptor), and ADP, giving strong evidence that 12-LOX activation is important to platelet dense granule secretion. To determine if inhibition of dense granule secretion was due to an inhibition of 12-LOX signaling or a potential indirect effect on thromboxane signaling, platelets were treated without or with 100 μM aspirin (COX-1 inhibitor) or 100 nM SQ29548 (TPα inhibitor) followed by stimulation with 10 nM thrombin, 20 μM PAR1-AP, or 200 μM PAR4-AP. Neither inhibition

of COX-1 nor TPα resulted in a significant change in agonist-mediated dense granule secretion (Figure 6.2B).

Role of 12-HETE in dense granule release. To determine if 12-HETE, or its precursor, 12-HpETE, plays a role in regulation of agonist-induced secretion, dense granule secretion was measured following PAR1-AP stimulation either in the presence or absence of exogenous metabolite (250 nM 12-HETE or 12-HpETE). Exogenous 12-HETE and 12-HpETE significantly increased PAR1-AP-mediated dense granule secretion by 20% compared to agonist alone (Figure 6.2C) while metabolite alone did not induce dense granule secretion. These data are in agreement with other published work implicating 12-HETE as a potential modulator of platelet function [320] and our current observation that absence of 12-LOX activity and 12-HETE results in inhibition of agonist-mediated dense granule secretion. Exogenously added 12-HpETE resulted in an even larger increase in PAR1-AP-induced dense granule secretion of 60% compared to control suggesting a potential role for other 12-LOX metabolites in modulating agonist-mediated platelet activation.

12-Lipoxygenase does not regulate α-granule secretion. Secretion of α-granules from human platelets is induced by both PAR1 and PAR4 [443,447]. To assess if 12-LOX specifically regulates dense granule secretion or both dense and α-granule secretion, platelets were treated with or without NCTT-956, NCTT-694, or baicalein for 10 minutes followed by stimulation with thrombin, PAR1-AP, or PAR4-AP. α-granule secretion was determined by measuring P-selectin levels on the platelet surface by flow cytometry (Figure 6.3). α-granule secretion was not significantly attenuated following 12-LOX inhibition in the presence of all three agonists. These

data suggest that α-granule secretion is not regulated via the 12-LOX pathway.

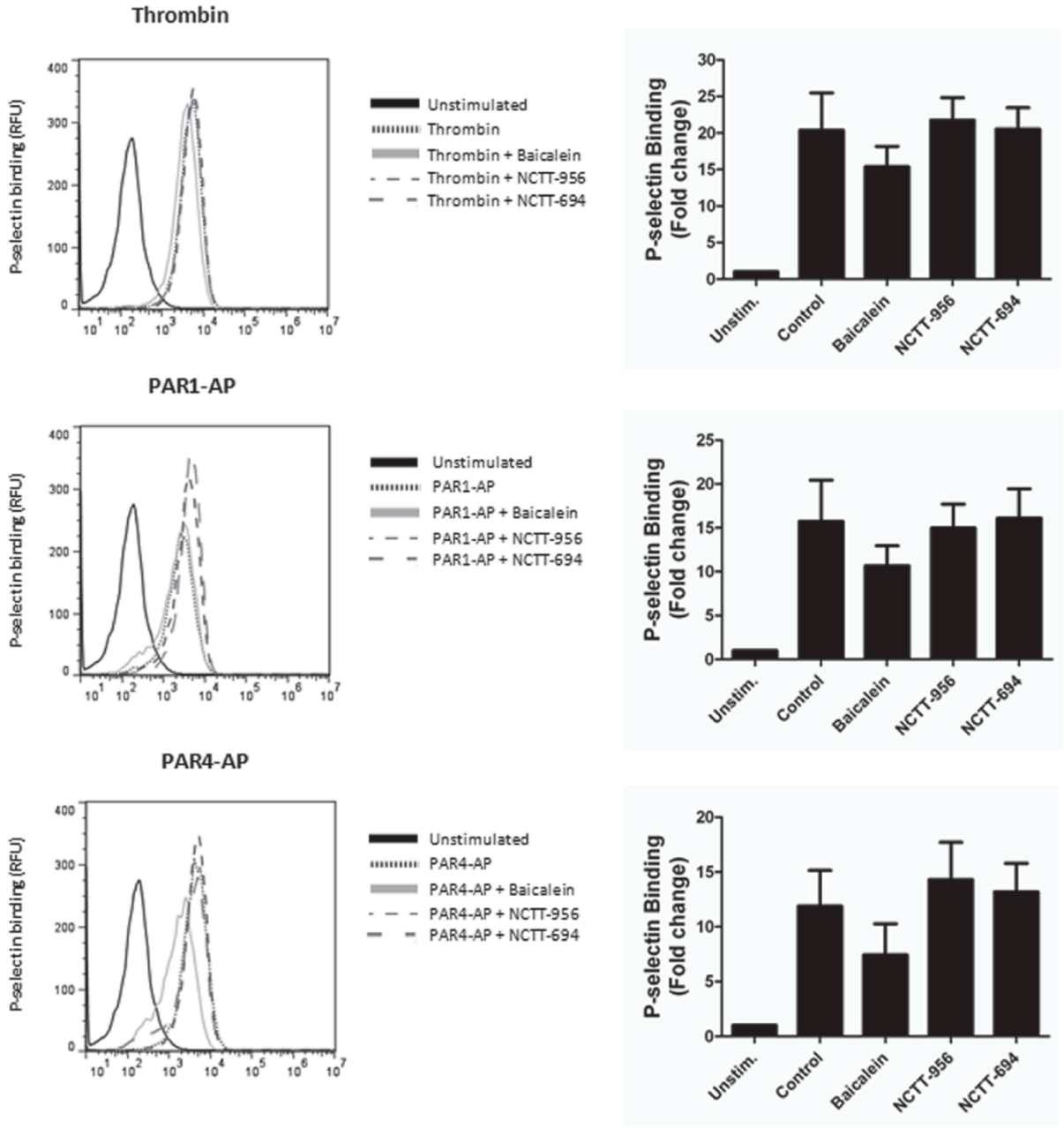
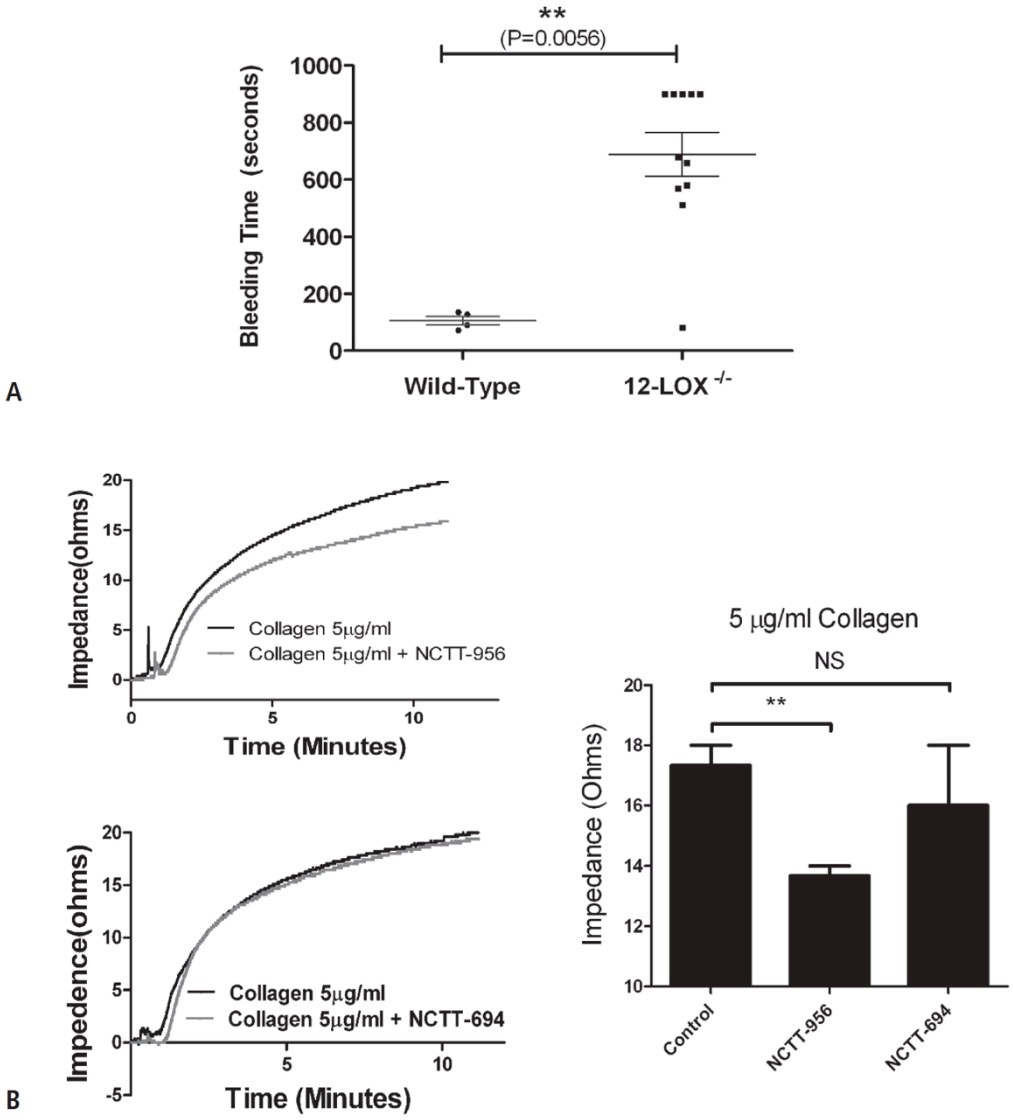


Figure 6.3 12-LOX is not critical for agonist-mediated α-granule secretion.
α-granule secretion was determined following stimulation of platelets with 10 nM thrombin, 20 μM PAR1-AP, or 200 μM PAR4-AP in the presence or absence of active and inactive 12-LOX inhibitors (N=4). P-selectin expression following treatment with 25 μM NCTT-956, 50 μM baicalein, or 25 μM NCTT-694 was not significantly different from control.

Regulation of platelet function in vivo by 12-LOX. To determine if 12-LOX plays a role in platelet function in vivo, bleeding time was assessed in 12-LOX$^{-/-}$ and strain-matched wild-type mice using the tail-bleeding assay [446] (Figure 6.4A). While wild-type mice showed a normal cessation time for bleeding following clipping of the tail vein (N=4), the time to cessation of bleeding in 12-LOX$^{-/-}$ mice was significantly prolonged (N=11).



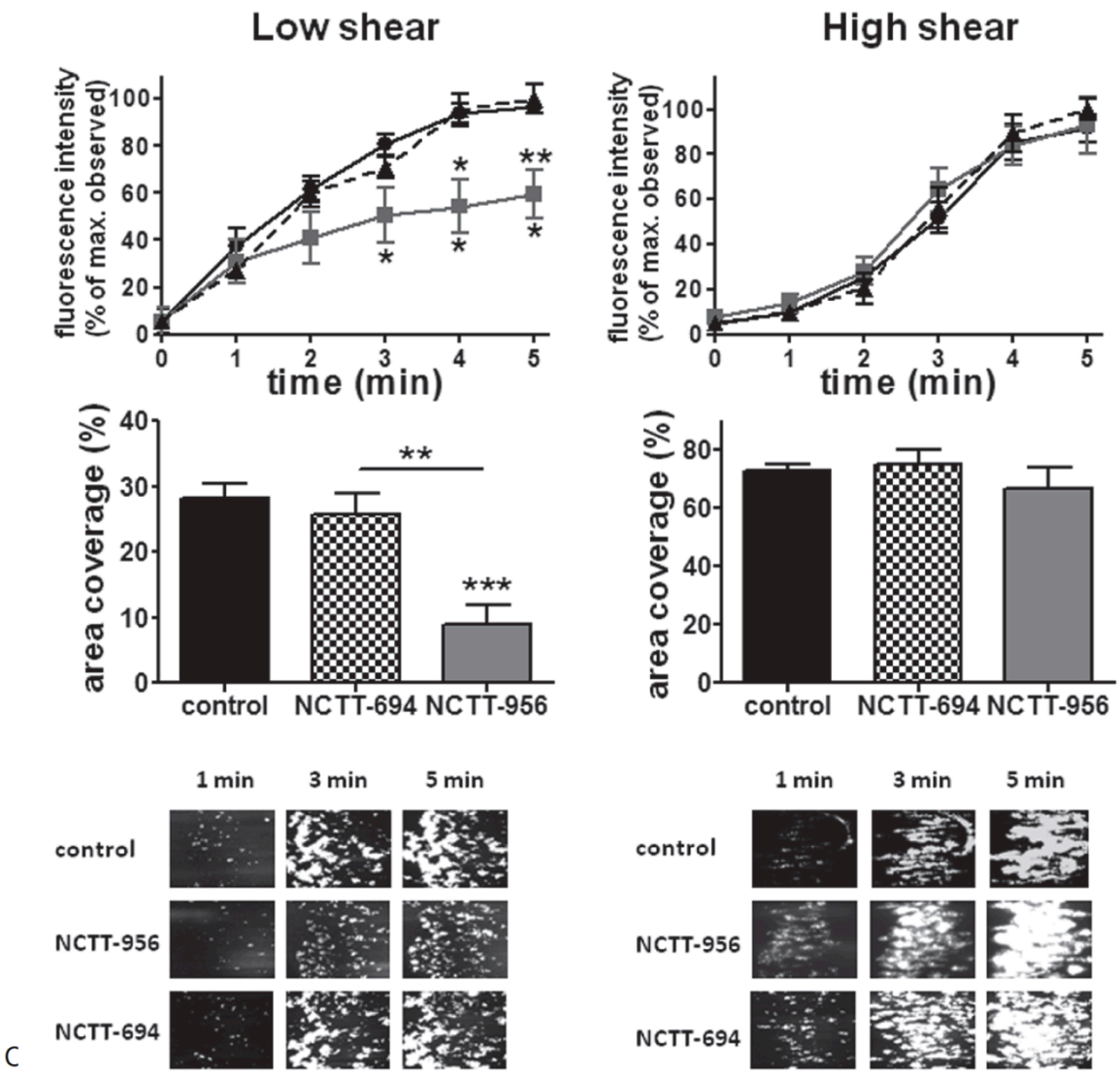


Figure 6.4 Inhibition of 12-LOX in whole blood attenuates platelet aggregation and adhesion.
Platelet aggregation and clot formation was measured in whole blood to determine the role of 12-LOX in regulating ex vivo platelet activation in the presence of confounding factors present in the blood. A) Whole blood aggregometry was performed in the absence or presence of the 12-LOX inhibitor, NCTT-956 or the negative analog, NCTT-694. Blood was treated with or without 100 μM NCTT-956 or -694, stimulated with 5 μg/ml collagen, and impedance aggregometry was measured for 10 minutes post-stimulation (N=3). A significant decrease in impedance was observed in the presence of NCTT-956 but not NCTT-694 (**, $P<0.01$; NS, not significant). B) Platelet clot formation over a collagen strip under low or high shear conditions (400 sec^{-1} or 2000 sec^{-1}, respectively) was measured either with (□) or without (•) 100 μM NCTT-956 or NCTT-694 (Δ) (N=4). The rate of clot formation was significantly delayed and attenuated following inhibition of 12-LOX compared to control. This figure was co-provided by Moritz Stolla, Jennifer Yeung, and Joanne Vesci

Regulation of whole blood aggregation and adhesion by 12-LOX. Although aggregation in washed platelets shows sensitivity to 12-LOX activation (Figure 6.1), its role in whole blood has not been well defined. To determine if platelet aggregation is altered in whole blood, freshly drawn human blood was treated with or without NCTT-956 or NCTT-694 for 10 minutes

followed by stimulation with collagen (the first step in platelet activation following vessel injury). Using impedance aggregometry a significant decrease in platelet aggregation was observed in the presence of the 12-LOX inhibitor compared to untreated platelets or platelets treated with the negative analog (Figure 6.4B). To determine if 12-LOX plays a role in platelet adhesion and clot formation under flow, whole blood treated with or without NCTT-956 or NCTT-694 was flowed over a collagen strip at low and high shear rates (400 and 2000 sec^{-1}) (Figure 6.4C). Although untreated blood showed significant clot growth within one to three minutes, inhibition of 12-LOX at low shear resulted in a significant delay in platelet adhesion as well as attenuation of the overall area and intensity of adhesion to collagen. Blood treated with the inactive analog NCTT-694 however, exhibited no delay in adhesion or clot growth. At high shear, 12-LOX activation did not appear to be essential as there was no observable difference in area coverage or clot formation at any time point measured. These data provide strong evidence that platelet reactivity and haemostasis rely in part on the enzymatic activity of platelet 12-LOX and supports a potential role for 12-LOX in the recruitment and activation of platelets into a growing clot.

12-LOX activity is important for haemostasis and thrombosis in the mouse. While murine 12-LOX is highly homologous to human, its enzymatic activity is less selective and is known to form at least two products from AA, 12-HETE and 15-HETE. Due to its altered enzymatic activity, the 12-LOX inhibitors directed toward the human enzyme do not bind with high efficacy to mouse 12-LOX. To determine if the observed 12-LOX effects identified in human platelets with the 12-LOX inhibitor are due to inhibition of 12-LOX activity, platelets from 12-$LOX^{-/-}$ and strain-matched wild-type mice were assessed for αIIbβ3 activity. Washed platelets were stimulated with either PAR4-AP or convulxin (Figure 6.5) and the level of αIIbβ3 was

measured. Mice deficient in 12-LOX showed a significant attenuation of αIIbβ3 activation for both agonists compared to wild-type mice. This data confirms an important role for 12-LOX in regulating platelet activation, thrombosis, and haemostasis.

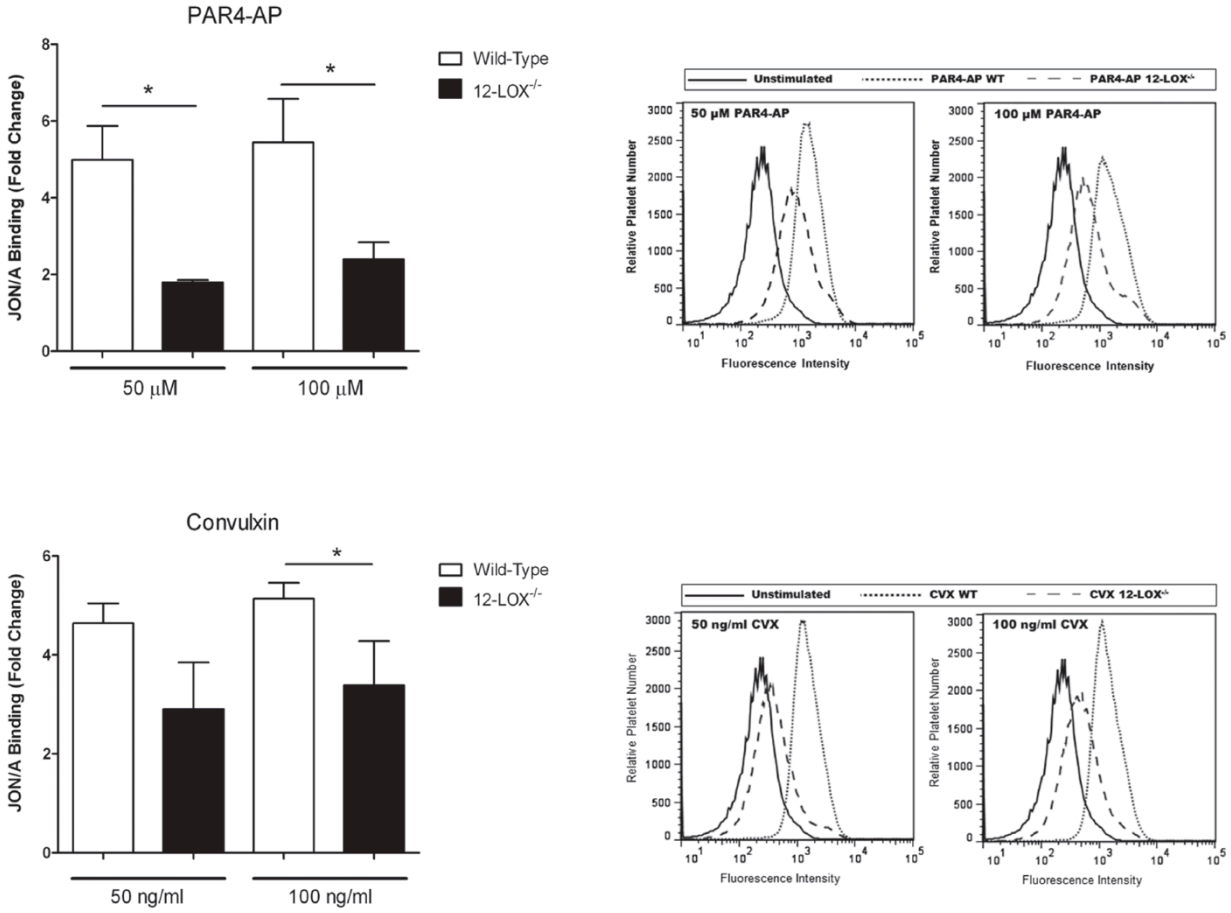
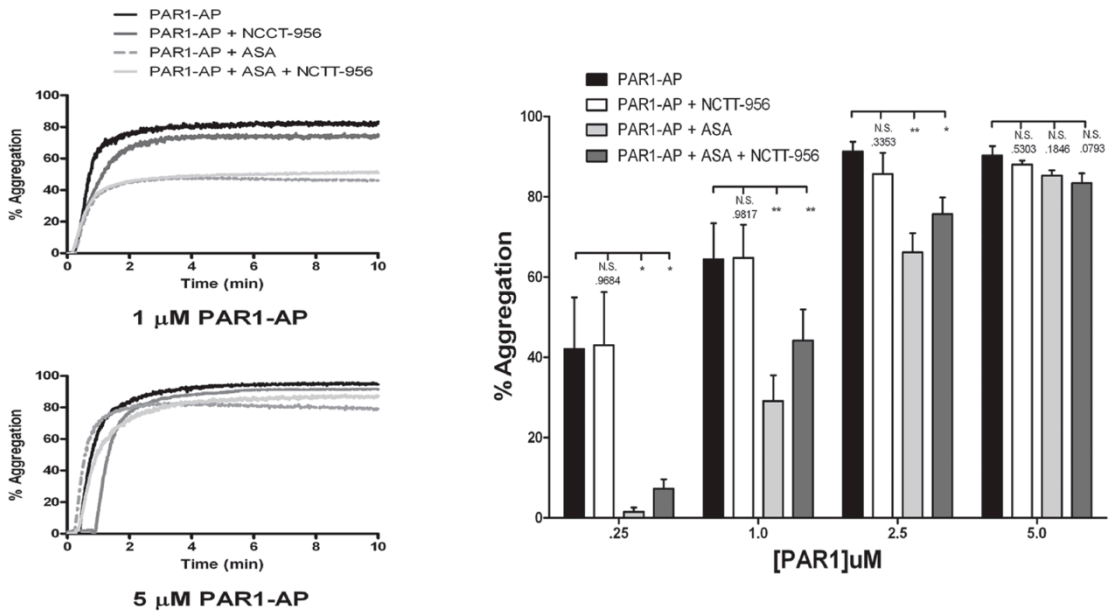
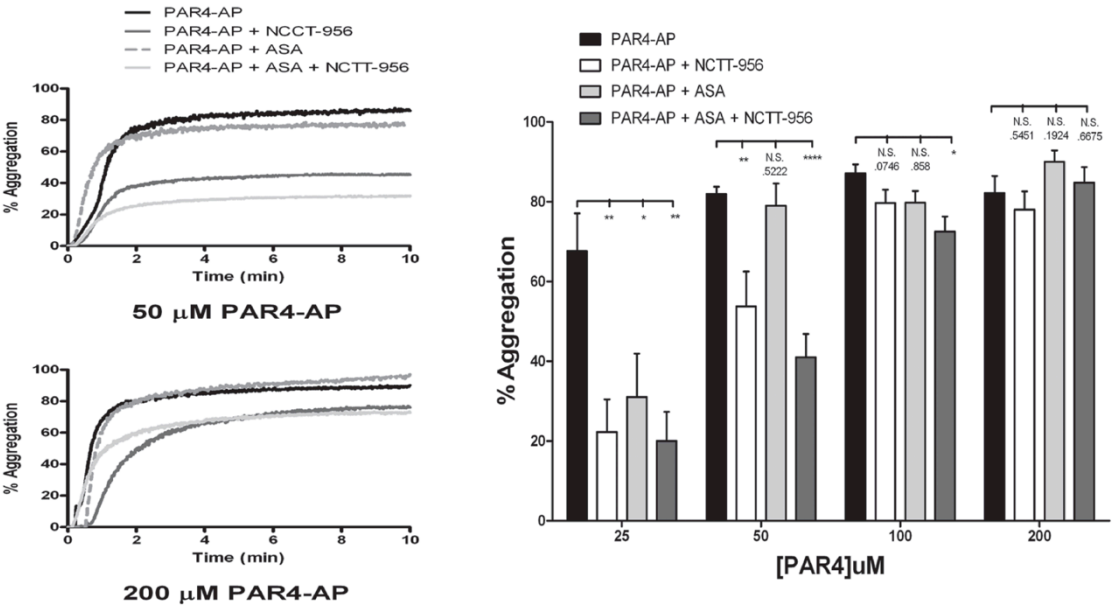


Figure 6.5 12-LOX$^{-/-}$ mice have impaired platelet activation and hemostasis.
Platelet function was measured in wild-type and 12-LOX$^{-/-}$ mice. Washed platelets were stimulated with either PAR4-AP (50 μM and 100 μM) or convulxin (CVX) (50 ng/ml or 100 ng/ml) for 10 minutes and αIIbβ3 activity was measured by flow cytometry using the JON/A antibody (N=3).

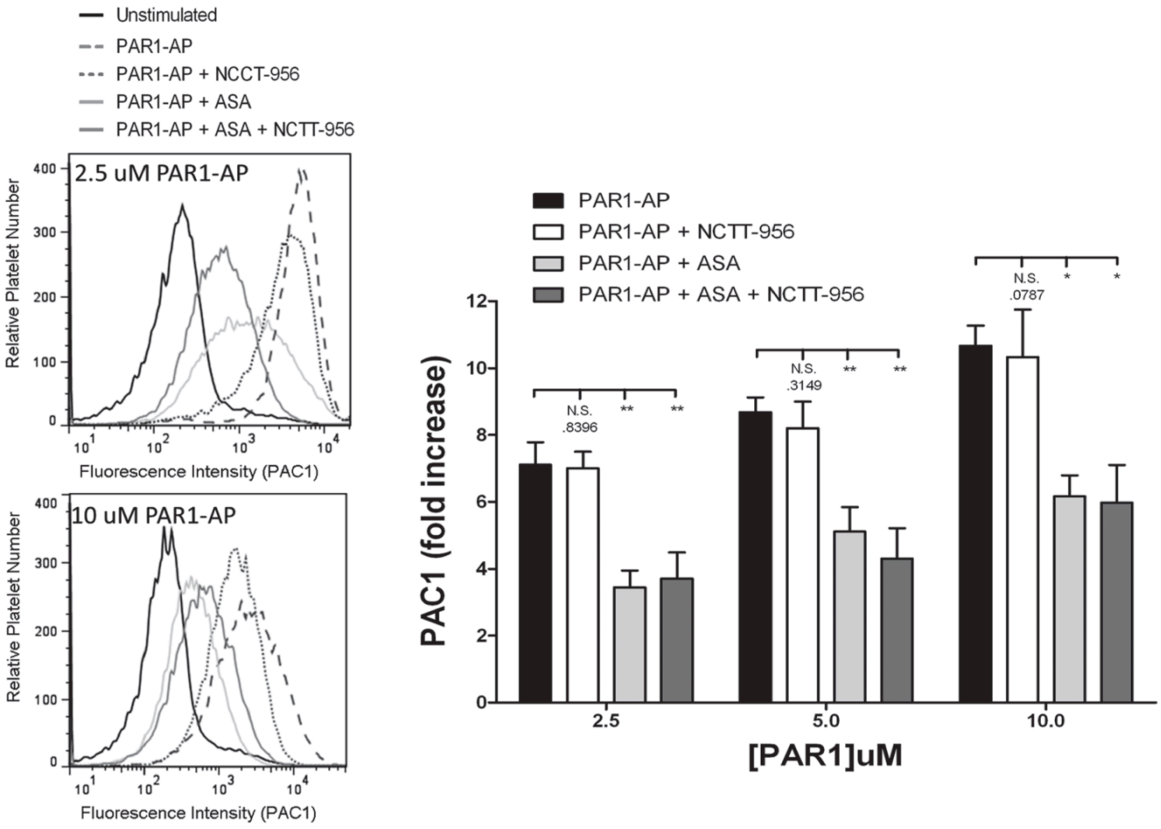
Agonist-dependent regulation of platelet activation through COX-1 and 12-LOX. Previously, we have shown that inhibition of thromboxane formation perturbs PAR1-mediated platelet activation to a much larger extent compared to PAR4 suggesting that PAR4 may rely on a 12-LOX signal for normal platelet activation as opposed to COX-1 [398]. To assess if this is the case, platelet aggregation and αIIbβ3 activation were measured in response to PAR1-AP or PAR4-AP in the presence of aspirin, NCTT-956, or both (Figure 6.6). PAR1-AP showed no sensitivity to

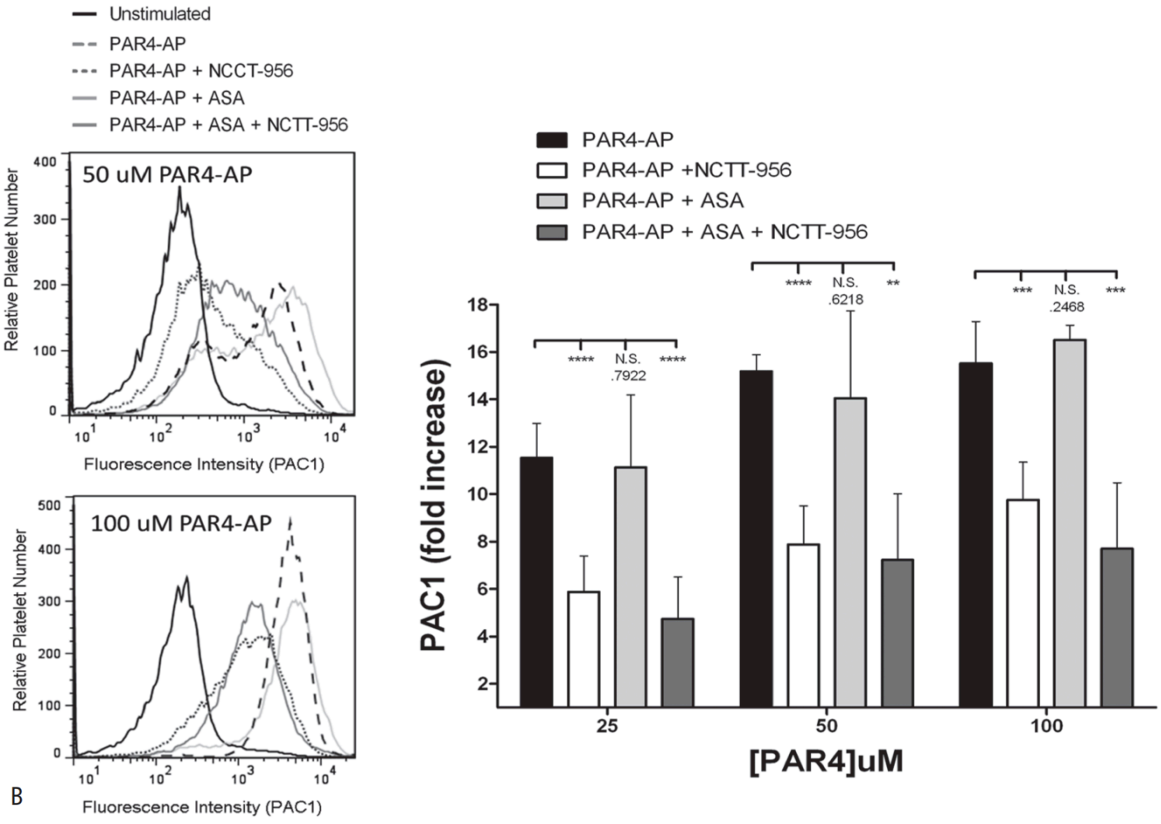
NCTT-956 even at low agonist concentrations but aggregation was significantly shifted to the right in the presence of aspirin. Treatment with both aspirin and NCTT-956 was not significantly different from aspirin alone. PAR4 showed no significant shift in the presence of aspirin at any concentration tested while treatment with NCTT-956 significantly shift the dose-response for aggregation to the right. Treatment with both aspirin and NCTT-956 was no different than NCTT-956 alone (Figure 6.6A). To confirm the observed selective inhibition of PAR1 and PAR4 signaling with aspirin and NCTT-956, respectively, integrin αIIbβ3 activation was measured under the same conditions (Figure 6.6B). Similar to what was observed for aggregation, PAR1-AP-induced αIIbβ3 activation was significantly attenuated in the presence of aspirin, but not NCTT-956, while PAR4-AP-induced αIIbβ3 activation was significantly attenuated in the presence of NCTT-956, but not aspirin. Dual treatment in either case had no additional inhibitory effect on either PAR-AP suggesting these signaling pathways are not synergistic in nature.


PAR1-AP
PAR1-AP + NCCT-956
PAR1-AP + ASA
PAR1-AP + ASA + NCTT-956
% Aggregation
Time (min)
1 μM PAR1-AP
5 μM PAR1-AP
PAR1-AP
PAR1-AP + NCTT-956
PAR1-AP + ASA
PAR1-AP + ASA + NCTT-956
% Aggregation
[PAR1]uM
.25
1.0
2.5
5.0
N.S. .9684
N.S. .9817
N.S. .3353
N.S. .5303
N.S. .1846
N.S. .0793


PAR4-AP
PAR4-AP + NCCT-956
PAR4-AP + ASA
PAR4-AP + ASA + NCTT-956
% Aggregation
Time (min)
50 μM PAR4-AP
200 μM PAR4-AP
PAR4-AP
PAR4-AP + NCTT-956
PAR4-AP + ASA
PAR4-AP + ASA + NCTT-956
% Aggregation
[PAR4]uM
25
50
100
200
N.S. .5222
N.S. .0746
N.S. .858
N.S. .5451
N.S. .1924
N.S. .6675

A


Unstimulated
PAR1-AP
PAR1-AP + NCCT-956
PAR1-AP + ASA
PAR1-AP + ASA + NCTT-956
2.5 uM PAR1-AP
10 uM PAR1-AP
Relative Platelet Number
Fluorescence Intensity (PAC1)
PAR1-AP
PAR1-AP + NCTT-956
PAR1-AP + ASA
PAR1-AP + ASA + NCTT-956
PAC1 (fold increase)
N.S. .8396
N.S. .3149
N.S. .0787
[PAR1]uM


Unstimulated
PAR4-AP
PAR4-AP + NCCT-956
PAR4-AP + ASA
PAR4-AP + ASA + NCTT-956
50 uM PAR4-AP
100 uM PAR4-AP
Relative Platelet Number
Fluorescence Intensity (PAC1)
PAR4-AP
PAR4-AP +NCTT-956
PAR4-AP + ASA
PAR4-AP + ASA + NCTT-956
PAC1 (fold increase)
N.S. .7922
N.S. .6218
N.S. .2468
[PAR4]uM

B

Figure 6.6 COX-1 and 12-LOX regulation of PAR-mediated platelet activation.
Washed platelets were assessed for differences in sensitivity to COX-1 and 12-LOX following stimulation with either PAR1-AP or PAR4-AP. A) Platelet aggregation was measured following 10 minute stimulation with increasing concentrations of either PAR1-AP or PAR4-AP in the absence or presence of 25 μM NCTT-956, 100 μM aspirin (ASA), or both (N=4-11). On the left are representative aggregation curves and on the right is the composite data for each agonist. B) αIIbβ3 activation was measured under the same conditions (N=3-11). On the left are representative histograms and on the right is the composite data for each agonist. N.S., not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

6.5 Discussion

Regulation of platelet activation is crucial in the treatment of a number of pathophysiological conditions including cardiovascular disease and diabetes and positive feedback plays a significant role in stable clot formation *in vivo* [423]. Although anti-platelet therapies targeting positive reinforcement by inhibition of COX-1 (aspirin) or the $P2Y_{12}$ receptor (clopidogrel) have succeeded in reducing morbidity and mortality due to clot formation and stroke, new approaches are warranted to further decrease the level of platelet reactivity. We have investigated an alternative approach to attenuating platelet activation and clot formation and have shown that targeting 12-LOX activation with selective platelet 12-LOX inhibitors may represent a viable approach to anti-platelet therapy. Inhibition of 12-LOX in human platelets resulted in agonist-specific attenuation of platelet aggregation and agonist-independent inhibition of dense granule secretion (Figures 6.1, 6.2). Since dense granule is thought to be a major reinforcement pathway leading to secretion of platelet agonists such as ADP, 5-HT, and epinephrine [423,447], 12-LOX inhibition of dense granule secretion may partially explain the observed deficit in normal platelet aggregation and adhesion as well as an inability to form stable clots under shear conditions. Further, attenuation of platelet adhesion and clot growth indicates a potential benefit inhibition of 12-LOX may present as an approach to anti-platelet therapy. Finally, while PAR1-mediated platelet activation is sensitive to inhibition of COX-1 but not 12-LOX, PAR4-mediated platelet activation is significantly attenuated in the absence of 12-LOX activity but not COX-1. Taken together, these data yield strong evidence that selective 12-LOX inhibitors have the potential to

decrease the severity of platelet clot formation and minimize vessel occlusion, which in turn may lead to a reduction in the incidence and severity of cardiovascular events.

Lipoxygenases play a role in a number of pathophysiological processes ranging from atherosclerosis and clot formation to cancer and essential hypertension. Elucidating the role of 12-LOX in regulation of platelet reactivity, however, has posed a significant challenge based in part on the lack of specific inhibitors [243,250]. Baicalein was initially used in human platelets to pharmacologically show that 12-LOX plays a role in calcium mobilization and aggregation [196]. This work was the first evidence that 12-LOX may play an important role in the regulation of platelet reactivity. Although baicalein is now viewed as a relatively non-specific 12-LOX inhibitor [326,441], the observation that inhibition of 12-LOX activity resulted in attenuation of calcium mobilization and perturbed resultant platelet activation steps helped to form the basis for the current study. The development of selective inhibitors targeting platelet 12-LOX has allowed us to investigate its potential role in the progression of platelet-mediated clot formation commonly observed in cardiovascular disease [326,395,441]. While the current study strongly supports a primary role for 12-LOX in regulation of normal platelet function through a number of signaling pathways including collagen and PAR4, the underlying mechanisms mediating the differential regulation of PAR1 versus PAR4 by COX-1 and 12-LOX, respectively, will also need to be delineated in order to fully appreciate the unique roles these receptors play in regulating platelet activation following injury.

Secretion and positive feedback are thought to be important events in clot formation and irreversible platelet activation. Our data suggest that 12-LOX regulates dense granule secretion in an agonist-independent manner but selectively attenuates platelet aggregation by collagen and PAR4-AP, while sparing the PAR1 pathway. This observation is in line with previous reports

showing dense granule secretion being more important for PAR4-induced aggregation compared to PAR1 [444]. Similarly, 12-LOX has recently been shown to regulate PKC activity which plays a role in dense granule secretion [326,446,448]. The current study also showed that while aspirin does not inhibit dense granule secretion in platelets, it prevents formation of thromboxane A_2 which is important for PAR1, but not PAR4-mediated platelet aggregation [398]. Hence, PAR1 appears to require COX-1 for normal platelet activation while PAR4 requires 12-LOX (Figure 6.6). The complex interactions between these receptor pathways and the oxygenases in the platelet may represent unique approaches for regulating these disparate pathways and an opportunity for the development of highly targeted anti-platelet therapeutics. The mechanistic differences mediating COX-1 and 12-LOX regulation of platelet activity are currently being investigated.

While inhibition of 12-LOX blocks dense granule secretion and 12-HETE formation, our data support the hypothesis that other 12-LOX metabolites formed following platelet activation may play an important role in regulating secretion since addition of 12-HpETE, the direct product of 12-LOX, exerts an even stronger effect than its reduced form. Several products may be formed following 12-LOX metabolism of AA, including 12-HETE and hepoxilins [449,450]. Furthermore, 12-LOX can oxidize any number of fatty acids present on the platelet membrane following cleavage with $cPLA_2$ [333,441], suggesting that other metabolites may be contributing to the regulation of platelet activation and thrombosis.

To differentiate between regulation of dense granule secretion and alpha granule secretion, P-selectin was measured as a surrogate for α-granule secretion (Figure 6.3). Our results show that 12-LOX was not required for α-granule secretion in the platelet. The potential role for 12-LOX in P-selectin secretion has been postulated by others [430] and agrees with the recently proposed model suggesting that a number of discrete pools of α-granules exist in the

platelet which are differentially secreted in an agonist-dependent manner [451]. This is an active area of investigation which will need to be elucidated in order to understand the extent to which 12-LOX regulates platelet function.

Although 12/15-LOX knockout mice have been generated to study lipoxygenase function in mice [320], this study is the first to identify the role of 12-LOX for regulation of platelet reactivity in regulation of aggregation, secretion, and adhesion in human platelets. The mouse homolog of 12-LOX is similar in structure to that of the human protein; however significant species differences do exist. For example, 12-LOX in the human solely produces 12-HETE from arachidonic acid, while 12/15-LOX in the mouse is known to produce both 12-HETE and 15-HETE, the latter product recently being shown to be essential for ischemia-induced angiogenesis [452]. With these limitations in mind, it is likely that the murine 12-LOX-deficient mouse shares some functional similarities to pharmacological inhibition of 12-LOX in the human as we show here that platelets isolated from 12-LOX$^{-/-}$ mice show significant attenuation of αIIbβ3 activation following PAR4-AP or convulxin compared to wild-type mice.

Our data support a physiologically important role for 12-LOX in mediating a number of pro-thrombotic processes, which are key in the formation of a stable platelet clot formation. Adequate regulation of platelet activation has been a central focus in clinically treating a number of diseases including acute coronary syndrome and cardiovascular disease as well as the cardiovascular complications often associated with metabolic syndrome and diabetes. Although several inhibitors of the reinforcement pathways are currently in use including aspirin and clopidogrel, a need exists for the development of alternative strategies to control platelet reactivity and minimize the occurrence of thrombotic events in patients diagnosed with a number of cardiovascular risks [76]. Hence, 12-LOX inhibition may represent a new approach to anti-

platelet therapy as it not only targets platelet aggregation, dense granule secretion, and adhesion to collagen under shear, but shows significant agonist-dependence specifically targeting collagen and PAR4 while sparing PAR1 signaling [326] (figure 6.6). Taken together, these findings provide impetus for studies to further identify how 12-LOX regulates platelet activity at a mechanistic level through PAR4 and collagen, and further probe the potential for 12-LOX inhibitors as a next generation of anti-platelet therapeutic agents either alone or in combination with existing anti-platelet approaches [453-456]

CHAPTER 7

12-Lipoxygenase Modulates FcγRIIa Signaling in Platelets[6]

7.1 Summary

Platelets are essential in maintaining hemostasis following inflammation or injury to the vasculature. Dysregulated platelet activity often results in thrombotic complications leading to myocardial infarction and stroke. Activation of the FcγRIIa receptor leads to immune-mediated thrombosis which is often life-threatening in patients undergoing heparin-induced thrombocytopenia or sepsis. Inhibiting FcγRIIa-mediated activation in platelets has been shown to limit thrombosis and is the principal target for prevention of immune-mediated platelet activation. Here we show for the first time that platelet 12(S)-lipoxygenase (12-LOX), a highly expressed oxylipin-producing enzyme in the human platelet, is an essential component of FcγRIIa-mediated thrombosis. Pharmacological inhibition of 12-LOX in human platelets resulted in significant attenuation of FcγRIIa-mediated aggregation. 12-LOX was shown to be essential for FcγRIIa-induced PLCγ2 activity leading to activation of calcium mobilization, Rap1 and PKC activation, and subsequent activation of the integrin αIIbβ3. Additionally, platelets from transgenic mice expressing human FcγRIIa but deficient in platelet 12-LOX failed to form normal platelet aggregates and exhibited deficiencies in Rap1 and $\alpha_{IIb}\beta_3$ activation. These results

[6] This has been published in Blood. Yeung J, Tourdot BE, Fernandez-Perez P, Vesci J, Ren J, Smyrniotis CJ, Luci DK, Jadhav A, Simeonov A, Maloney DJ, Holman TR. Platelet 12-LOX is essential for FcγRIIa-mediated platelet activation. Blood. 2014 Oct 2;124(14):2271-9.

support an essential role for 12-LOX in regulating FcγRIIa-mediated platelet function and identify 12-LOX as a potential therapeutic target to limit immune-mediated thrombosis.

7.2 Introduction

Platelet activation is essential for maintaining normal hemostasis following vascular insult or injury [457]. While formation of the platelet plug is a required step in primary hemostasis, under certain conditions activation of platelets with the surrounding environment results in the formation of an occlusive thrombus resulting in myocardial infarction and stroke [423]. One mode of platelet activation involves the platelet signaling through an immune response via immunoreceptors on the platelet surface [423,458-461]. Human platelets express a number of receptors containing or associated with immunoreceptor tyrosine-based activation motif (ITAM) containing transmembrane receptors including glycoprotein VI (GPVI) [462], C-type lectin-like receptor 2 (CLEC-2) [463], and the IgG immune complex receptor FcγRIIa [50]. Ligation of ITAM-containing receptors on the platelet has been previously shown to lead to a shared downstream signaling pathway resulting in platelet activation [463-465]. While activation of each of these receptors contributes in distinct ways to physiologic hemostasis and thrombosis [57,466-469], they have non-redundant pathophysiological functions. In particular FcγRIIa, which is present on the surface of human but not mouse platelets [470], is best known for its pathophysiological role in immune-mediated thrombocytopenia and thrombosis, a family of disorders including thrombocytopenia associated with sepsis, thrombosis due to certain therapeutic monoclonal antibodies and heparin-induced thrombocytopenia (HIT) [460,471,472]. Selectively inhibiting the FcγRIIa signaling pathway in platelets for prevention of immune-mediated thrombocytopenia and thrombosis has been a long sought approach for prevention of HIT [473].

Platelet 12(S)-lipoxygenase (12-LOX), an oxygenase highly expressed in platelets, has been shown to potentiate the activation of select signaling pathways including protease-activated receptor 4 (PAR4) and an ITAM-containing receptor complex (GPVI-FcRγ) [196,325,474,475]. The most well understood function of 12-LOX is production of oxylipins, most notable being the conversion of arachidonic acid (AA) to 12-hydroxyeicosatretraenoic acid (12-HETE) upon agonist stimulation of platelets through both GPCR- and non-GPCR-mediated pathways [475]. 12-HETE is an oxylipin that has been shown to be pro-thrombotic in platelets [476,477]. While the mechanism by which 12-LOX regulates platelet activity is not fully understood, previous publications have demonstrated the ability of 12-LOX activity to augment key signaling components of platelet activation including Rap1, Ca^{2+} mobilization, αIIbβ3 activation, and dense granule secretion [196,325,474,477]. As 12-LOX activity was recently shown to be required for normal GPVI-mediated platelet activation [325,474], we sought to determine if 12-LOX activity is an essential component of FcγRIIa signaling in platelets [464].

In this study, human platelets were treated with the selective 12-LOX inhibitor ML-355 [478] or vehicle control prior to FcγRIIa stimulation to determine if 12-LOX plays a role in the FcγRIIa signaling pathway. Pharmacological inhibition of 12-LOX activity in human platelets attenuated FcγRIIa-mediated platelet aggregation. Consistent with our human studies, murine platelets isolated from mice expressing human FcγRIIa in their platelets and deficient in 12-LOX had an attenuated response to FcγRIIa stimulation compared to littermates expressing 12-LOX. The activity of 12-LOX was further demonstrated to be essential for a number of biochemical steps known to be essential for FcγRIIa signaling in the platelet. Hence, this study is the first to identify 12-LOX activity as a critical component of normal FcγRIIa signaling in platelets.

Further, the results of this study suggest for the first time that 12-LOX may represent a novel therapeutic target to treat immune-mediated thrombocytopenia and thrombosis.

7.3 Materials and Methods

Preparation of washed human platelets: Prior to blood collection written informed consent was obtained under approval of the Thomas Jefferson University Institutional Review Board. Washed platelets were resuspended in Tyrodes buffer as previously described [325] at a concentration of 3 $x10^8$ platelets/mL unless otherwise indicated.

Mice and platelet preparation: FcγRIIA transgenic mice (hFcR/ALOX12$^{+/+}$) on C57BL/6J background [52] were bred with platelet 12-lipoxygenase knockout (ALOX12$^{-/-}$) mice [320,325] on C57BL6/129S2 background to generate FcγRIIA transgenic mice deficient in platelet 12-lipoxygenase (hFcR/ALOX12$^{-/-}$). The newly generated hFcR/ALOX12$^{-/-}$ mice appear phenotypically normal compared to hFcR/ALOX12$^{+/+}$ with similar body size, platelet counts, WBCs and RBCs profiles (Supplemental 1). All mice were housed in the AALAC-approved mouse facility of Thomas Jefferson University (TJU). Experimental procedures were approved by the Animal Care and Use Committee of TJU. Blood was drawn from the inferior vena cava of 12 week-old anesthetized mice using a syringe containing sodium citrate. Mouse platelet preparation was prepared as previously described [325]. Murine platelets of 2.5 $x10^8$ platelets/mL were resuspended in Tyrodes buffer containing human fibrinogen (75 μg/mL) and $CaCl_2$ (1 mM).

Reagents: Human FcγRIIa (IV.3 (CD32), StemCell Technologies), human fibrinogen (type I) (Sigma-Aldrich), goat anti-mouse IgG (Fab'2, Santa Cruz, Biotechnology), mouse anti-CD9 (BD Biosciences), Fluo-4AM (Life Technologies), PAC1-FITC (BD Biosciences), P-Selectin-PE (CD62P, BD Biosciences), antibody (Cell Signaling), ATP standard (Chronolog), Chronolume

(Chronolog), Accur C6 (BD Biosciences), Secondary rabbit and mouse antibodies (LI-COR), Y759 PLCγ2 antibody (Cell Signaling), glutathione beads for Rap1 pull down (GE-Healthcare), Fibrinogen from human plasma (Sigma).

FcγRIIa-mediated platelet activation: FcγRIIa-mediated platelet activation was initiated by two distinct models; either 1) FcγRIIa antibody cross-linking or 2) CD9 monoclonal antibody stimulation. To cross-link FcγRIIa, washed platelets were incubated with IV.3, an FcγRIIa mouse monoclonal antibody, for one minute followed by the addition of a goat anti-mouse (GAM) IgG antibody to cross-link FcγRIIa. The concentration of FcγRIIa cross-linking antibodies used for each experiment is indicated in the text. Alternatively, washed human platelets were stimulated with an anti-CD9 monoclonal antibody to activate FcγRIIa. Due to inter-individual variability in anti-CD9 response, a range of anti-CD9 concentrations (.25 – 1 μg/ml) was used to achieve aggregation at each donor's EC_{80}. In studies using the 12-LOX inhibitor (ML355), washed platelets were incubated with either ML355 (20 μM), or DMSO (vehicle control) for 15 minutes prior to FcγRIIa stimulation.

Platelet aggregation: Platelet aggregation was measured with a lumi-aggregometer (Chronolog Corp, Model 700D, Havertown, PA) under stirring conditions at 1,100 rpm at 37°C.

Quantification of 12 HETE: 12-D8-HETE (1 ng) was added to the samples as an internal standard. Platelets pretreated with DMSO or ML355 were stimulated with FcγRIIa cross-linking and quenched with 1% formic acid in acetonitrile at the indicated time points. To prepare the sample for LC/MS injection, the sample was extracted twice with hexane, dried under N_2 gas, reconstituted with ACN/water (1:1, v/v), and centrifuged at 20,000 g for 5 min at 4° C.

LC/MS/MS analysis of the samples was performed on an ACQUITY UPLC system coupled to a Xevo TQ-S MS/MS (Waters Corp), an electrospray ionization triple quadrupole mass analyzer

system. The instrument was operated in the negative mode. The ion source voltage was set at -2.0 kv and desolvation temperature was set at 550° C. 12-HETE was quantified by monitoring specific multiple reaction monitoring (MRM) transitions. The MRM transitions of 12-HETE and D-8-12-HETE, the internal standard, were m/z 319 to m/z 179 and m/z 327 to m/z 184. Chromatographic analysis was performed on the ACQUITY UPLC® BEH C18 column (1.7 mm, 100 x 2.1 mm internal diameter). LC mobile phase was composed of 0.25% acetic acid in water solution (solvent A) and isopropanol/acetonitrile (90:10, v/v) (solvent B). Solvent B was increased from 30% to 44% at 1 min, 60% at 6.3 min and 7.3 min, 30% at 8 min. The detection limit of 12-HETE was 0.2 pg/l. The linear range of 12-HETE was from 0.5 pg/l to 50 pg/l.

PLCγ2 phosphorylation: Washed human platelets were adjusted to $5x10^8$ platelets/mL and stimulated in an aggregometer by antibody cross-linking of FcγRIIa and lysed at designated times with 5X Laemmli reducing buffer (1.5 M Tris-Cl pH 6.8, glycerol, β-mercaptoethanol, 10% SDS, 1% bromophenol blue) to stop the reaction. Samples were separated on a 7.5% SDS-PAGE gel. Antibodies to PLCγ2 and phospho-Y759 PLCγ2, (Cell Signaling Technology, Danvers, MA) a marker of PLCγ2 activation, were used to evaluate the relative levels of total and active PLCγ2, respectively.

Calcium mobilization: Intracellular calcium release was measured as previously described [326]. Briefly, washed human platelets were resuspended at 1.0×10^6 platelets/mL in Tyrodes buffer containing 1 mM calcium. Platelets were incubated with Fluo-4-AM (Life Technologies, Carlsbad, CA), a cell permeable calcium sensitive dye, for ten minutes prior to stimulation. Platelets were stimulated by FcγRIIa antibody cross-linking and fluorescence intensity was measured in real-time by flow cytometry in an Acurri C6 flow cytometer. Data are reported as

the fold change in the fluorescence intensity comparing maximum fluorescence intensity relative to fluorescence intensity prior to platelet stimulation.

Rap1 activation: Washed human platelets were stimulated by FcγRIIa antibody cross-linking for five minutes and aggregation was stopped with 2X platelet lysis buffer. RalGDS-Rap binding domain (RalGDS-RBD), a truncated form of RalGDS (788-884aa) that contains the Rap1 binding domain specific for the GTP bound form of Rap1, was used to selectively precipitate the active conformation of Rap1 from the platelet lysate as previously described [479]. Total platelet lysate and Rap1 pull-down samples were run on a SDS-PAGE gel and identified by Western blot with a Rap1 antibody. The levels of active Rap1 were normalized to the amount of total Rap1 contained in each sample.

PKC activation assay: Washed platelets were stimulated by FcγRIIa antibody cross-linking under stirring conditions (1100 rpm) in an aggregometer at 37°C. Reactions were stopped by the addition of 5X Laemmli sample buffer at the indicated times. As a positive control platelets were treated with PMA (1 mM), a direct PKC agonist, for one minute. Samples were run on an SDS-PAGE gel and Western blots were performed using antibodies specific for PKC substrate phosphorylation and pleckstrin.

Dense granule secretion: ATP release was measured from washed platelets as surrogate for dense granule secretion. Prior to activation washed platelets were incubated with Chronolume Reagent, an ATP sensitive dye, for one minute. Platelets were stimulated with an FcγRIIa antibody under stirring conditions and fluorescence was measured in real-time using a lumi-aggregometer.

Alpha granule release and αIIbβ3 activation: Prior to stimulation, human washed platelets were pre-incubated with either FITC-conjugated P-selectin antibody or FITC-conjugated PAC-1, an

antibody specific for the active conformation of αIIbβ3. Platelets were stimulated with an FcγRIIa antibody and reactions were stopped by the addition of 2% formaldehyde at indicated times. Fluorescence intensity was measured by flow cytometry. Results are reported as mean fluorescence intensity.

Western Blotting: Standard Western blots for Rap1 activation, PKC substrates, and PLCγ2 phosphorylation were used and band intensity were quantified with the Odyssey Infrared Imaging System (LIC-OR Biosystems).

Statistical analysis: Where applicable, the data represents the mean ± S.E.M. Statistical significance was determined with unpaired one-tailed t-test using GraphPad Prism software. A p value less than 0.05 was considered significant.

7.4 Results

12-LOX regulates FcγRIIa-mediated platelet aggregation.

Our group previously showed that pharmacological inhibition of 12-LOX activity resulted in attenuation of GPVI- or PAR4-mediated platelet activation [325,474]. In platelets, FcγRIIa utilizes many of the same downstream signaling effectors as GPVI-FcRγ [464]. To determine if 12-LOX plays a role in FcγRIIa-mediated platelet aggregation, washed human platelets were treated with increasing concentrations of ML355, a recently identified highly selective 12-LOX inhibitor [478], or DMSO (vehicle control) prior to FcγRIIa stimulation. Treatment of platelets with ML355 at a concentration of 10 or 20 μM resulted in significant attenuation of FcγRIIa-mediated platelet

aggregation via FcγRIIa antibody cross-linking (Figure 7.1A). To assess if the

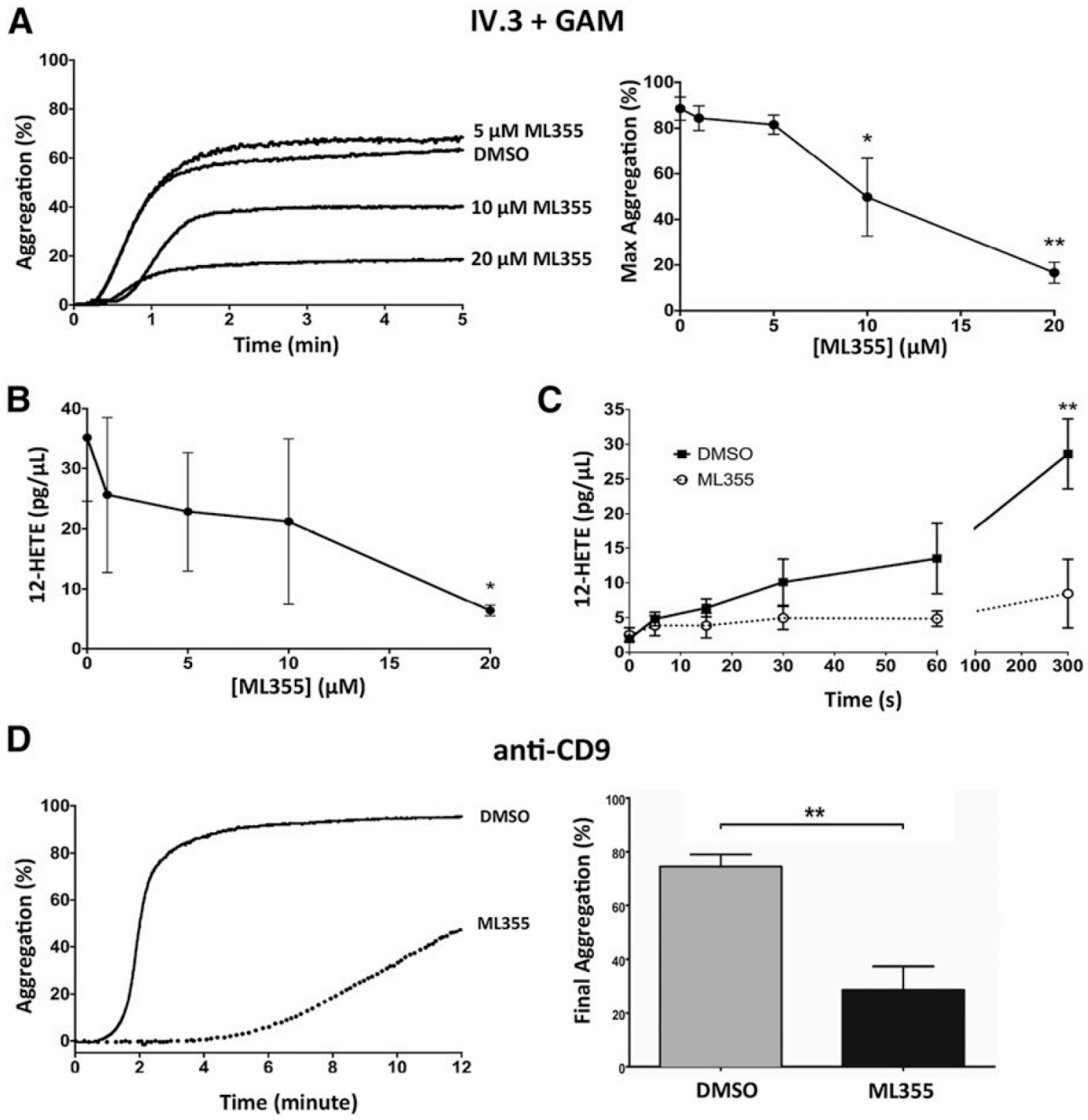


Figure 7.1 12-LOX modulates FcγRIIa-mediated platelet aggregation.
A) The aggregation of washed human platelets was measured following FcγRIIa crosslinking (IV.3 + GAM) in the presence of increasing concentrations of ML355, a 12-LOX inhibitor, ranging from 1-20 μM (n= 5) or DMSO (vehicle control, n= 5). The left panel shows the representative dose response of ML355 affecting FcγRIIa-induced aggregation. The right panel is a composite of ML355 doses. B) Following FcγRIIa cross-linking (IV.3 + GAM), the production of 12-HETE, the predominant 12-LOX oxylipin, was measured in platelets pretreated with concentrations of ML355 ranging from 1 – 20 μM (n= 4) or DMSO (n= 4). C) 12-HETE production was measured in FcγRIIa cross-linked platelets at increasing time points in the presence of DMSO or ML355 (20 μM) (n=4). D) Washed human platelets were pretreated with DMSO (n=4) or ML355 (20 μM) (n=8) and platelet aggregation was measured following FcγRIIa stimulation (anti-CD9). Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$. This figure was co-provided by Jennifer Yeung and Johnny Yu (12-HETE measurement)

attenuation of aggregation observed in figure 7.1A correlated with the ability of ML355 to inhibit 12-LOX activity, 12-HETE (the predominant 12-LOX product) production was measured in FcγRIIa-stimulated platelets pre-treated with DMSO or ML355. Stimulation of FcγRIIa with GAM + IV.3 stimulated platelets with ML355 (20 μM) significantly decreased 12-HETE production (Figure 7.1B). We observed 20 mM of ML355 as the lowest concentration that efficiently blocked both FcγRIIa-induced platelet aggregation and 12-HETE production independent of inter-individual variability in response to the inhibitor, therefore this concentration of inhibitor was used in all subsequent assays in this study. The kinetics of 12-HETE production in PAR stimulated platelets was previously determined [398]; however, the kinetics of 12-HETE production in FcγRIIa stimulated platelets is unknown. Therefore, we sought to determine the kinetics of 12-HETE production in FcγRIIa stimulated platelets. 12-HETE formation is significantly enhanced at 15 seconds in DMSO treated platelets stimulated with FcγRIIa agonist (P=.0143) and continues to increase through all times measured (Figure 1C). The production of 12-HETE in FcγRIIa stimulated platelets is significantly attenuated by ML355 (20 μM) (Figure 7.1C). FcγRIIa-mediated platelet aggregation in response to a second agonist for FcγRIIa, anti-CD9, was also found to be attenuated in the presence of 20 μM of ML355 (Figure 7.1D). To confirm the decrease in FcγRIIa-mediated platelet aggregation was due to pharmacological inhibition of 12-LOX and not a potential off-target effect of the ML355, *ex vivo* platelet aggregation was measured following anti-CD9 stimulation in humanized FcγRIIa (hFcR) transgenic mice expressing 12-LOX (ALOX12$^{+/+}$) or deficient in 12-LOX (ALOX12$^{-/-}$). Platelets from mice deficient in 12-LOX (hFcR/ALOX12$^{-/-}$) showed a delayed (Figure 7.2B) and attenuated (Figure 7.2C) aggregation in response to anti-CD9 stimulation (.125 and .25 μg/mL) when compared to platelets from mice expressing functional 12-LOX (hFcR/ALOX12$^{+/+}$). These

data suggest that platelets lacking 12-LOX activity through pharmacological or genetic ablation exhibit a significantly attenuated platelet aggregation response to FcγRIIa activation.

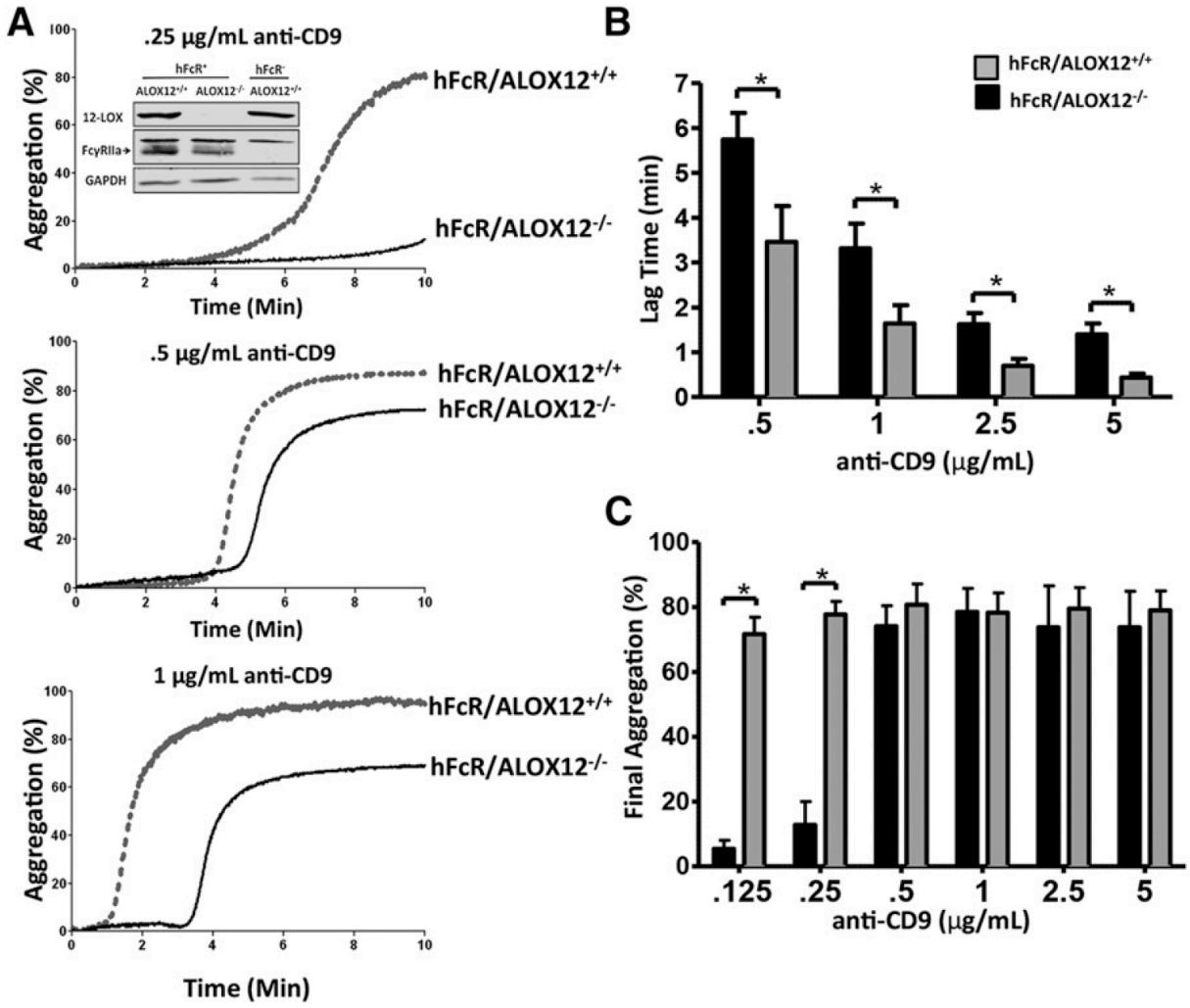


Figure 7.2 Murine platelets require 12-LOX for normal FcγRIIa-induced platelet aggregation.
A dose response of anti-CD9-induced platelet aggregation was performed with washed platelets from hFcR/ALOX12$^{+/+}$ or hFcR/ALOX12$^{-/-}$ mice. Prior to aggregation, fibrinogen (75 µg/mL) and $CaCl_2$ (1 mM) were added to platelets. A) Washed platelets from hFcR/ALOX12$^{+/+}$ (grey tracings) and hFcR/ALOX12$^{-/-}$ (black tracings) were measured for aggregation in response to .25, .5 and 1 µg/mL of mouse anti-CD9 for 10 minutes. Inset: Western blots for 12-LOX, FcγRIIa, and GAPDH were performed with platelet lysate from hFcR/ALOX12$^{+/+}$ or hFcR/ALOX12$^{-/-}$ mice. The B) lag time and C) final aggregation was measured in hFcR/ALOX12$^{+/+}$ (grey bars) and hFcR/ALOX12$^{-/-}$ (black bars) washed platelets stimulated with increasing doses of anti-CD9 (n = 3-6 per group). Error bars indicate SEM. *, $p<0.05$

12-LOX regulates αIIbβ3 and Rap1 activity in FcγRIIa stimulated platelets.

Figures 1 and 2 suggest 12-LOX is essential for normal FcγRIIa-mediated platelet aggregation; however, the component(s) in the FcγRIIa pathway regulated by 12-LOX remain unclear. As the

activation of integrin αIIbβ3 is required for platelet aggregation [480,481], the potential role of 12-LOX in mediating αIIbβ3 activation in FcγRIIa-stimulated platelets was investigated. αIIbβ3 activation was measured by PAC1-FITC binding to FcγRIIa-stimulated platelets treated with DMSO or ML355 in flow cytometry. FcγRIIa activation resulted in a large increase in active αIIbβ3 on the platelet surface. Treatment with ML355 prior to stimulation resulted in a significant reduction in PAC1 binding to platelets (Figure 7.3A) relative to the DMSO control.

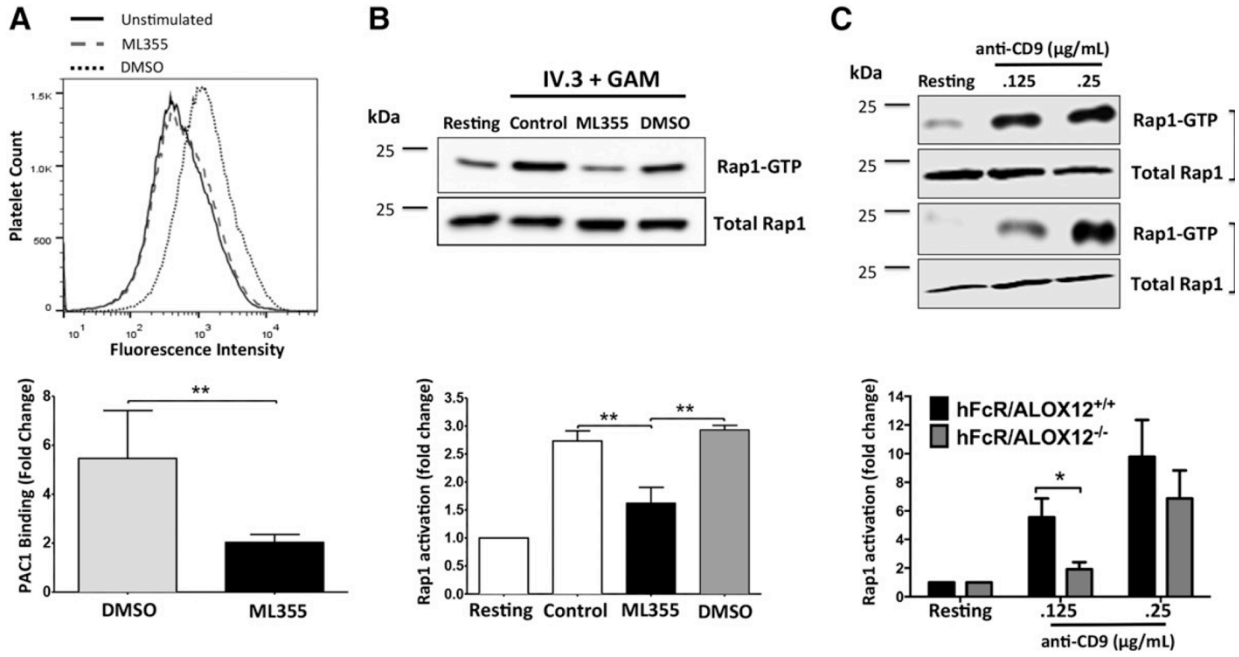


Figure 7.3 FcγRIIa-mediated Rap1 and integrin αIIbβ3 activation are potentiated by 12-LOX.
A) Washed human platelets pre-treated with ML355 or DMSO were stimulated by FcγRIIa crosslinking (IV.3 + GAM) and αIIbβ3 integrin activation (n=3 for DMSO and n=6 for ML355) and B) Rap1 activation (n=4) were assessed. C) hFcR/ALOX12$^{+/+}$ and hFcR/ALOX12$^{-/-}$ platelets were stimulated with .125 and .25 μg/mL of mouse anti-CD9 and replicates of n=5 were assessed for Rap1 activation. PAC1-FITC was used to measure αIIbβ3 activation by flow cytometry. A composite bar graph of PAC1-FITC fold changes relative to the unstimulated PAC1-FITC fluoresence. Activated Rap1 was pulled down using Ral-GDS and blotted with a Rap1 antibody. Active Rap1 was measured using LI-COR and then normalized to total Rap1 and unstimulated for fold change in Rap1 activity. Error bars indicate SEM. **, $p<0.01$

While αIIbβ3 activation is under the control of a complex milieu of signaling proteins including Talin, RIAM1, and Rap1 [482]. Rap1, a small G protein regulated by calcium and PKC, is known to play a central role in inside-out activation of αIIbβ3 [465,483-486]. Therefore, Rap1 activation was measured following FcγRIIa stimulation for five minutes in washed human

platelets treated with or without ML355 (Figure 7.3B). FcγRIIa stimulation of platelets (control) resulted in a significant increase in active Rap1 (Rap1-GTP). However, platelets treated with ML355 and subsequently stimulated through FcγRIIa failed to activate Rap1. To confirm that the inhibition of Rap1 was not due to the vehicle, platelets were treated with DMSO and stimulated with FcγRIIa. Treatment with DMSO did not result in attenuation in Rap1 activation compared to untreated control.

While unlikely, it is possible that ML355 inhibits Rap1 in a 12-LOX-independent manner. To determine if this is the case, Rap1 activation was measured in platelets from mice expressing human FcγRIIa and 12-LOX (hFcR/ALOX12$^{+/+}$) or deficient in 12-LOX (hFcR/ALOX12$^{-/-}$). FcγRIIa-mediated Rap1 activation in the platelet was measured following stimulation with anti-CD9 (Figure 7.3C). Rap1 activity was significantly increased in mouse platelets expressing both human FcγRIIa and endogenous 12-LOX. Genetic ablation of 12-LOX however (hFcR/ALOX12$^{-/-}$) resulted in a significant attenuation of anti-CD9-mediated Rap1 activation confirming the importance of 12-LOX in this process.

12-LOX regulates dense and alpha granule secretion in FcγRIIa activated platelets.

The release of alpha and dense granules from activated platelets serves to amplify platelet aggregation [487-490] and are important components in normal agonist-induced platelet activation. To determine if 12-LOX plays a role in FcγRIIa-mediated granule secretion, surface expression of P-selectin was used as a surrogate marker to measure alpha granule secretion in FcγRIIa-stimulated platelets and measurement of ATP release was assessed as a measure of agonist-induced dense granule secretion. FcγRIIa-stimulated platelets treated with ML355 showed a significant decrease in the percentage of platelets that expressed P-Selectin on their surface compared to DMSO treated platelets (Figure 7.4A). Additionally, FcγRIIa-stimulated platelets

treated with ML355 showed a significant attenuation in ATP release compared with DMSO treated platelets (Figure 7.4B). The data strongly supports a role for 12-LOX regulation of FcγRIIa-mediated platelet secretion through release of dense and partial regulation of alpha granule secretion.

12-LOX modulates proximal signaling components of the FcγRIIa pathway in human platelets.

As shown in Figures 7.1-7.3, 12-LOX activity is required for the normal activation of distal signaling components of the FcγRIIa pathway (Rap1, αIIbβ3, and platelet aggregation). We sought to identify the earliest biochemical steps in the FcγRIIa pathway which are regulated by 12-LOX. The earliest signaling observed following FcγRIIa activation is ITAM phosphorylation which has been shown to result in Syk activation [491-494]. Syk activation leads to activation of phospholipase gamma 2 (PLCγ2) resulting in calcium release and PKC activation, both of which are critical for platelet activation [394,495]. To determine where in this complex signaling milieu 12-LOX plays an essential role in FcγRIIa signaling in the platelet, a number of the signaling components directly downstream of FcγRIIa activation were assessed in the presence or absence of ML355. To investigate whether 12-LOX directly regulated phosphorylation of the FcγRIIa receptor, the first step in FcγRIIa-mediated platelet activation, FcγRIIa was immunoprecipitated from IV.3+GAM-stimulated platelets treated with ML355 or DMSO and immunoblotted for phosphorylation of FcγRIIa. Stimulation of FcγRIIa resulted in a noticeable increase in receptor phosphorylation. Treatment with ML355 failed to cause a reduction in FcγRIIa phosphorylation (Figure 7.5). This data suggests that 12-LOX activity is not required for phosphorylation of FcγRIIa itself.

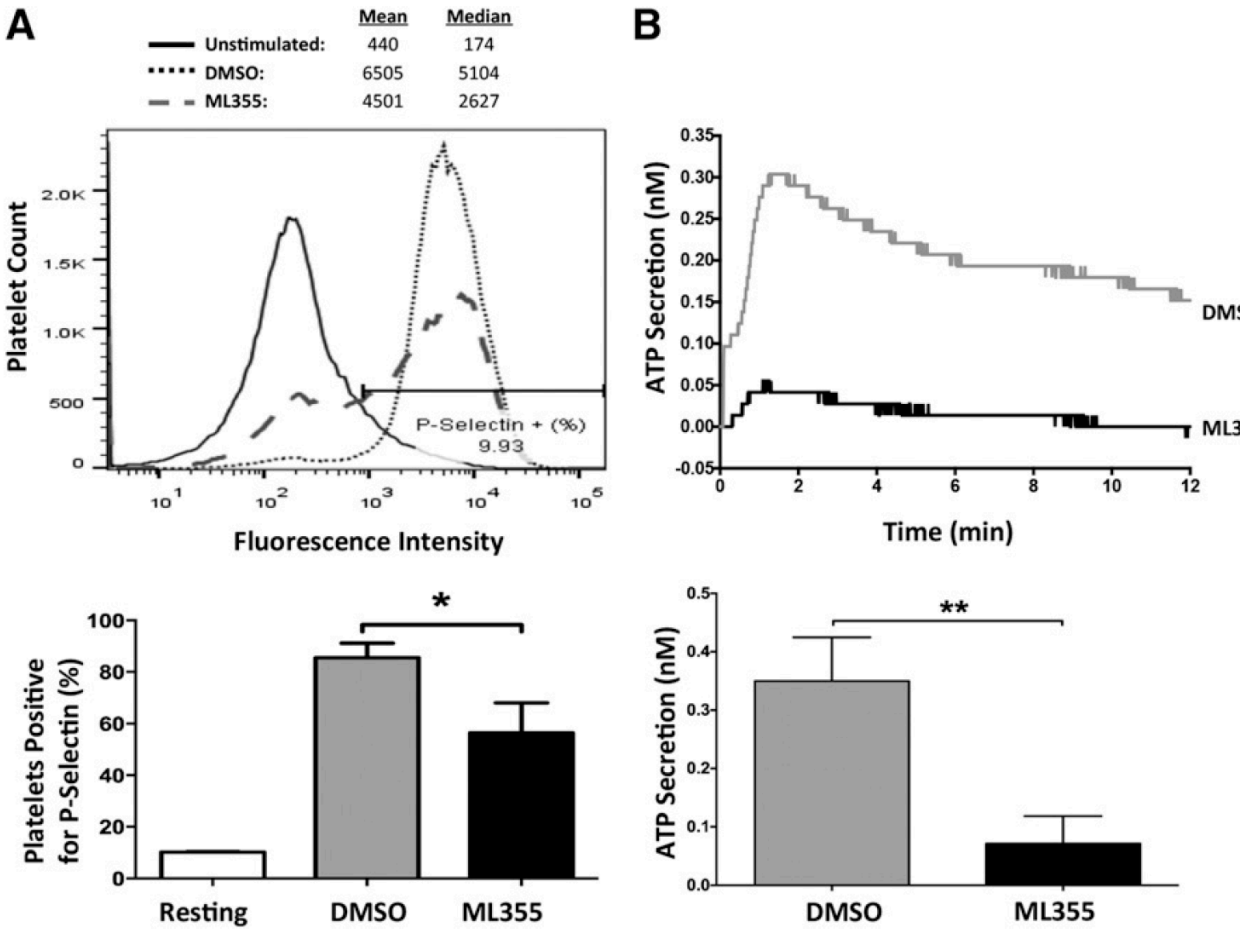


Figure 7.4 Granule secretion mediated by FcγRIIa activation is regulated by 12-LOX.
Washed human platelets treated with DMSO or ML355 were stimulated by FcγRIIa crosslinking in which IV.3 (2 μg/mL) + GAM (10 μg/mL) were used for A) alpha granule secretion. Alpha granule secretion was measured by using P-selectin-PE conjugated antibody in a flow cytometer. To obtain the percentage of platelets that were positive for P-Selectin, approximately 10% of the unstimulated platelet population was gated (shown in the histogram) and then applied to ML355 and DMSO treated platelets in order to quantify percentage of platelets that were positive for P-Selectin. A composite bar graph shows the percent of platelets positive for P-selectin in ML355 and DMSO treated platelets (n=5). B) IV.3 (2 μg/mL) + GAM (5 μg/mL) were used to stimulate ATP secretion as a surrogate marker for dense granule secretion in a lumi-aggregometer. A bar graph of DMSO or ML355 treated platelets measured for ATP secretion following FcγRIIa crosslinking (n=4). Error bars indicate SEM. **, $p<0.01$.

To assess if 12-LOX regulates PLCγ2 activation downstream of FcγRIIa, phosphorylation of PLCγ2 was measured in washed human platelets in the presence or absence of ML355. Platelets stimulated through FcγRIIa were phosphorylated within 15 seconds following stimulation. Platelets treated with ML355; however, showed significantly attenuated PLCγ2 phosphorylation at 15 seconds compared to control conditions (Figure 7.6A).

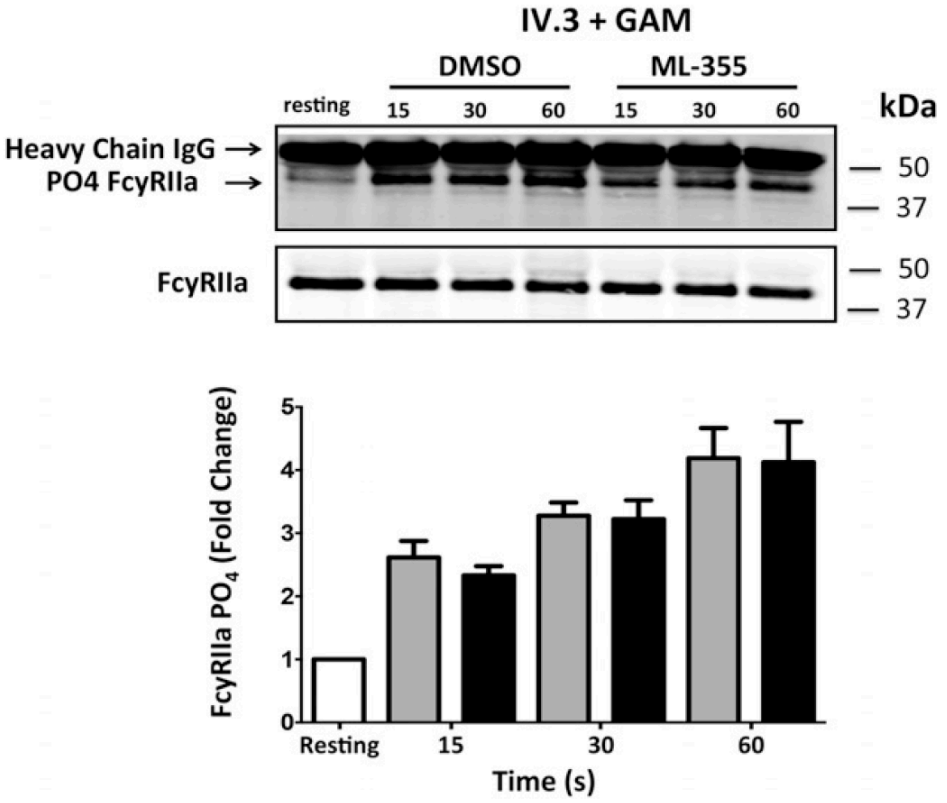


Figure 7.5 The role of 12-LOX in regulating the FcγRIIa signaling complex.
Washed human platelets were treated with ML355 (20 μM) or vehicle control prior to stimulation by crosslinking (IV.3 + GAM). Immunoprecipitation of FcγRIIa was conducted at 15, 30, and 60 seconds post crosslinking and phosphorylation of FcγRIIa was measured via Western blot. A bar graph of immunoprecipitated FcγRIIa that had been treated with DMSO or ML355 following FcγRIIa crosslinking were assessed for phosphorylation (n=7). Error bars indicate SEM. This figure was provided by Dr. Benjamin Tourdot.

Since 12-LOX attenuated FcγRIIa-mediated PLCγ2 activation, its effects on intracellular calcium release were measured. FcγRIIa was stimulated in washed human platelets treated with or without ML355 and calcium release was measured. Platelets treated with ML355 exhibited a decrease in intracellular calcium following platelet stimulation compared to DMSO treated platelets (Figure 7.6B) supporting a physiological role for both PLCγ2 and calcium in 12-LOX regulation of FcγRIIa. As PLCγ2 activation leads to activation of PKC in platelets through calcium mobilization, the potential regulation of FcγRIIa-mediated PKC activity by 12-LOX was also evaluated. Platelets stimulated through the FcγRIIa pathway showed a high level of PKC-mediated phosphorylation as measured by a phospho-substrate PKC antibody. A significant

decrease in PKC activation was observed in platelets treated with ML355 at 30 and 60 seconds compared to platelets treated with the vehicle control (Figure 6.7C). This regulation of PKC by ML355 was determined to be indirect since PKC activation by PMA, a direct PKC activator, showed no difference in its ability to activate PKC either in control or ML355 treated platelets.

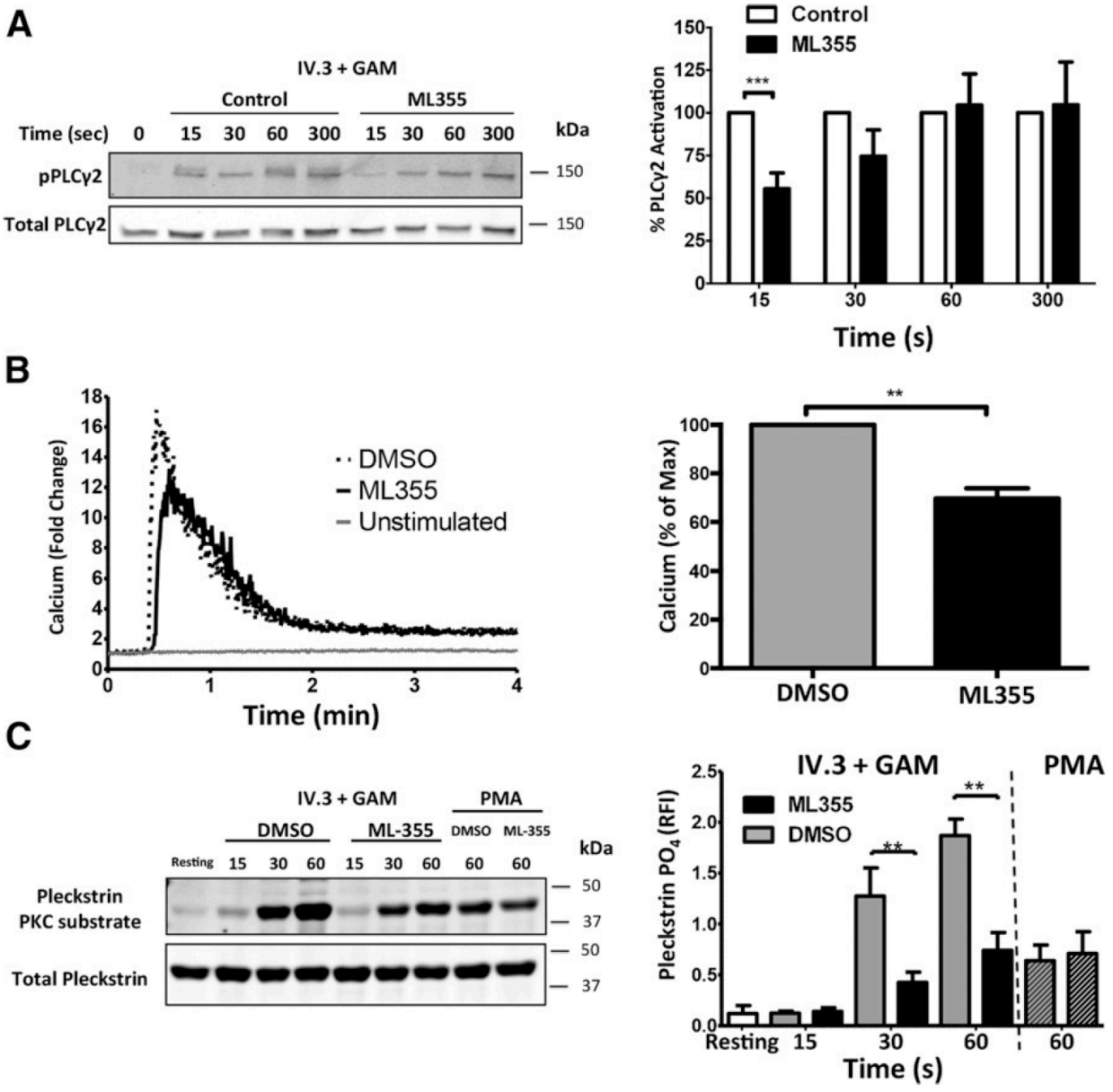


Figure 7.6 12-LOX modulates early signaling components of the FcγRIIa pathway in human platelets.
Washed human platelets were treated with ML355 (20 μM) or vehicle control prior to stimulation by crosslinking (IV.3 + GAM). A) A time course of PLCγ2 phosphorylation at site Y759 was assessed by western blot analysis. All samples were normalized to

total PLCγ2 and fold changes were quantified relative to the unstimulated condition. The bar graph comprises of n=7 individuals. B) Following crosslinking (IV.3 + GAM), calcium mobilization was measured by flow cytometry. Representative curves were quantitated in fold change of free calcium relative to the unstimulated condition over four minutes. Bar graphs represent the ratio of the fold change in calcium mobilization (n=5). C) N=7 stimulated human platelets with or without ML355 were analyzed for PKC activity following CD32 crosslinking and PMA rescue comprises of n=3. A PKC substrate was blotted as a surrogate for PKC activation and Pleckstrin phosphorylation. Data represents mean ± S.E.M. *, $p<0.05$; **, $p<0.01$. This figure was co-provided by Jennifer Yeung (PLCγ2 phosphorylation and calcium flux) and Dr. Benjamin Tourdot (PKC substrate phosphorylation)

7.5 Discussion

12-LOX has recently been shown to be an important regulator of GPVI-mediated platelet activation [325,474]. As FcγRIIa and GPVI are purported to signal via a conserved pathway, we postulated that 12-LOX may play an essential role in the regulation of FcγRIIa signaling in human platelets. This study is the first to demonstrate that 12-LOX is an essential component of FcγRIIa immune-mediated platelet activation. Human platelets treated with a highly selective 12-LOX inhibitor, ML355 [478], or FcγRIIa transgenic mouse platelets deficient in 12-LOX, showed significantly attenuated aggregation in response to FcγRIIa-mediated activation. To investigate the underlying mechanism by which 12-LOX regulates FcγRIIa-mediated platelet activation, the activity of multiple signaling intermediates in the FcγRIIa pathway were assessed in the presence of the 12-LOX inhibitor ML355. Following stimulation, platelets treated with ML355 were significantly attenuated along multiple signaling steps in the immune-mediated FcγRIIa activation pathway including αIIbβ3, Rap1, Ca^{2+}, PLCγ2, PKC, and granule secretion (Figure 7.7).

The best studied function of 12-LOX is to produce oxylipins (such as 12(S)-HETE) from free fatty acids in the cell membrane. While previous work showed that selective 12-LOX inhibitors significantly reduced oxylipin production and were associated with reduced platelet-mediated reactivity, the role of these bioactive metabolites in platelet activation remains unclear. Our study demonstrates that a significant increase in 12-HETE formation is reproducibly

observed within 15 seconds following FcγRIIa crosslinking-mediated 12-LOX oxidation of AA (P=0.0143) (Figure 7.1C). Our data suggest that 12-LOX may partially regulate FcγRIIa-induced platelet activation either through oxylipin formation and subsequent metabolite signaling [329,330], or directly through a protein complex formation within the cell [496-498]. While both of these regulatory mechanisms are plausible, the kinetics by which 12-LOX inhibition attenuates FcγRIIa signaling may favor a direct role for 12-LOX in regulating the FcγRIIa pathway through non-enzymatic regulation of the FcγRIIa signaling cascade.

Although 12-LOX activity is required for normal FcγRIIa-mediated platelet activation, the direct molecular component by which 12-LOX activity is required has yet to be determined. 12-LOX was not required for FcγRIIa phosphorylation, suggesting 12-LOX activity is not directly regulating Src family kinase activity. However, 12-LOX activity was shown to be important for early PLCγ2 activation, indicating 12-LOX may be an important regulator in the kinetics of PLCγ2 activation affecting downstream effectors. The delay in PLCγ2 activation due to 12-LOX inhibition may be attributed to direct regulation of PLCγ2 or regulation of upstream effectors such as LAT or BTK. The data presented here narrows the scope of where 12-LOX impinges on the FcγRIIa pathway to a proximal point in the signaling pathway between the receptor and PLCγ2.

The role of FcγRIIa signaling is well established in the pathogenesis of immune-mediated thrombosis such as heparin-induced thrombocytopenia and thrombosis (HIT), an iatrogenic disorder characterized by immune-mediated platelet activation that can lead to life threating thrombosis. HIT is a paradigm of the ITT disorders, immune-mediated thrombocytopenia and thrombosis. Alternative therapeutic interventions, such as direct thrombin inhibitors (DTIs), have been considered for anti-coagulation therapy; however, complications of bleeding remains a

primary concern [499]. Even with this potentially fatal complication, heparin remains the standard anticoagulant for prevention and treatment of thrombosis. Therefore, there is a need for novel therapeutic approaches that directly treat the pathogenesis of HIT. The activation of platelets by pathogenic HIT immune complexes requires FcγRIIa signaling, which makes it an attractive target. While the pathogenesis of HIT is complex, we have provided strong evidence supporting 12-LOX as a key regulator of FcγRIIa-mediated platelet activation. Hence, this study has delineated for the first time a novel approach in regulation of HIT and potentially other ITT disorders through inhibition of human platelet 12-LOX.

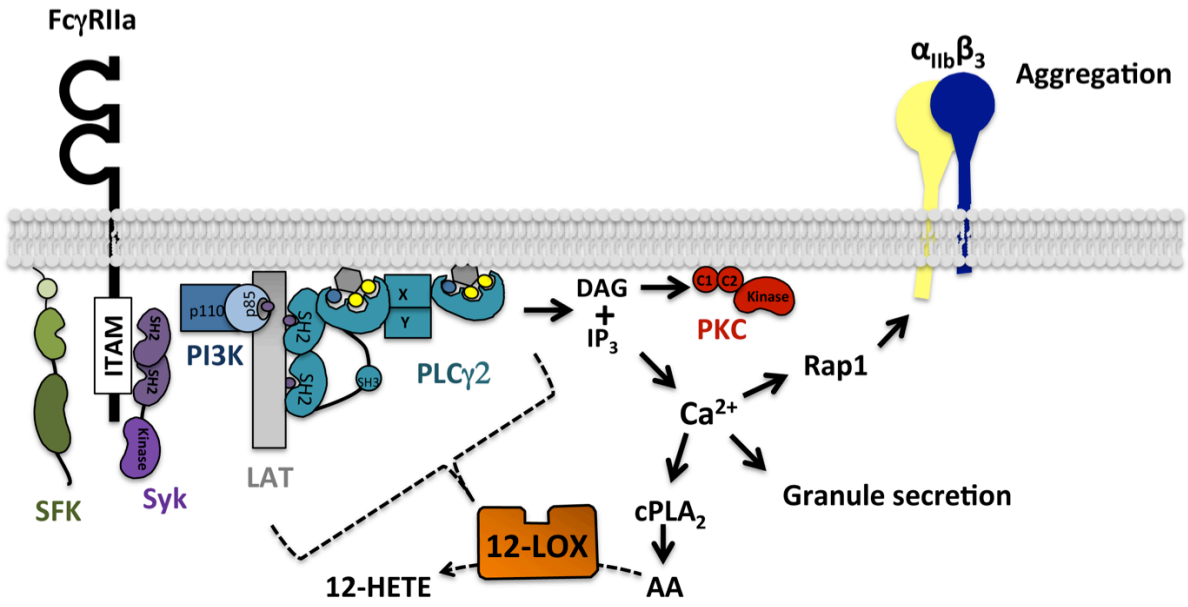


Figure 7.7 Schematic model of 12-LOX role in the regulation of FcγRIIa pathway.
12-LOX regulates early PLCγ2 activation-mediated by FcγRIIa stimulation, which is essential for full calcium release in the platelets. Calcium flux is required for $cPLA_2$ activity to generate free fatty acids, such as arachidonic acid (AA). Subsequently, Rap1 activation is also dependent on 12-LOX activity in order to activate integrin αIIbβ3 for platelet aggregation. This illustration was generated by Dr. Benjamin Tourdot.

CHAPTER 8

Investigations of Human Platelet-type 12-Lipoxygenase: Role of Lipoxygenase Products in Platelet Activation[7]

8.1 Summary

Human platelet-type 12-lipoxygenase (12-LOX) has recently been shown to play an important role in regulation of human platelet function by reacting with arachidonic acid (AA). However, a number of other fatty acids are present on the platelet surface which, when cleaved from the phospholipid, can be oxidized by 12-LOX. We sought to characterize the substrate specificity of 12-LOX against six essential fatty acids: AA, dihomo-gamma-linolenic acid (DGLA), eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA), eicosadienoic acid (EDA) and linoleic acid (LA). Three fatty acids were comparable substrates (AA, DGLA and EPA), one was 5-fold slower (ALA), and two showed no reactivity with 12-LOX (EDA and LA). The bioactive lipid products resulting from 12-LOX oxidation of DGLA, 12-(S)-hydroperoxy-8Z,10E,14Z-eicosatrienoic acid (12(S)-HPETrE), and its reduced product, 12(S)-HETrE, resulted in significant attenuation of agonist-mediated platelet aggregation, granule secretion, αIIbβ3 activation, Rap1 activation, and clot retraction. Treatment with DGLA similarly inhibited PAR1-mediated platelet activation as well as platelet clot retraction. These observations are in surprising contrast to our recent work showing 12(S)-HETE is a pro-thrombotic bioactive lipid

[7] This section has been published in Journal of Lipids Research. Ikei KN, Yeung J, Apopa PL, Ceja J, Vesci J, Holman TR, Holinstat M. Investigations of human platelet-type 12-lipoxygenase: role of lipoxygenase products in platelet activation. Journal of lipid research. 2012 Dec 1;53(12):2546-59.

and supports our hypothesis that the overall effect of 12-LOX oxidation of fatty acids in the platelet is dependent on the fatty acid substrates available at the platelet membrane.

8.2 Introduction

In the human platelet, hydroperoxidation of polyunsaturated fatty acids using molecular oxygen is accomplished by the human lipoxygenase (LOX) isozyme family [500]. 5-LOX, 12-LOX and 15-LOX are the three main LOX's and are named according to their positional specificity on arachidonic acid (AA), producing their respective hydroperoxyeicosatetraenoic acid (HPETE) products. The HPETE products are subsequently reduced by cellular peroxidases to the bioactive lipid, hydroxyeicosatetraenoic acid (HETE). The LOX products are responsible for human inflammatory responses [501], and are also implicated in a variety of human diseases. 5-LOX is involved in asthma [502] and cancer [503,504]. 12-LOX is involved in psoriasis [505], hypertension [431,432], hemostasis [196,322,506,507], diabetes [508,509], and cancer [504,510,511], and 15-LOX is involved in atherosclerosis [512] and cancer [504,513].

Two 12-LOX isozymes are expressed in humans, platelet-type 12-LOX (12-LOX) [514] and epithelial 12-LOX [515]. The former isozyme (12-LOX), makes the S-product and is primarily expressed in megakaryocytes and platelets, while the latter makes the R-product and is primarily expressed in epithelial tissue. Recently, 12-LOX has been implicated in platelet activation, which is known to play a central role in the pathophysiology of cardiovascular disease [329,398,441,476,507]. Following initial activation of human platelets by primary signals such as thrombin and collagen, secondary signals mediated by bioactive lipids (thromboxane A_2 and 12(S)-HETE) and secreted molecules (ADP) are essential for recruitment and activation of platelets.

12-LOX acts upon fatty acid substrates that are released from the phospholipid membrane in the platelet by cytosolic phospholipase A_2 ($cPLA_2$) [516]. The composition of the phospholipids in

the platelet membrane is dynamic in nature and since many of the fatty acids that make up the phospholipid bilayer are not produced in the body, their content is primarily regulated by dietary intake [517-520]. Although AA makes up a large proportion of the phospholipid content, other fatty acids are also available from the platelet membrane and their content, relative to AA, has been shown to fluctuate depending on diet [518,520,521]. While the catalysis of 12-LOX with AA has been studied previously [398,514,522,523], little is known about the relative 12-LOX kinetic rates of the other fatty acid substrates and their physiological effects on platelet activation. LOX's are known to have promiscuous substrate selectivity and can react with a variety of fatty acids, ranging from 18 to 22 carbons long and having 2 to 6 sites of un-saturation. Based on this knowledge, the current study investigated the substrate specificity and reactivity of 12-LOX with six fatty acids. In addition, the substrates and their 12-LOX products were screened for the potential to regulate platelet reactivity and thrombosis. Interestingly, we were able to determine that some, but not all of the fatty acids and their bioactive metabolites, directly regulate the level of agonist-mediated platelet activation, albeit in a different manner than the AA product, 12(S)-HETE.

8.3 Materials and Methods

Materials: All commercial fatty acids were purchased from Nu-Check prep with purities of 99.0% or greater. HPLC grade solvents were used for both semi-preparative HPLC purification and analytical HPLC analysis of 12-LOX products. Large scale product purification was achieved by using a C18 HAIsil 250 x 10 mm semi preparative column, whereas the C18 HAIsil 250 x 4.6 mm analytical column was used for product separation in tandem with MS/MS analysis. Both columns were purchased from Higgins Analytical (Mountain View, Ca). All other chemicals were reagent grade or better and were used without further purification. PAR1-AP (SFLLRN) was purchased from GL Biochem (Shanghai, China). Aggregometers, chronolume

reagent, collagen, and other aggregation supplies were purchased from Chrono-Log Corp. (Havertown, PA). Anti-Rap1 primary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Thrombin was purchased from Enyzme Research Labs (South Bend, IN). Anti-Rabbit-800nM secondary antibody was purchased from Rockland Antibodies and Assays (Gilbertsville, PA). ADP and Acetylsalicylic acid (aspirin) was purchased from Sigma-Aldrich (St. Louis, MO). PAC1 and CD62P were purchased from BD Biosciences (San Jose, CA). Convulxin was purchased from Centerchem (Stamford, CT).

Large scale generation of 12-LOX products: LOX products were generated by reacting an individual substrate with 12-LOX in 1,000 mL of 25 mM HEPES (pH 8.0) with 25-50 μM substrate and run to completion. Reactions were quenched with 5 mL acetic acid, extracted three times with 30% volume of dichloromethane, evaporated to dryness, and reconstituted in MeOH for HPLC purification. The reduced products were generated by selectively reducing the hydroperoxide product to the alcohol, with trimethylphosphite. Products were purified by HPLC using a C18 HAIsil 250 x 10 mm semi prep column. Solution A was 99.9% MeOH and 0.1% acetic acid; Solution B was 99.9% H_2O and 0.1% acetic acid. An Isocratic gradient of 75% A and 25% B was used to purify products. Products were stored at -80^{o} C for a maximum of 6 months. The purity and identity of the products were confirmed by using a Thermo-Electron LTQ liquid chromatography-tandem mass spectrometer (LC-MS-MS).

Overexpression and Purification of 12-LOX. Human platelet-type 12-LOX was expressed as an N-terminally, His_6-tagged protein and purified to greater than 90% purity, as evaluated by SDS-PAGE analysis [524,525]. Concentration of 12-LOX was determined by using the method of Bradford, with a bovine serum albumin standard, where 1 mg/ml solutions were equivalent. Iron content of 12-LOX was determined with a Finnigan inductively coupled plasma mass

spectrometer (ICP-MS), using cobalt-EDTA as an internal standard. Iron concentrations were compared to standardized iron solutions and used to normalize enzyme concentrations. Protein expression yields and iron content of 12-LOX were similar to previously published results [522,523].

12-LOX Product Identification and Distribution. Product identification was achieved by using a Thermo-Electron LTQ liquid chromatography-tandem mass spectrometer (LC-MS/MS). Product separation was achieved by using a C18 HAIsil 250 x 4.6 mm analytical column from Higgins Analytical. Solution A was 99.9% acetonitrile and 0.1% acetic acid; Solution B was 99.9% H_2O and 0.1% acetic acid. An isocratic gradient of 55% A and 45% B was used to purify products. The identification of 12-LOX products was achieved by comparing MS-MS fragments with known standards at www.lipidmaps.org. In cases where MS-MS fragmentation standards were not available, products were identified by comparing fragment masses with predicted fragment masses mediated by the hydroxy group near an unsaturated carbon [526]. The mechanism is described as charge remote allylic fragmentation (Supplemental Figure S1) and gives rise to a negatively charged carboxylic fragment that can identify the regio-specificity of the product. Product distribution of the products was analyzed by comparing the total ion count of single oxygenated product versus double and triple oxygenated products by parent mass and fragment mass.

Steady-State Kinetic Measurements. LOX rates were determined by following the formation of the conjugated diene product at 234 nm with either a Perkin-Elmer Lambda 40 or a Shimadzu (UV-2401PC) UV-Vis spectrophotometer. Molar extinction coefficients for 12-LOX products of each substrate at 234 nm were measured in the enzymatic buffer and are as follows: 13(S)-HPOTrE (ALA product) $\varepsilon = 22{,}500\ M^{-1}\ cm^{-1}$, 12(S)-HPETE (AA product) $\varepsilon = 22{,}500\ M^{-1}\ cm^{-1}$, 12(S)-HPETrE (DGLA product) $\varepsilon = 14{,}300\ M^{-1}\ cm^{-1}$, 12(S)-HPEPE (EPA product) $\varepsilon = 18{,}400$

M^{-1} cm^{-1}. Molar extinction coefficients were calculated by first weighing the substrate on an analytical balance, then dissolving the substrate with a measured mass of HPLC grade methanol to achieve a stock substrate/methanol solution. Small aliquots of this solution were then diluted into a quartz cuvette containing a Teflon stir bar and 2 mLs of 25 mM HEPES buffer pH 7.5 at room temperature 22^{o} C. Diluted samples were mixed thoroughly for 20 minutes and baseline stability was monitored at 234 nm before proceeding. The substrate buffer solution was reacted with small amounts of purified 12-LOX and the final absorbance at 234 nm was determined. A small aliquot of soybean LOX-1 was added at the end of the reaction to assure all substrate was converted to product. At a minimum three independently weighed substrate measurements and UV-Vis assays were performed and averaged to achieve the extinction coefficients listed above. The extinction coefficient of 12(S)-HPETE determined by this method was within error of the published values by Graff et al., after correcting for differences in solvent conditions [527]. Kinetic assays were carried out in 25 mM HEPES buffer (pH 7.5) with substrate concentrations ranging from 1 μM – 20 μM, and were initiated by the addition of enzyme (approximately 20 nM 12-LOX). The 12-LOX displays variable behavior at low substrate concentrations (< 1 μM), resulting in large errors in the K_M values. To circumvent this inherent problem, we determined that adding the 12-LOX first, and then quickly initiating the reaction with the addition of the appropriate amount of substrate, yielded significantly more reproducible results. Substrate concentrations were quantitatively determined by allowing the enzymatic reaction to go to completion with soybean LOX-1. Kinetic data were obtained by recording initial enzymatic rates at each substrate concentration and then fitting them to the Michaelis-Menten equation using the Kaleida Graph (Synergy) program to determine k_{cat} and k_{cat}/K_M values. It should be noted that the steady state kinetics with AA were slightly higher than our previously published data [523], which

we ascribe to differences in the extinction coefficients used and a more active 12-LOX preparation.

Determination of LOX Product Stereochemistry. Determining the stereochemistry of the secondary alcohol LOX products was achieved by mosher ester analysis [528]. HPLC purified LOX product (evaporated in a glass vial) was reacted with 39 equivalents of anhydrous pyridine, 100 uL of anhydrous deuterated chloroform, and 16 equivalents of either (S)-(+)-α-Methoxy-α-trifluoromethylphenylacetyl chloride or (R)-(–)-α-Methoxy-α-trifluoromethylphenylacetyl chloride. The reaction was completed after one hour and esterfication was checked by thin layer chromatography. Samples were then diluted with deuterated chloroform to a final volume of 700 uL then transferred to a 5 mm NMR tube and both 1D proton and 2D COSY spectra were taken (Varian 600 MHz NMR). The non-modified LOX product was also analyzed in a similar manner for comparison and the proton assignments determined. The differences in proton chemical shifts between R and S mosher esterfied products were tabulated (Supplemental Figures S2-S22). Subtracting the chemical shifts between S and R spectra yields a positive or negative value, which indicates the priority of each side of the alcohol in regards to the Chan Ingold Prelog convention and thus allows the determination of the absolute configuration of the secondary alcohol. A LOX product with known stereochemistry, 13-(S)-HODE from soybean LOX-1 catalysis, was first analyzed by mosher ester analysis before proceeding with the analysis of 12(S)-HETrE and 13(S)-HOTrE. The stereochemistry of the vinyl groups were also assigned based on the proton coupling constants, with the Z-vinyl protons having J-values of approximately 10 Hz and those of the E-vinyl protons having approximately 16 Hz. These coupling values along with the proton signal positions were consistent with the values found for our standard LOX product, 13(S)-HODE.

Human Platelets. Human platelets were obtained from healthy volunteers from within the Thomas Jefferson University community and the Philadelphia area. These studies were approved by the Thomas Jefferson University Institutional Review Board and informed consent was obtained from all donors before blood draw. Washed platelets were prepared as previously described [398,444]. The final platelet concentration of washed platelets in Tyrodes buffer was adjusted to a physiological concentration of 3 X 10^8 platelets/ml. Reported results are the data obtained using platelets from at least three different subjects.

Platelet Aggregation. 250 µl of washed platelets were pretreated with or without varying concentrations (0 to 40 µM) of fatty acid metabolites for 7 minutes followed by stimulation with 20 µM protease-activated receptor-activating peptide (PAR1-AP), 20 µM ADP, or 100 ng/ml convulxin. The aggregation response was measured in real-time using a lumi-aggregometer with stirring at 1100 rpm at 37°C.

Human Platelet Dense Granule Secretion. ATP release was used as a measure of dense granule secretion in washed platelets. For ATP studies, 240 µl aliquots of washed platelets were pretreated with or without varying concentrations of fatty acid metabolites for 7 minutes. After addition of 10 µl of chronolume reagent, ATP release in response to 20 µM PAR1-AP was measured in real-time using a Lumi-aggregometer at 37°C with stirring at 1100 rpm.

Measurement of Rap1 Activity. Rap1 activity was measured using GST-RalGDS-Rap1-binding domain that specifically interacts with activated Rap1 as described elsewhere [479,529]. Activated Rap1 was detected by immunoblotting with the anti-Rap1 antibody. In parallel experiments using whole platelet lysate, Rap1 expression was analyzed to confirm equal protein loading.

Flow cytometric measurements of αIIbβ3 and P-selectin. αIIbβ3 activity and surface expression of P-selectin were measured by flow cytometry with the FITC- PAC1 (an antibody

which only recognizes the active conformation of αIIbβ3) and PE-conjugated CD62P, which recognizes P-selectin on the surface of the cell. 40 µl aliquots of washed platelets adjusted to a final concentration of 2.5 X 10^7 platelets/ml were pretreated with metabolites for 15 minutes followed by addition of 2 µl PAC1 and 2µl CD62P. Platelets were stimulated with agonist for 10 minutes and diluted to a final volume of 500 µl using tyrodes buffer. The fluorescence intensity of platelets was immediately measured using an Accuri flow cytometer.

Clot Retraction Assay. Clot retraction experiments were performed as previously described [530]. Briefly, platelet rich plasma (PRP) was adjusted to a platelet count of 3 X 10^8 platelets/ml in glass tubing. Following treatment with fatty acids or metabolites, clot formation and retraction was initiated with 10 nM thrombin. Pictures were taken of the clots at 10 minute intervals and the size of the clot was quantified using Image-J software from NIH. Statistical significance was determined by student t-test.

Statistical Analysis. Comparison between experimental groups was made using *paired t-test or 2-way ANOVA* with post-test analysis with the software program, Prism. Differences in mean values ± standard error of the mean were considered significant at $p < 0.05$.

8.4 Results

12-LOX Product Identification and Distribution

Of the six fatty acids tested as substrates for 12-LOX catalysis, two fatty acids, linoleic acid (LA) and eicosapentaenoic acid (EDA), were found not to be suitable as substrates for 12-LOX. This is possibly due to improper positioning of the activated methylene, whose position near the active site iron is critical for hydrogen atom abstraction. As shown in Figure 8.1, the substrates are positioned with the methyl-end of the substrate entering the cavity first, as previously determined [531]. In this configuration, the activated methylene moieties for EDA and LA are not

positioned properly due to length for the iron-hydroxide moiety to abstract a hydrogen atom. However, it should be noted that other factors, such as conjugation could also play an important part in substrate activity. Of the four other fatty acids that were screened, AA, dihomo-gamma-linolenic acid (DGLA), eicosapentaenoic acid (EPA) and alpha-linolenic acid (ALA), they all produced over 90% of a single oxygenated product, whose retention time, parent peak mass, and main fragmentation peaks are listed in Tables 8.1 and 8.2. Interestingly, ALA produces predominately one product, 13(S)-HPOTrE, but it is unclear how it is positioned for hydrogen atom abstraction (*vide infra*). The methyl-end first binding of ALA positions it in a similar manner to that of LA and EDA, however those fatty acids are not substrates.

Substrate	Product Name
AA	12(S)-HPETE (99.0%)
DGLA	12(S)-HPETrE (99.0%)
EPA	12(S)-HPEPE (99.0%)
ALA	13(S)-HPOTrE (99.0%)
EDA	No reaction
LA	No reaction

Table 8.1 Primary product distribution.
The primary products for 12-LOX are singly oxygenated species. The percent distribution was determined by tabulating total ion count of the singly oxygenated species in comparison with double or triply oxygenated species. It should be noted that the S-configuration of 12(S)-HPEPE was not directly determined but assumed, since the configuration of 12(S)-HPETrE and 13(S)-HPOTrE was determined directly.

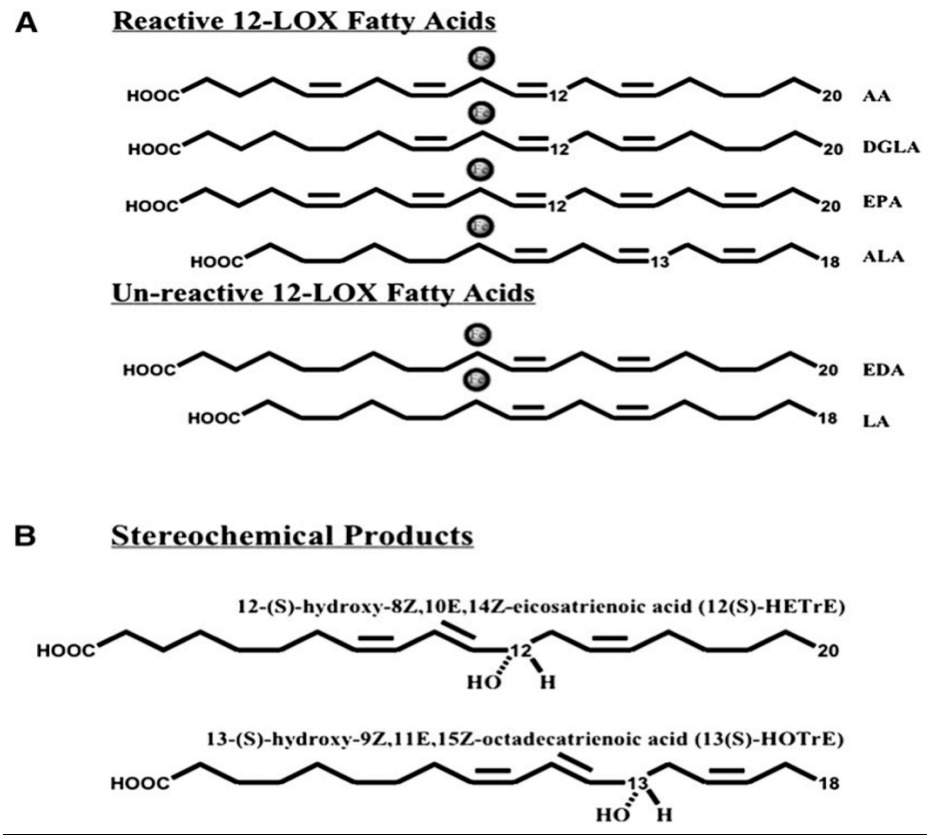


Figure 8.1 12-LOX product regio-specificity and substrate comparison.
A) The substrates are positioned methyl-end first, relative to the active site iron. The carbon atoms that are labeled with numbers indicate location of oxygenation. **B)** Stereochemical structures of 12(S)-HETrE and 13(S)-HOTrE. This illustration was provided by our collaborator Dr. Theodore Holman's group.

Kinetics of 12-LOX

The kinetics of 12-LOX with the four active substrates were measured and reported in Table 3. The k_{cat} and k_{cat}/K_M values indicate that the substrates can be categorized into two groups. The longer substrates, with 20 carbon atoms (AA, DGLA, and EPA) have over 10-fold greater k_{cat} and k_{cat}/K_M values than the shorter substrate, with 18 carbon atoms (ALA), suggestive that length is a key factor in the rate of substrate capture and product release. These data could be due to the fact that ALA appears not to have an activated methylene positioned for hydrogen atom abstraction (*vida* supra, Figure 8.1), and thus ALA may have a less than optimal binding conformation.

Determination of LOX Product Stereochemistry

LOX's typically generate one stereospecific product due to the antrafacial oxygen insertion [532]. It has been previously reported that 12-LOX generates an S-configured product (12(S)-HPETE) when AA is used as a substrate [525]. DGLA is similar to AA with respect to length, and one would predict that the oxygenated DGLA product (12(S)-HPETrE) would have the same stereochemistry as the oxygenated AA product (12(S)-HPETE), due to the stereospecific nature of the active site. Mosher ester analysis of the reduced 12(S)-HETrE product indicates that this is indeed the case and it does have an S-configuration, secondary alcohol. For 13(S)-HPOTrE, the 12-LOX product of ALA, the stereochemistry was determined in the same manner and found to also be of an S-configuration. The stereochemistry of the vinyl groups were assigned based on the proton signal positions and their coupling constants, as compared to our standard, 13(S)-HODE. The 12(S)-HETrE configuration was determined to be 12-(S)-hydroxy-8Z,10E,14Z-eicosatrienoic acid and the 13(S)-HOTrE configuration was 13-(S)-hydroxy-9Z,11E,15Z-octadecatrienoic acid (Figure 8.1b).

12-LOX Reduced Product (Substrate)	Retention Time (Min)	Mass (Da)	MS-MS Fragment Mass (Da)
12(S)-HETE (AA)	44.97	319	179
12(S)-HETrE (DGLA)	54.53	321	181
12(S)-HEPE (EPA)	28.09	317	179
13(S)-HOTrE (ALA)	21.73	293	195

Table 8.2 Primary Product Identification by Mass Spectrometry.
The retention times are for the method described in the text, with subsequent MS/MS analysis. The mass of the primary identifying fragment is listed.

Human Platelet Aggregation Dependence of Fatty Acid Metabolites

Fatty acid metabolites play an important role in regulation of platelet activation and normal hemostasis. Although AA is widely studied in platelets, 12-LOX may oxidize a number of other fatty acids on the platelet membrane following stimulation [476,533]. Since thrombin is the most

potent activator of platelet function, we investigated the ability of these metabolites to modulate platelet activation following stimulation of the thrombin receptor, protease-activated receptor-1 (PAR1). The hydroperoxide metabolites of 12-LOX catalysis are reduced to hydroxymetabolites in the presence of peroxidases, therefore the hydroxymetabolites were evaluated for their potential regulation of human platelet function. Platelets were treated with increasing concentrations of each fatty acid metabolite followed by stimulation with 20 μM PAR1-activating peptide (PAR1-AP) and subsequent platelet aggregation was measured. PAR1-AP alone resulted in more than 80% aggregation in less than one minute, which was stable for the duration of the experiment (Figure 8.2). Platelet aggregation was sensitive to treatment with 12(S)-HETrE, showing inhibition in the presence of 40 μM 12(S)-HETrE (Figure 8.2a), a clinically relevant concentration following fatty acid supplementation [534,535]. Not all metabolites were shown to attenuate platelet aggregation, as 12(S)-HEPE and 13(S)-HOTrE had no observable effect on PAR1-AP-mediated aggregation. Even at higher concentrations, only 12(S)-HETrE was shown to attenuate PAR1-AP-induced platelet aggregation (Figure 8.2b). In comparison, exposure to increasing concentrations of 12(S)-HETE, the predominant product of AA and 12-LOX in human platelets, did not result in attenuation of PAR1-AP-mediated platelet aggregation at any concentration tested. This is consistent with the observation that 12(S)-HETE is pro-thrombotic in human platelets [329].

Human Platelet Dense Granule Secretion is Sensitive to Fatty Acid Metabolites

Thrombin activation of human platelets involves a number of biochemical steps. One of the biochemical steps important for the stability of clot formation is secretion of the dense granule which contains small molecules that are essential for autocrine and paracrine reinforcement of the platelet clot (including ATP, ADP, and 5-HT). PAR1-AP-mediated ATP secretion was

measured as a surrogate for dense granule secretion (Figure 8.3). Only the presence of increasing concentrations of 12(S)-HETrE resulted in complete inhibition of dense granule secretion clearly showing 12(S)-HETrE signals in a specific, dose-dependent manner, unlike the other metabolites tested.

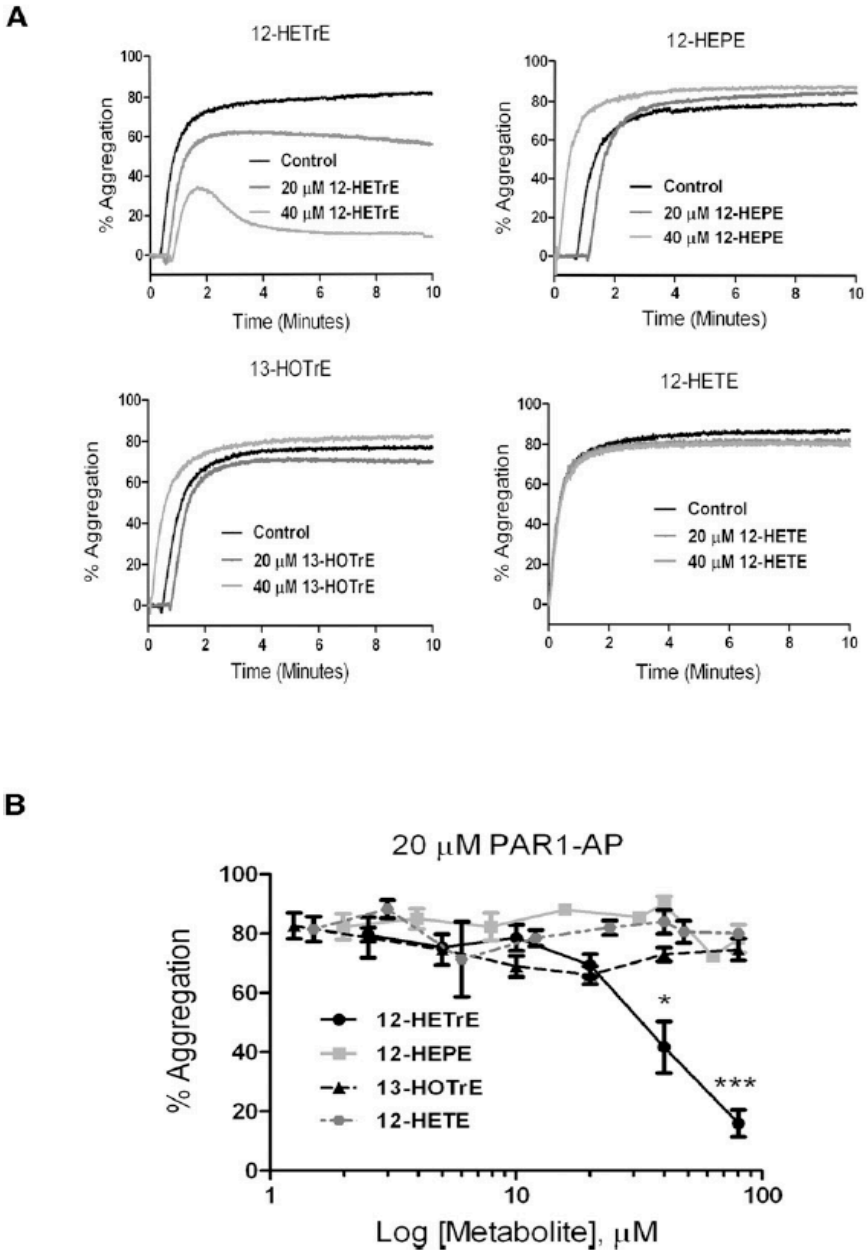


Figure 8.2 Fatty acid metabolites regulate platelet aggregation.

Washed human platelets were treated with or without increasing concentrations of the exogenously added fatty acid metabolites 12(S)-HETrE, 12(S)-HEPE, 13(S)-HOTrE, or 12(S)-HETE. a) Representative curves for platelets treated with 20 μM PAR1-AP in the absence (control) or presence of each metabolite. The level of platelet aggregation was measured for 10 minutes post-stimulation (N=3 for each condition). b) Composite for all replicates in the presence of increasing concentrations of each metabolite ranging from 0 to 80 μM (N=3). Replicates were graphed for the maximal level of platelet aggregation 10 minutes post-stimulation for each condition. *, P<0.05; ***, P=0.002.

Substrate	k_{cat} (sec^{-1})	k_{cat}/K_M (sec^{-1} μM^{-1})
AA	12 (0.23)	18 (1.9)
DGLA	19 (0.91)	9.3 (1.8)
EPA	8.4 (0.30)	19 (4.8)
ALA	1.3 (0.08)	0.36 (0.08)
EDA	No reaction	
LA	No reaction	

Table 8.3 Steady state kinetics of substrates.
Kinetic measurements were measured in 25 mM HEPES buffer (pH 7.5) at room temperature. Error values are shown in parentheses.

DGLA Inhibits PAR-Mediated Platelet Activation

It is possible that although the bioactive metabolite 12(S)-HETrE inhibits platelet aggregation, the substrate responsible for its formation, DGLA, is not oxidized by 12-LOX in the platelet and therefore has no effect on platelet function. To confirm that the observed inhibition of platelet activation in the presence of 12(S)-HETrE is due to 12-LOX oxidation of DGLA, 5 μM DGLA was added to washed platelets followed by addition of 20 μM PAR1-AP (Figure 8.4a). When the platelets were incubated with DGLA for 10 minutes followed by addition of PAR1-AP, maximal platelet aggregation was attenuated and quickly reversed within 1 minute following addition of PAR1-AP. This observation is similar to an earlier study which showed that addition of DGLA to the human platelet resulted in a partial inhibition of collagen-mediated platelet aggregation [536]. Likewise, DGLA completely inhibited PAR1-AP-mediated dense granule secretion similar to what was observed when treating platelets with the reduced 12-LOX oxidation product of DGLA, 12(S)-HETrE (Figures 8.2 and 8.3). Finally, to confirm DGLA regulates platelet aggregation in part through 12-LOX, washed platelets were stimulated with PAR1-AP following treatment with DGLA or DGLA in combination with baicalein (12-LOX

inhibitor) or aspirin. The absence of either 12-LOX or COX-1 activity resulted in a partial rescue of platelet aggregation (Figure 8.4a).

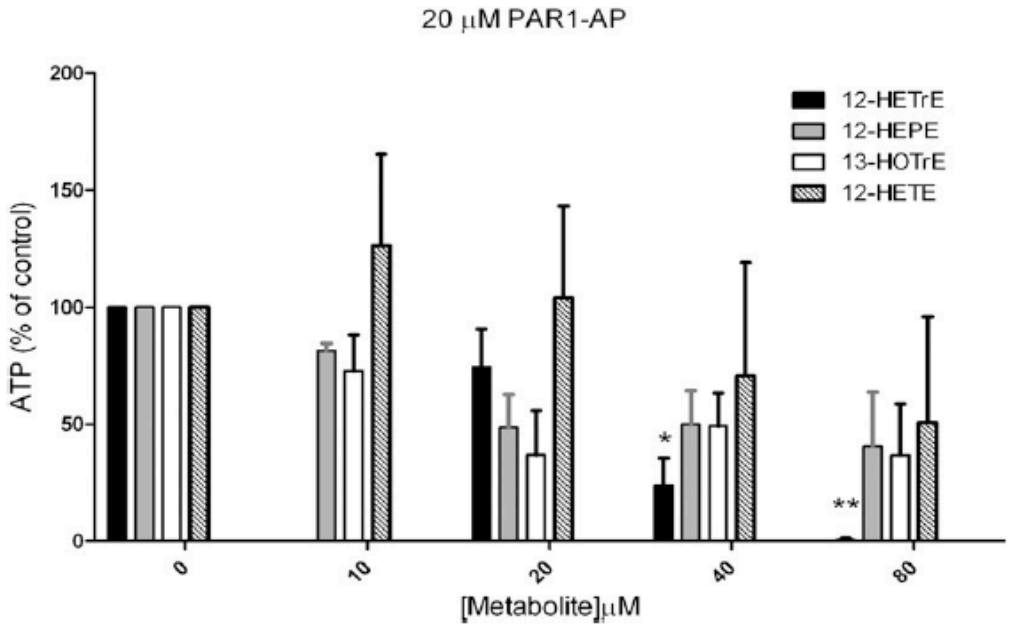


Figure 8.3 Fatty acid regulation of platelet dense granule secretion.
Washed human platelets were treated with or without increasing concentrations of the exogenously added fatty acid metabolites 12(S)-HETrE, 12(S)-HEPE, 13(S)-HOTrE, or 12(S)-HETE. The maximal level of ATP secretion was measured post-stimulation (N=3 independent donors for each condition). *, P<0.05; **, P<0.01.

12-LOX metabolite inhibition of ADP- and GPVI-induced platelet activation

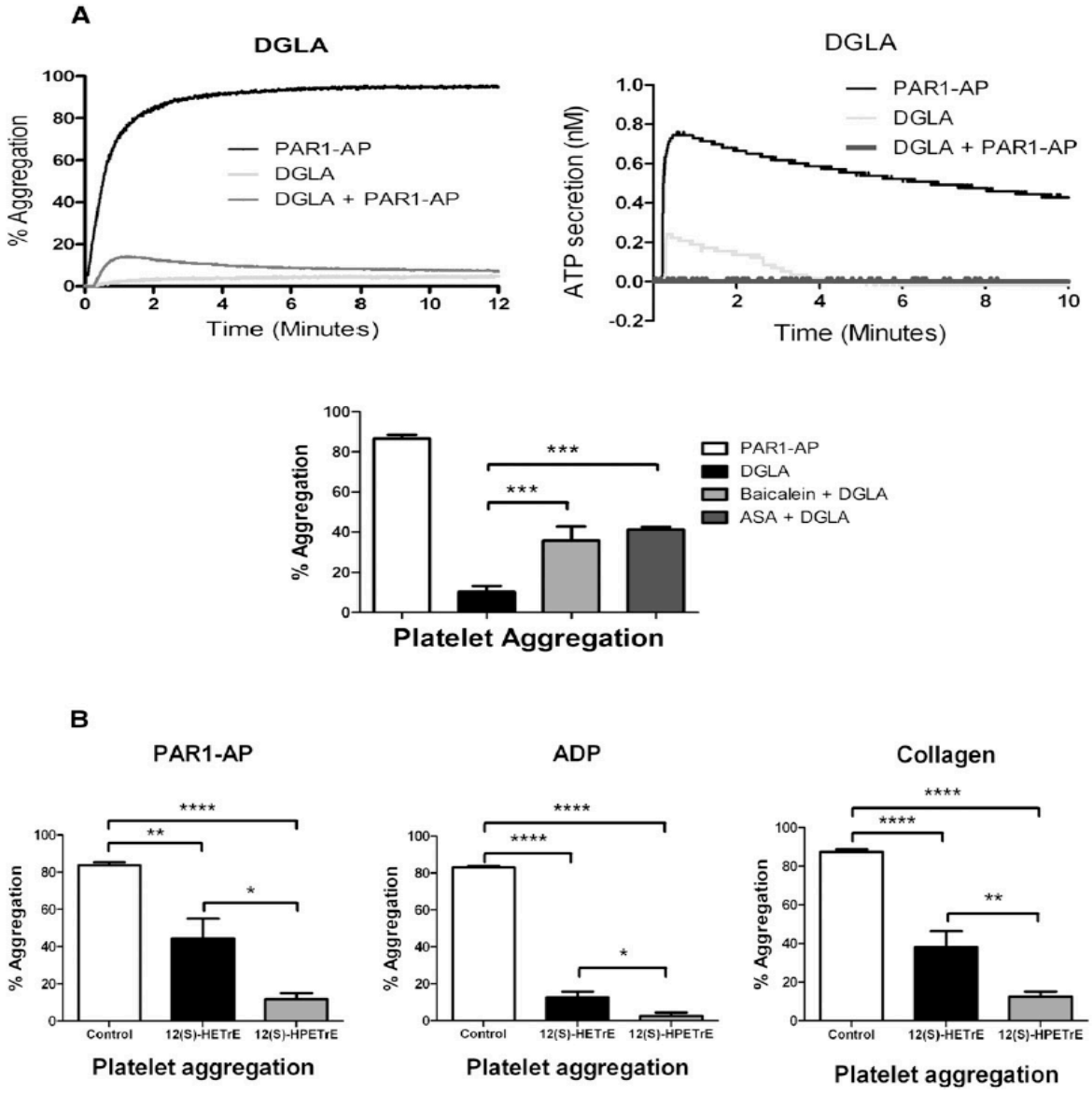
To identify if 12-LOX eicosanoids produced from DGLA regulate platelet activation through multiple agonist pathways, platelets were treated with either 12(S)-HETrE or its peroxidated precursor, 12(S)-HPETrE, followed by stimulated with PAR1-AP, collagen, or ADP (Figure 8.4b-d). Similar to the observed sensitivity to 12(S)-HETrE in Figure 8.2, platelet aggregation induced by ADP and collagen were attenuated following treatment with 12(S)-HETrE. Additionally, treatment with 12(S)-HPETrE significantly attenuated platelet aggregation following stimulation with ADP, collagen, or PAR1-AP (Figure 8.4b). To determine if these eicosanoids affected activation of the integrin αIIbβ3, an essential integrin in the activation of platelets, platelets treated with either eicosanoid were stimulated with ADP, PAR1-AP, or

convulxin (direct activator of the collagen receptor GPVI). αIIbβ3 activation was attenuated in the presence of either 12(S)-HETrE or 12(S)-HPETrE (Figure 8.4c). Finally, to identify if alpha granule secretion is affected by the presence of 12(S)-HETrE or 12(S)-HPETrE, P-selectin (a marker of alpha granule secretion) surface expression was measured following treatment with either eicosanoid (Figure 8.4d). Treatment with either eicosanoid attenuated alpha granule secretion following stimulation with ADP, PAR1-AP, or convulxin. While both metabolites attenuate platelet function, 12(S)-HPETrE was found to have a lower IC_{50} compared to 12(S)-HETrE for inhibition of platelet aggregation (5 μM versus 40 μM, respectively). Considering the only difference between 12(S)-HPETrE and 12(S)-HETrE is the their oxidation state, it is possible that either the structural difference between the hydroperoxide and the alcohol affect receptor binding or that the increased reactivity of the hydroperoxide could generate a different species (i.e. oxidation to the ketone). These two explainations are currently being investigated further.

Bioactive Metabolite Regulation of Rap1 Activity

Platelet activation is regulated in large part by the small GTPase, Rap1 [529]. To identify if 12-LOX metabolites from the various fatty acids regulate the level of Rap1 activation, washed platelets treated with or without 40 μM of each metabolite (12(S)-HETE, 12(S)-HETrE, 12(S)-HEPE, or 13(S)-HOTrE) or 10 μM fatty acid (AA, DGLA, EPA, LA, or EDA) followed by stimulation with PAR1-AP for 5 minutes (Figure 8.5). The level of Rap1 activation was measured as a fold change relative to the unstimulated condition. While PAR1-AP induced a large increase in the level of active Rap1, treatment with 12(S)-HETrE prior to PAR1-AP stimulation fully inhibited Rap1 activation (Figure 8.5a). No significant difference in the level of

Rap1 activity was observed with the other metabolites tested (N=3). Similarly, when platelets were treated with fatty acids prior to stimulation with PAR1-AP, DGLA completely inhibited Rap1 activation while treatment with the other fatty acids did not appear to significantly alter the level of PAR1-AP-induced Rap1 activation (Figure 8.5b).



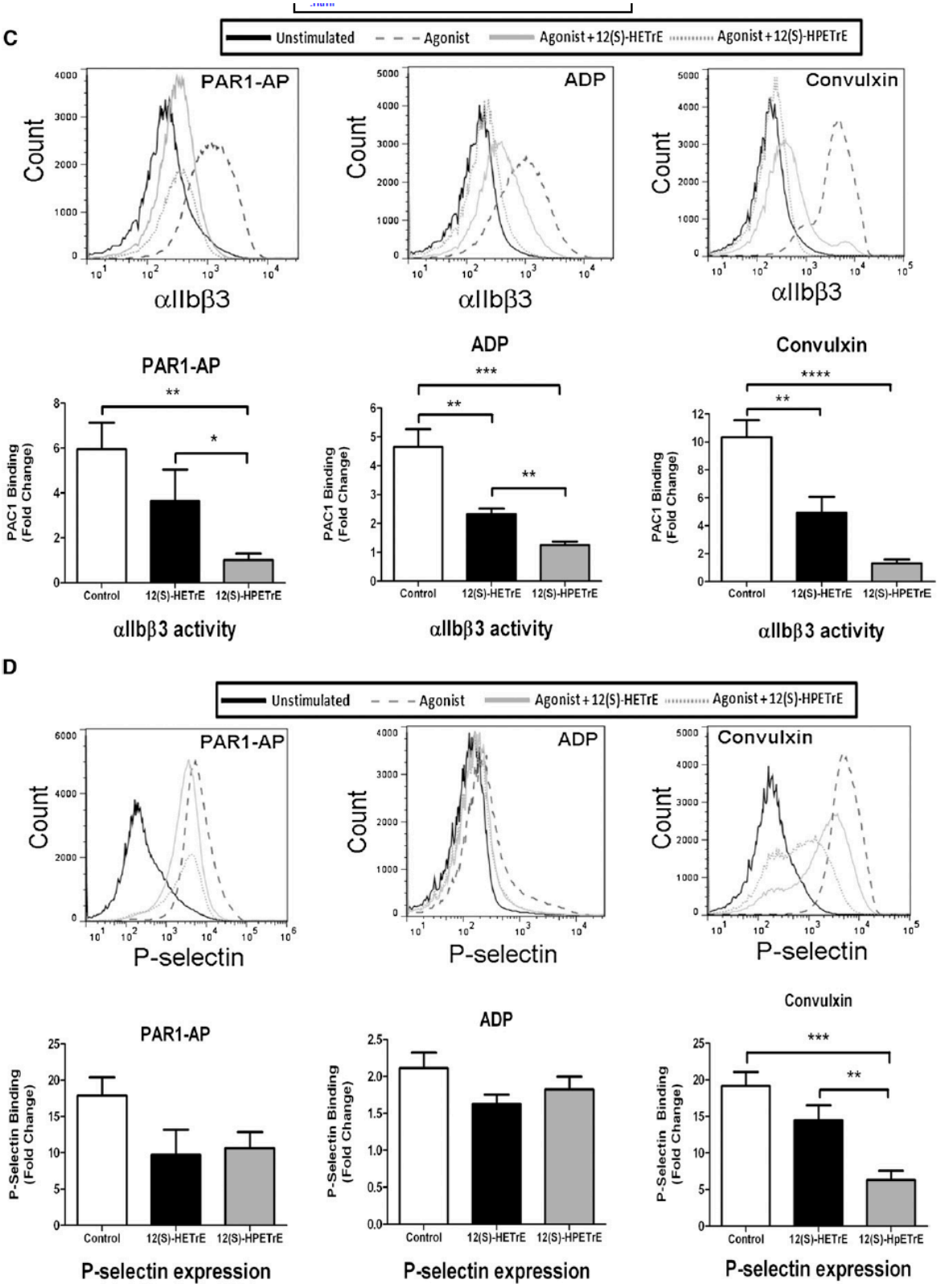


Figure 8.4 Agonist-independent regulation of platelet activity by 12-LOX metabolites.

a) Washed human platelets were treated with or without 5 μM DGLA for 10 minutes followed by stimulation with 20 μM PAR1-AP (PAR1-AP) for 10 minutes. PAR1-AP induced an immediate and stable platelet aggregation when added to platelets. Addition of vehicle (DMSO) had no effect on platelet activation. Maximal and final platelet aggregation following treatment with DGLA was significantly attenuated. Additionally, treatment of DGLA resulted in inhibition of dense granule secretion in the presence of PAR1-AP (N=3). b) Platelet aggregation was measured following 10 minutes stimulation with PAR1-AP, 20 μM ADP, or 5 μg/ml collagen in the presence of 40 μM 12(S)-HPETrE or 40 μM 12(S)-HETrE (N=4-9). c) αIIbβ3 activation was measured by flow cytometry following 10 minutes stimulation with PAR1-AP, ADP, or 100 ng/ml convulxin in the presence of 12(S)-HPETrE or 12(S)-HETrE (N=4-10). d) α-granule secretion was assessed by measuring P-selectin surface expression on the platelet by flow cytometry following 10 minutes stimulation with PAR1-AP, ADP, or 100 ng/ml convulxin in the presence of 12(S)-HPETrE or 12(S)-HETrE (N=4-10). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

DGLA and EPA Prevent Clot Retraction

As Rap1 is an upstream regulator of integrin activation in platelets, we sought to determine if the fatty acids regulate integrin-dependent platelet-mediated clot retraction. To determine if any of the fatty acids play a role in clot retraction, which involves the consolidation of integrin-dependent retraction of platelets within the clot [537], platelet-rich-plasma (PRP) was treated with each fatty acid (Figure 8.6). 10 nM thrombin was added to the PRP for each condition in the presence or absence of 25 μM fatty acid (Figure 8.6a) and a picture of the clot was taken at several time points following stimulation. The size of the clot was quantified using NIH Image J software. The PRP clotted with thrombin in the absence of fatty acid within 60 minutes. Following treatment with DGLA or EPA, clot retraction was significantly delayed and attenuated. However, pre-treatment with AA, LA, or EDA had no effect. To identify if DGLA inhibition of clot retraction was due to COX-1 or 12-LOX activity, platelets were treated with aspirin, 12(S)-HPETrE, aspirin + 12(S)-HPETrE, or aspirin + DGLA, followed by stimulation with thrombin (Figure 8.6b). Inhibition of COX-1 with aspirin did not significantly inhibit thrombin-induced clot retraction. Additionally, aspirin did not rescue clot retraction in the presence of DGLA. However, treatment with high concentration of 12(S)-HPETrE (200 μM) in the presence or absence of aspirin significantly inhibited thrombin-induced clot retraction suggesting that 12-LOX oxidation of DGLA plays at least a partial role in DGLA inhibition of platelet clot retraction.

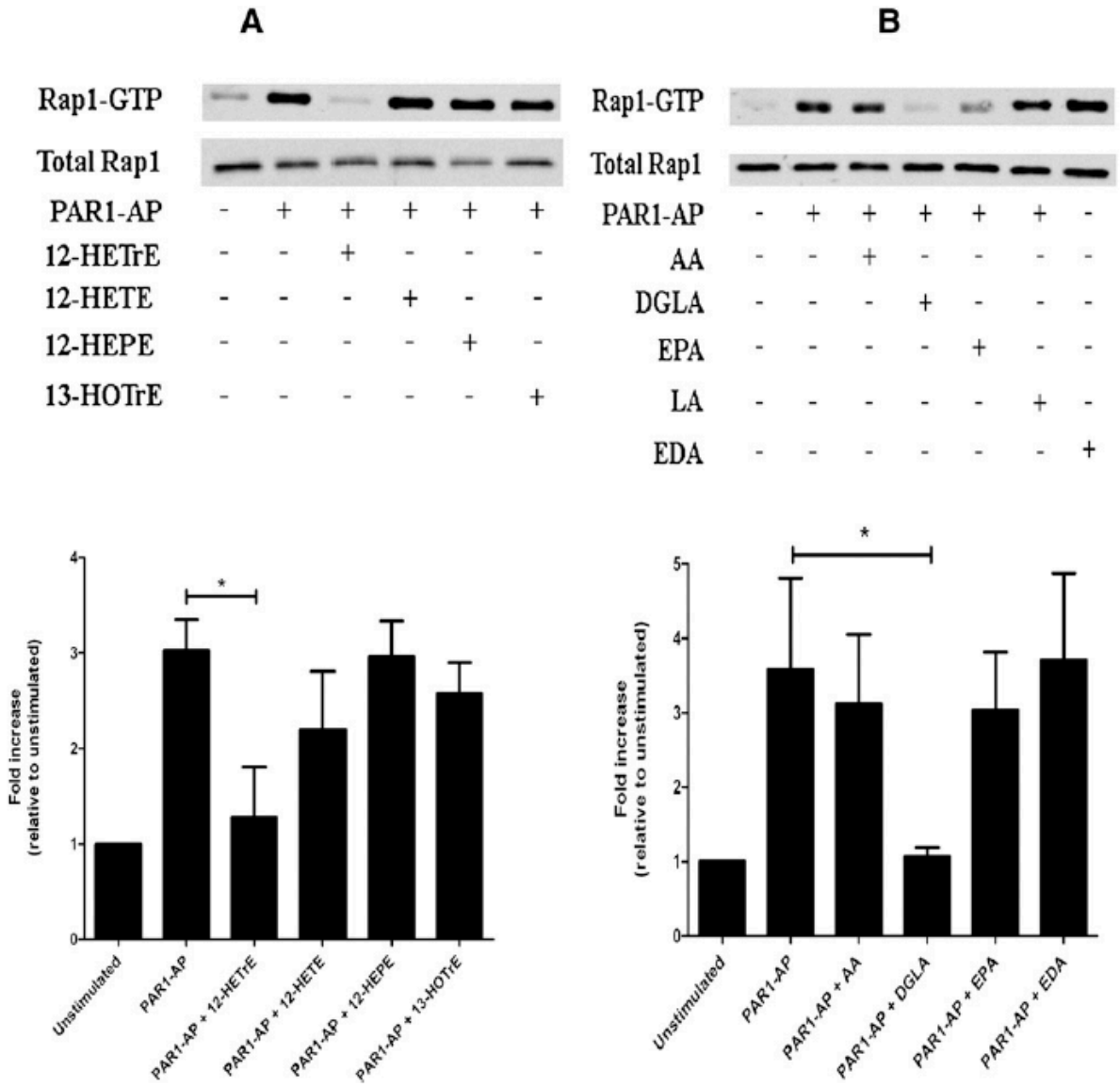


Figure 8.5 12-LOX metabolite regulation of Rap1.
Washed platelets were treated with 12-LOX metabolites or various fatty acids for 10 minutes prior to stimulation with 20 μM PAR1-AP in order to determine their regulatory effects on the level of PAR1-AP-mediated Rap1 activation. a) Platelets were treated with or without 40 μM 12(S)-HETrE, 12(S)-HETE, 12(S)-HEPE, or 13(S)-HOTrE for 10 minutes. Following 12-LOX metabolite treatment, Rap1 activation was measured after stimulation with PAR1-AP for 5 minutes (N=3 independent experiments). PAR1-AP alone induced a significant increase in Rap1-GTP levels. Treatment with 12(S)-HETrE significantly attenuated PAR1-AP-induced Rap1 activation. Treatment with 12(S)-HETE, 12(S)-HEPE, or 13(S)-HOTrE had no significant effect on PAR1-AP-induced Rap1 activation. b) Platelets were treated with or without 10 μM AA, DGLA, EPA, LA, or EDA for 10 minutes. Following fatty acid treatment, Rap1 activation was measured after stimulation with PAR1-AP for 5 minutes (N=6). Treatment with DGLA and EPA significantly attenuated PAR1-AP-induced Rap1 activation. Treatment with AA, LA, or EDA had no significant effect on the level of Rap1 activity. *, $P < 0.05$. This figure was co-provided by Jennifer Yeung and Joanne Vesci

8.5 Discussion

Although fatty acid regulation of hemostatic function, including platelets, has been studied by various research groups [519], a consensus is lacking on the importance of fatty acid intake to the role of platelets in regulating hemostasis. This is in part due to the small number of studies, small number of subjects in each study, and the differences in study design between research groups. However, human subject studies support an important role in the type of fatty acid supplemented in the diet to overall regulation in platelet function and hemostasis [518,538]. GLA supplementation has been shown to increase DGLA content in the serum by 3-fold, as GLA is quickly converted to DGLA enzymatically [518]. While a significant increase in the amount of DGLA was shown to be incorporated into the neutrophil membrane, no observable change in AA was measured suggesting DGLA incorporation does not displace AA on the membrane [518].

DGLA was initially identified as a fatty acid which produced potentially inhibitory metabolites such as PGE_1 through COX-1 and 12(S)-HETrE through 12-LOX [539,540]. Although PGE_1 is known to be a potent anti-platelet eicosanoid, its role in platelet function deriving from DGLA as a substrate is controversial with research groups identifying that while PGE_1 and thromboxane B_1 are produced by DGLA through COX-1, their levels are much smaller than that of 12-hydroxyheptadecadienoic acid and may not play a significant role in DGLA-induced inhibition of platelet activity [277,536]. 12(S)-HETrE formation through 12-LOX oxidation of DGLA, which has not been as widely studied, was shown here to play an important role in DGLA-mediated inhibition of platelet function by significantly attenuating a number of biochemical endpoints. Hence, inhibitory activity of DGLA is mediated through a number of eicosanoids produced from both 12-LOX and COX-1 and formation of these metabolites results in attenuation of agonist-induced platelet activation.

Polyunsaturated fatty acid content in the platelet membranes varies, depending on diet. The most abundant phospholipid in the platelet is phosphatidylcholine, containing a number of polyunsaturated fatty acids (PUFAs) including AA (13.5%), LA(7.9%), DGLA (2.1%), EDA (0.6%), docosahexaenoic acid (DHA) (0.6%), EPA (0.2%), ALA (0.1%), and gamma-linolenic acid (GLA) (0.07%) [517]. Our laboratories investigated the kinetic and biological effects of these fatty acids as potential 12-LOX substrates. Other PUFAs found in the platelet such as DHA and GLA, displayed multiple enzymatic products and due to this complexity will be the subject of subsequent investigations. From this work, it was determined that 12-LOX has selective substrate preferences, with two fatty acids not being substrates (LA and EDA) and 4 producing only one oxygenated fatty acid (AA, DGLA, ALA, EPA). The fatty acids with 20 carbon atoms and more than 2 sites of unsaturation had the fastest k_{cat}/K_M values. These relative kinetic values can be directly related to fatty acid metabolism in the platelet, where substrate concentration is low. ALA is 10-fold slower than the other four fatty acid substrates, which could be indicative of a distinct binding mode. In analysis of the placement of these substrates in the active site, with the substrate binding methyl-end first, as previously determined [531], there is no activated methylene close to the iron-hydroxide moiety for LA, ALA and EDA (Figure 8.1). This is in contrast to what is seen for AA, DGLA, and EPA, where an activated methylene is located in close proximity to the iron. Therefore, to position the C10 of ALA close to the iron to generate the 13-product, the substrate would have to be inserted only partially into the active site. In contrast, 12-LOX does not react with LA, so it appears that the additional ω-3 unsaturation for ALA affects its positioning in the active site, possibly by allowing the carboxylate or pi-bond active site interaction to supersede the methyl-end active site interaction [541], leading to partial insertion of ALA into the active site. Another possible explanation could be that ALA binds in

the opposite direction, with its carboxylate moiety entering the active site first producing the R-isomer of the product, which has been previously seen with soybean LOX-1 [542]. This hypothesis, however, is unlikely since only the S-isomer of 13(S)-HPOTrE is observed, which supports the partial substrate active site insertion hypothesis.

In addition to being catalyzed at different rates, the effects of these 12-LOX products on the platelets were also markedly different. Following treatment with PAR1-AP, 12(S)-HETrE significantly attenuated platelet aggregation, the release of ATP, and Rap1 activation at 40 μM concentration. This result is in contrast to comparable concentrations of 12(S)-HETE, where agonist-mediated aggregation and ATP release were potentiated, suggesting a substrate specific regulation of 12-LOX in platelet reactivity. Under ex vivo conditions, platelets spiked with high levels of DGLA or its 12-LOX metabolites inhibited platelet activation following stimulation with a number of agonists. While it may be possible to reach circulating fatty acid concentrations of 40 μM following fatty acid supplementation [534], it is unlikely that all of the DGLA would be incorporated into the platelet membrane and converted by 12-LOX to 12(S)-HETrE as several 12-LOX products have been previously identified through catalysis of DGLA [540]. Further, the readout of these assays may be overestimated due to the fact that endogenous AA is present in the platelet leading to production of 12(S)-HETE. The endogenous 12(S)-HETE could then compete with the exogenous 12(S)-HETrE, thus counterbalancing both of their effects and dampening the inhibitory effect of 12(S)-HETrE on the platelet. Nonetheless, 12(S)-HPETrE is over 8-fold more potent than that of 12(S)-HETrE, making its concentration biologically relevant. However, due to the high level of peroxidases present in the platelet, the biological effect of 12(S)-HPETrE on the platelet clot may be limited to localized regions of metabolite production. Taken together, these observations further support a potential role for unique pools

of AA and DGLA in the platelet which are independently regulated to mediate either a pro-thrombotic or anti-thrombotic environment dependent on a number of factors including 12-LOX localization, fatty acid compartmentalization and specific agonist used [398].

The mechanism by which these fatty acids regulate platelet function through catalysis by 12-LOX is not well understood. While the receptor for 12(S)-HETE has been recently identified [543], the underlying mechanism by which other metabolites, such as 12(S)-HETrE, regulate the platelet are unknown. Here we have shown that 12(S)-HETrE, as well as its substrate, DGLA, inhibit Rap1 activation. Rap1 is an essential GTPase involved in regulation of platelet function and inhibiting its activation in the presence of a strong activating agonist such as PAR1-AP suggests an important regulatory role for 12-LOX metabolites such as 12(S)-HETrE in mediating this process. Our previous work showing direct inhibition of 12-LOX activity attenuates calcium mobilization [507], a key step in activation of Rap1, further supports a role for 12-LOX products in regulation of platelet function through Rap1. Additionally, since Rap1 is known to be important for integrin activation, it is not surprising that addition of DGLA results in attenuation of thrombin-induced clot retraction [530]. Finally, 12-LOX metabolite formation from catalysis of DLGA attenuates platelet aggregation, integrin αIIbβ3 activation, and alpha granule secretion. Inhibition of these biochemical endpoints in the presence of 12(S)-HPETrE and 12(S)-HETrE supports a potentially protective role for 12-LOX metabolites in preventing unwanted platelet activation resulting in occlusive thrombotic events. As both the peroxidated and non-peroxidated products of DGLA attenuate platelet function and DGLA inhibition of platelet activity is stronger than either of the 12-LOX products, it is likely that multiple 12-LOX-dependent and independent products of DGLA are involved in protection against platelet activation and thrombosis [540,544,545].

It has long been postulated that fatty acid content in the diet can affect human health [546]. Dietary fatty acid formulation has been shown to inhibit leukotriene biosynthesis [547] and DGLA specifically can be converted to the anti-inflammatory prostaglandin, PGE_1, by COX [548], which is implicated in the inhibition of platelet activation [545] and in diminishing atherosclerosis [549,550] and ocular discomfort symptoms [551]. In this study, we have shown a direct anti-platelet phenotype of the 12-LOX product of DGLA, which supports the role of this and potentially other fatty acids as protective agents against unwanted platelet activation and thrombosis. Considering the recent de-orphanization of GPR31 as the 12(S)-HETE receptor [543], the data presented here supports the possibility that a unique receptor may exist on the surface of the platelet which, like PGE_1, may act as a protective signal inhibiting excessive platelet activation and thrombosis. Given the similar 12-LOX kinetics against AA and DGLA and the fact that the concentration of DGLA can become elevated in the blood with specific diets [518,552], it is possible that the level of coagulation and thrombosis in circulating blood could be regulated in part by modifications in the diet [553,554]. Currently, we are testing this hypothesis in animal models in the hopes of confirming both the beneficial and detrimental roles of 12-LOX in blood coagulation as well as the level of metabolite produced in vivo.

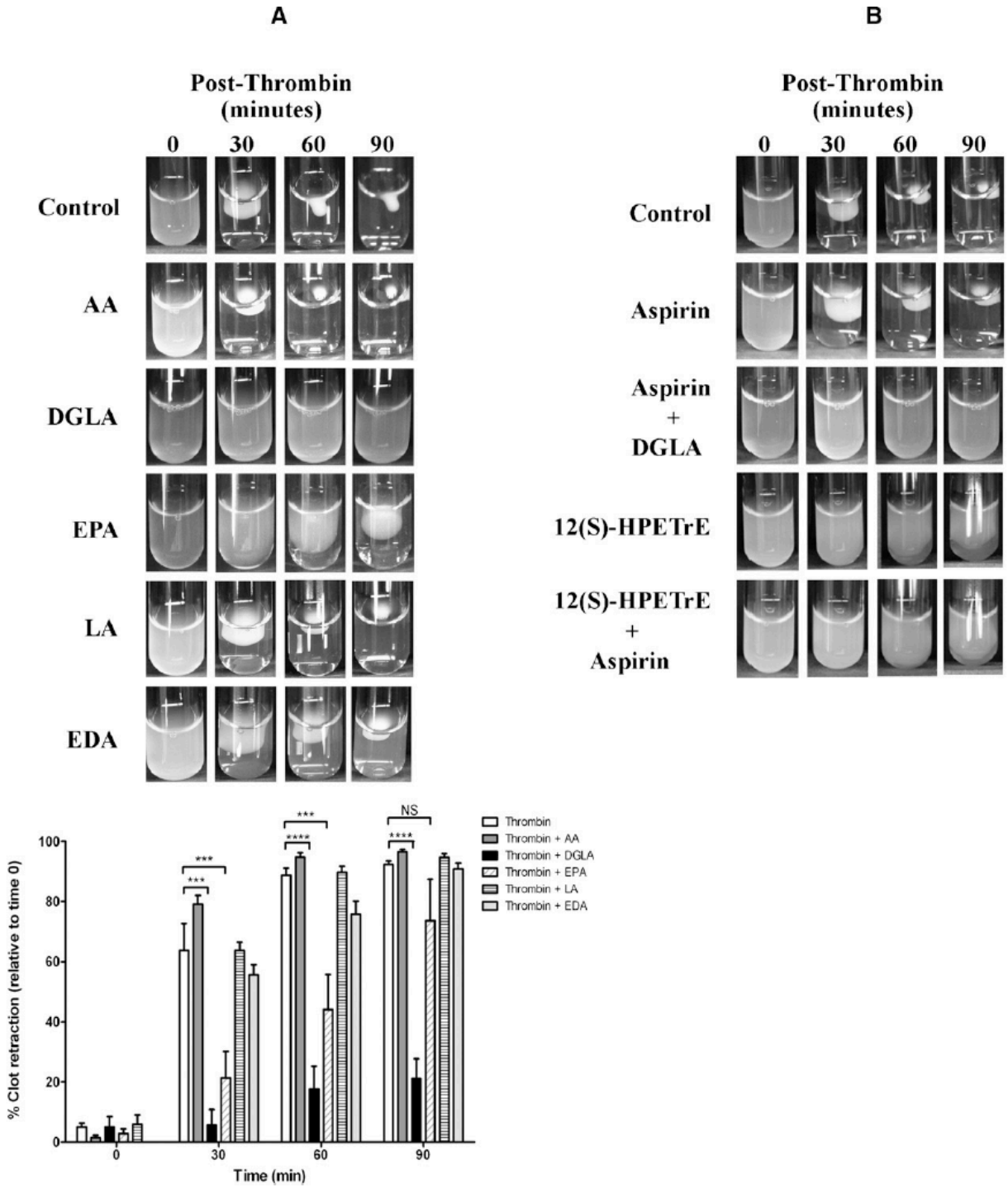


Figure 8.6 Fatty acid regulation of platelet-mediated clot retraction.
Platelet rich plasma (PRP) was treated with fatty acids, inhibitors, or eicosanoids followed by stimulation with thrombin. The rate at which thrombin caused a platelet-dependent clot retraction of the PRP was determined. Pictures were taken at several time points following stimulation (0, 30 minutes, 60 minutes, and 90 minutes). The size of the clot was quantified using NIH Image J. PRP formed a complete clot retraction within 60 minutes without fatty acid treatment. a) PRP treated with 25 μM fatty acid (AA, DGLA, EPA, LA, or EDA) followed by stimulation with 10 nM thrombin. The rate of clot retraction was not affected by treatment with AA, LA, or EDA, but was significantly attenuated by DGLA and EPA (N=4-6). b) PRP treated with 100 μM aspirin for 40 minutes, 25 μM DGLA for 10 minutes, or 200 μM 12(S)-HPETrE for 10 minutes, were stimulated with thrombin (N=3-5). NS= not significant; ***, P<0.001; ****, P<0.0001. This figure was provided by Joanne Vesci.

CHAPTER 9

12-HETrE, a 12-Lipoxygenase Oxylipin of Dihomo-γ-Linolenic Acid, Inhibits Thrombosis via $G\alpha_s$ Signaling in Platelets

9.1 Summary

Dietary supplementation with polyunsaturated fatty acids (PUFAs) has been widely used for primary and secondary prevention of CVD in individuals at risk; however, the cardioprotective benefits of PUFAs remain controversial due to lack of mechanistic and *in vivo* evidence. We present direct evidence that an omega-6 PUFA, dihomo-γ-linolenic acid (DGLA), exhibits *in vivo* cardioprotection through 12-lipoxygenase (12-LOX) oxidation of DGLA to its reduced oxidized lipid form, 12(S)-HETrE, inhibiting platelet activation and thrombosis.

DGLA inhibited *ex* vivo platelet aggregation and Rap1 activation in wild-type mice, but not in mice lacking 12-LOX expression (12-$LOX^{-/-}$). Similarly, wild-type mice treated with DGLA were able to reduce thrombus growth (platelet and fibrin accumulation) following laser-induced injury of the arteriole of the cremaster muscle, but not 12-$LOX^{-/-}$ mice, supporting a 12-LOX requirement for mediating the inhibitory effects of DGLA on platelet-mediated thrombus formation. Platelet activation and thrombus formation were also suppressed when directly treated with 12(S)-HETrE. Importantly, two hemostatic models, tail bleeding and arteriole rupture of the cremaster muscle, showed no alteration in hemostasis following 12(S)-HETrE treatment. Finally, the mechanism for 12(S)-HETrE protection was shown to be mediated via a $G\alpha_s$-linked GPCR pathway in human platelets.

This study provides the first direct evidence that an omega-6 PUFA, DGLA, inhibits injury-induced thrombosis through its 12-LOX oxylipin, 12(S)-HETrE, which strongly supports the potential cardioprotective benefits of DGLA supplementation through its regulation of platelet function. Furthermore, this is the first evidence of a 12-LOX oxylipin regulating platelet function in a $G\alpha_s$-linked GPCR-dependent manner.

9.2 Introduction

Platelet activation plays a critical role in the thrombotic complications associated with life-threatening cardiovascular ischemic events, such as myocardial infarction and stroke. Inhibiting platelet activation in individuals at risk for thrombotic events through the use of aspirin and $P2Y_{12}$ receptor antagonists has significantly decreased morbidity and mortality associated with these debilitating conditions [555,556]. Nonetheless, the fact that the rate of ischemic events still remains high in individuals on antiplatelet agents [557] stresses the need to investigate alternative therapies that reduce occlusive thrombotic events without promoting an increased risk of bleeding. Dietary supplementation with polyunsaturated fatty acids (PUFAs) are commonly used for their potential cardioprotective effects including their antiplatelet effects; but, the evidence supporting this claim *in vivo* remains unclear.

Dihomo-γ-linolenic acid (DGLA), an ω-6 polyunsaturated fatty acid (PUFA), has been suggested to play a role in inhibiting platelet aggregation *ex vivo* [545,558,559]. While these studies support DGLA as a potential inhibitor of platelet function, the underlying mechanism by which DGLA elicits its antiplatelet effect and the *in vivo* relevance of this inhibition have remained elusive. PUFAs are primarily thought to exert their regulatory effects on platelet function through their conversion into bioactive lipids (oxylipins) by oxygenases [281]. In platelets, DGLA can be oxidized by cyclooxygenase-1 (COX-1) or platelet 12-lipoxygenase (12-LOX) [540]

following its release from the phospholipid bilayer predominately through the actions of cytoplasmic phospholipase A_2 [560,561]**.** While both COX-1 and 12-LOX are able to oxidize DGLA to their respective metabolites, the relative contributions of these oxylipin products to the inhibitory effects of DGLA on platelet function remain unclear. Further, the antiplatelet effects of DGLA have been primarily attributed to the COX-1-derived metabolites that have been shown to inhibit platelet activation [545,558,559,562]. However, the DGLA derived products of COX-1 (TXA_1 and PGE_1) are liable and produced in low amounts in platelets [277,563-565]. Additionally, a recent study demonstrated for the first time that 12(S)-hydroxyeicosatetrienoic acid (12-HETrE), the 12-LOX-derived oxylipin of DGLA, exhibits a potential antiplatelet effect *ex vivo* [333]. Hence, it is important to delineate if 12-LOX is required for DGLA-mediated inhibition of platelet function *in vivo* and whether DGLA and 12-HETrE play an essential role in regulation of thrombosis.

This study showed for the first time that an ω-6 PUFA, DGLA, inhibited platelet thrombus formation *in vivo* following an insult to the vessel wall. Interestingly, DGLA was unable to inhibit thrombus formation in 12-$LOX^{-/-}$ mice suggesting the antithrombotic effects of DGLA were mediated by 12-LOX. The 12-LOX-derived oxylipin of DGLA, 12-HETrE, potently impaired thrombus formation following vessel injury irrespective of 12-LOX expression. Importantly, the antithrombotic effects of 12-HETrE did not disrupt primary hemostasis or result in increased bleeding. Finally, the antiplatelet effect of 12-HETrE was delineated here for the first time and shown to inhibit platelet function through activation of the $G\alpha_s$ signaling pathway leading to formation of cAMP and PKA activation in the platelet. Hence, these findings are the first to demonstrate an antithrombotic role of DGLA 12-HETrE at both the mechanistic and *in vivo* levels.

9.3 Materials and Methods

Preparation of washed murine platelets. The 12-LOX null C57BL/6 (12-LOX$^{-/-}$) mice, generated using homozygous breeding pairs, and wild-type (WT) C57BL/6 (12-LOX$^{+/+}$) mice were purchased from Jackson Laboratory. The Institutional Animal Care and Use Committees (IACUC) at the University of Michigan and Thomas Jefferson University approved all experimental procedures in this study involving mice. Blood was drawn from the inferior vena cava of anesthetized 8-12 week old mice with a 21-gauge needle attached to a 1 mL syringe containing 100 μl of 3.8% sodium citrate. Mouse blood was diluted with equal volumes of Tyrode's buffer and centrifuged at 200 *g*. Platelet-rich-plasma (PRP) was transferred to a tube containing 10x acid citrate dextrose solution (ACD) and apyrase (.02 U/mL) and centrifuged at 2000 *g*. Platelet count was adjusted to 3x10^8 platelets/mL with Tyrode's buffer for all studies.

Preparation of washed human platelets. University of Michigan and Thomas Jefferson University Institutional Review Boards approved all protocols involving human subjects. In accordance with the Declaration of Helsinki, written informed consent was obtained from healthy donors prior to each blood draw. Blood was collected as previously described [333] and washed platelets were resuspended in Tyrode's buffer at 3x10^8 platelets/mL, unless otherwise specified.

Extraction, liquid chromatography, and mass spectrometry (LC/MS) quantification of oxylipins. Washed human platelets were treated with agonists in the presence of vehicle control or specific fatty acids and acidified with 40 μL of 1M hydrochloric acid to pH of 3. Extraction standards were then added to the samples: 20ng each of PGE_1-d_4 and 13(S)HODE-d_4. Oxylipids were extracted with dichloromethane, reduced with trimethylphosphite, and dried under nitrogen gas.

The samples were reconstituted in methanol containing 10 ng of each of the internal standards and transferred to MS vials with inserts.

Chromatographic separation was performed on a Dionex UltiMate 3000 UHPLC with a C_{18} column (1.7 μm, 150 mm x 2.1mm). The autosampler was held at 4°C and injection volume was 30 μL. Mobile phase A consisted of water with 0.1% (v/v) formic acid and mobile phase B was acetonitrile with 0.1% formic acid. Flow rate was 0.400mL/min. The initial condition (30% B) was maintained for 2.33 minutes. Mobile phase B was then ramped to 65% over 28.67 minutes, held at 65% for 1 minute, ramped to 100% over 0.1min, held at 100% for 7 minutes, and finally returned to 30% to equilibrate for 7 minutes. The chromatography system was coupled to a Velos Pro linear ion trap for mass analysis. Analytes were ionized via heated electrospray ionization with -4.0kV spray voltage, 60, 10, and 0 arbitrary units for sheath, auxiliary, and sweep gas, respectively. All analyses were performed in negative ionization mode at normal resolution setting. MS^2 was performed in both a data dependent and targeted manner, simultaneously from ions detected in a full scan with a mass-to-charge ratio (m/z) range of 200-400. The targeted MS^2 were selected for the parent ions of the analytes that were contained in a mass list –TxB_1(m/z = 371.2), TxB_2(m/z = 369.2), TxB_2-d_4(m/z= 373.3), PGE_1(m/z = 353.2), PGE_2(m/z = 351.2), PGE_1-d_4(m/z = 357.2), PGE_2-d_4(m/z = 355.2), 12HETE(m/z = 319.2) , 12HETrE(m/z =321.2), 12(S)HETE-d_8(m/z = 327.3), 13(S)HODE-d_4(m/z = 299.3).

Mass spectrometry analysis, normalization, and relative quantitation of oxylipins. Retention times and fragmentation patterns of all analytes were determined with lipid standards prior to sample analyses. Total ion counts (TIC) of the m/z transitions of each analyte peak were used for relative quantitation. The m/z transitions of all analytes and standards were as follows: TxB_1(m/z = 371.2→197), TxB_2(m/z = 369.2→195), TxB_2-d_4(m/z= 373.3→199), PGE_1(m/z = 353.2→317),

PGE_2(m/z = 351.2→315), PGE_1-d_4(m/z = 357.2→321), PGE_2-d_4(m/z = 355.2→319), 12HETE(m/z = 319.2→179) , 12HETrE(m/z =321.2→181), 12(S)HETE-d_8(m/z = 327.3→184), and 13(S)HODE-d_4(m/z = 299.3→198). Nanograms of 12-HETE and 12-HETrE present in sample were estimated based on TICs of 12-HETE and 12-HETrE relative to the TIC of 10 ng of 12-HETE-d_8. These nanogram estimates were then corrected for extraction efficiency by the percent recovery of 13-HODE-d_4 in each sample. The TIC of TxB_1, TxB_2, PGE_1, and PGE_2 were normalized to PGE_1-d_4 for extraction. For ionization, TxB_1 and TxB_2 were normalized to TxB_2-d_4, and PGE_1 and PGE_2 were normalized to PGE_2-d_4.

Platelet aggregation. A lumi-aggregometer (Chrono-log model 700D) was used to measure platelet aggregation under stirring conditions (1100 rpm) at 37°C. Prior to agonist stimulation platelets were incubated with a preselected PUFA or oxylipin for 10 minutes.

Rap1 activation. Washed platelets were incubated with PUFAs or oxylipins for 10 minutes prior to stimulation with PAR4-AP. Following 1 minute of stimulation, platelets were lysed and Rap1 activity was assessed by western blot analysis as previously described[333].

Laser-induced cremaster arteriole thrombosis model. WT or 12-$LOX^{-/-}$ mice (12 weeks of age) were anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg) prior to the exposure of the cremaster muscle arterioles under a dissecting microscope with constant perfusion of preheated bicarbonate-buffered saline[566-568]. Anti-platelet (DyLight 488 anti-GPIb, 1 μg/g) and anti-fibrin (Alexa Fluor 647, 0.3 μg/g) antibodies were administered via jugular vein catheter prior to intravital microscopy. DGLA or DMSO were dissolved in a formulation of 5% DMSO and 45% PEG300 in sterile 1X PBS and intravenously injected into mice 10 minutes prior to induction of thrombosis. 12-HETrE or equal volume of DMSO was intravenously injected into mice 10 minutes prior to induction of thrombosis. Multiple thrombi were induced in

the arterioles (30-50 μm diameter) in each mouse by a laser ablation system (Ablate! photo-ablation system; 3I). Images of thrombus formation were acquired in real-time under 63X water-immersion objective with a Zeiss Axio Examiner Z1 fluorescent microscope equipped with solid laser launch system and high-speed sCMOS camera. All captured images were analyzed on Slidebook.

Tail bleeding assay. Mice were anesthetized with ketamine/xyzaline and placed on a heating pad in prone position, the tip of the tail (5 mm) was excised with a sterile scalpel, and the tails were immediately immersed into isotonic saline solution (.9%) warmed to 37°C. Bleeding time was assessed until cessation of blood flow from the tail for 1 minute.

Cremaster muscle arterial puncture model of hemostasis. Mice were anesthetized, tail vein injected with anti-platelet and anti-fibrin antibodies and their cremaster muscle arterioles were prepared as described above. A high intensity laser pulse from the laser ablation system was used to puncture a hole in the cremaster muscle arteriole wall as visualized by red blood cell (RBC) leakage from the vessel. Images of RBCs leakage and hemostatic plug formation were acquired in real-time with a fluorescent microscope as described above. Arterial bleeding time was defined as the time from laser pulse injury until cessation of RBC leakage from the vessel.

Liquid chromatography extraction and mass spectrometry (LC/MS) quantification of cAMP. Washed human platelets were treated with the specific ligand or vehicle control for 1 min at room temperature, and quenched with an equal volume of ice-cold 2x platelet lysis buffer containing protease and phosphatase inhibitors. Lysed platelet samples were centrifuged to pellet cytoskeleton and the supernatant was stored in -80°C.

To prepare the sample for LC/MS injection, 200 μL of the supernatant were spiked with 400 pg of adenosine-3',5'-cyclic-13C5 monophosphate ($^{13}C_5$-cAMP) and 600 μL of LC/MS grade

acetonitrile. The sample was vortexed and centrifuged for 10 min. The supernatants were dried and reconstituted in 200 μL of LC/MS grade water, centrifuged for 5 min at 4°C, and used for LC/MS injection.

The separation and detection of cAMP was performed on a Waters ACQUITY UPLC system equipped with a Xevo Triple Quadrupole Mass Spectrometer (TQ-S MS/MS). The extraction/purification of cAMP from the sample was carried out using a Waters HSS C18 column (1.8 μm, 2.1*100mm). The sample (10 μL) was then injected.

The mass spectrometer was operated with an ESI source in positive mode. The electrospray voltage was 3.9kv. The source temperature was maintained at 150°C, and the desolvation temperature was 525 °C with a nitrogen desolvation gas flow of 1000L/h. cAMP was quantitated using $^{13}C_5$-cAMP as the internal standard. For cAMP monitoring in the MRM mode with a collision energy of 22 volts, 330/136 mass transition was used, and for $^{13}C_5$-cAMP, 335/136 was used.

VASP phosphorylation in human platelets. Washed platelets were treated with PUFA or PUFA metabolite for one minute then directly lysed in 5x Laemmeli sample buffer. The samples were boiled for five minutes and assayed by western blot for total and phospho-VASP (serine 157).

Membrane preparation and [^{35}S]GTPγS binding. PRP was isolated from whole blood as described above and incubated with 1 mM aspirin for 1 hour at 37°C. Washed platelets were prepared as previously described. The platelets were flash frozen with liquid nitrogen and resuspended in cold detergent-free TME buffer (50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 2 mM EDTA, and 100 mM NaCl) with 1 μM GDP and protease and phosphatase inhibitors to lyse the platelets. The lysed platelets were centrifuged at 1500 *g* at 4°C for 5 min. The supernatant was

collected and centrifuged at 100,000 *g* for 30 min at 4°C. The pelleted membranes were resuspended in TME buffer with 1 μM GDP and stored at -80°C prior to use.

Fatty acid metabolites, agonists, or DMSO and [^{35}S]GTPγS (10 nM) were added to platelet membranes and the tubes were immediately transferred to a 30°C water bath shaker for 20 min. The reaction was terminated by the addition of ice-cold IP buffer (50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 150 mM NaCl, .5% Nonidet P-40, .33% aprotinin, .1 mM GDP, and .1 mM GTP). The samples were pre-cleared with Protein A agarose beads and normal rabbit IgG and aliquoted equally into two tubes containing either normal rabbit IgG or a Gas antibody that had been conjugated to Protein A agarose beads. The samples were incubated on a nutator for 1 hr at 4°C and washed 4 times with IP buffer and 1 time in TME buffer. The samples were boiled in .5% SDS for 30 sec and the supernatants were collected following brief centrifugation. The supernatants were analyzed in 8 mL of scintillation fluid. The background counts for the normal rabbit IgG (50-200 cpm) for each sample were subtracted from the anti-G_s immunoprecipitated samples prior to analyzing the data.

Statistics. Unpaired, paired two-tailed student t-tests, and two-way analysis of variance (ANOVA) were used to compare between experimental groups with Prism 6.0 software (GraphPad). Where appropriate the statistical test used is contained in the figure legend. Data represents mean values +/- SEM.

9.4 Results

DGLA inhibits platelet aggregation and thrombus growth in a 12-LOX dependent manner

Treatment of human platelets with either DGLA [545,558,559] or its 12-LOX-derived metabolite, 12-HETrE, potently inhibited platelet aggregation [333]; however, the relative contribution of 12-LOX to DGLA-mediated inhibition of platelet activation was unclear. To assess the role of 12-LOX in

DGLA-mediated platelet inhibition, washed platelets from WT or 12-LOX$^{-/-}$ mice were stimulated with an EC_{80} concentration of either protease-activated receptor-4-activating peptide (PAR4-AP) or collagen in the presence or absence of DGLA. As previously reported, platelets from 12-LOX$^{-/-}$ mice were hypoactive compared to platelets from WT mice [325], hence, requiring a higher concentration of agonist to reach EC_{80}. Pretreatment of platelets from WT mice with DGLA resulted in significant inhibition of aggregation compared to DMSO treated platelets in response to PAR4-AP or collagen stimulation (Figure 9.1A and 9.1B). Conversely, DGLA treatment of platelets from 12-LOX$^{-/-}$ mice failed to inhibit platelet aggregation in response to PAR4-AP or collagen stimulation (Figure 9.1A and 9.1B). To fairly compare the concentrations of the higher PAR4-AP concentration used on 12-LOX$^{-/-}$ platelets, WT platelets treated with DGLA were also stimulated with higher PAR4-AP concentration, resulting in significant inhibition of platelet aggregation compared to vehicle control (data not shown). As the observed DGLA-mediated inhibition of aggregation may be due to the modification of the lipid membrane structure thus affecting platelet signaling or activation, other PUFAs including linoleic acid (LA) and AA were used as controls to rule out a lipid-membrane insulating effect in platelet activation [569]. Pretreatment of platelets with either LA or AA had no inhibitory effect on PAR4-AP or collagen-mediated platelet aggregation compared to vehicle alone **(**Figure 9.1A and 9.1B).

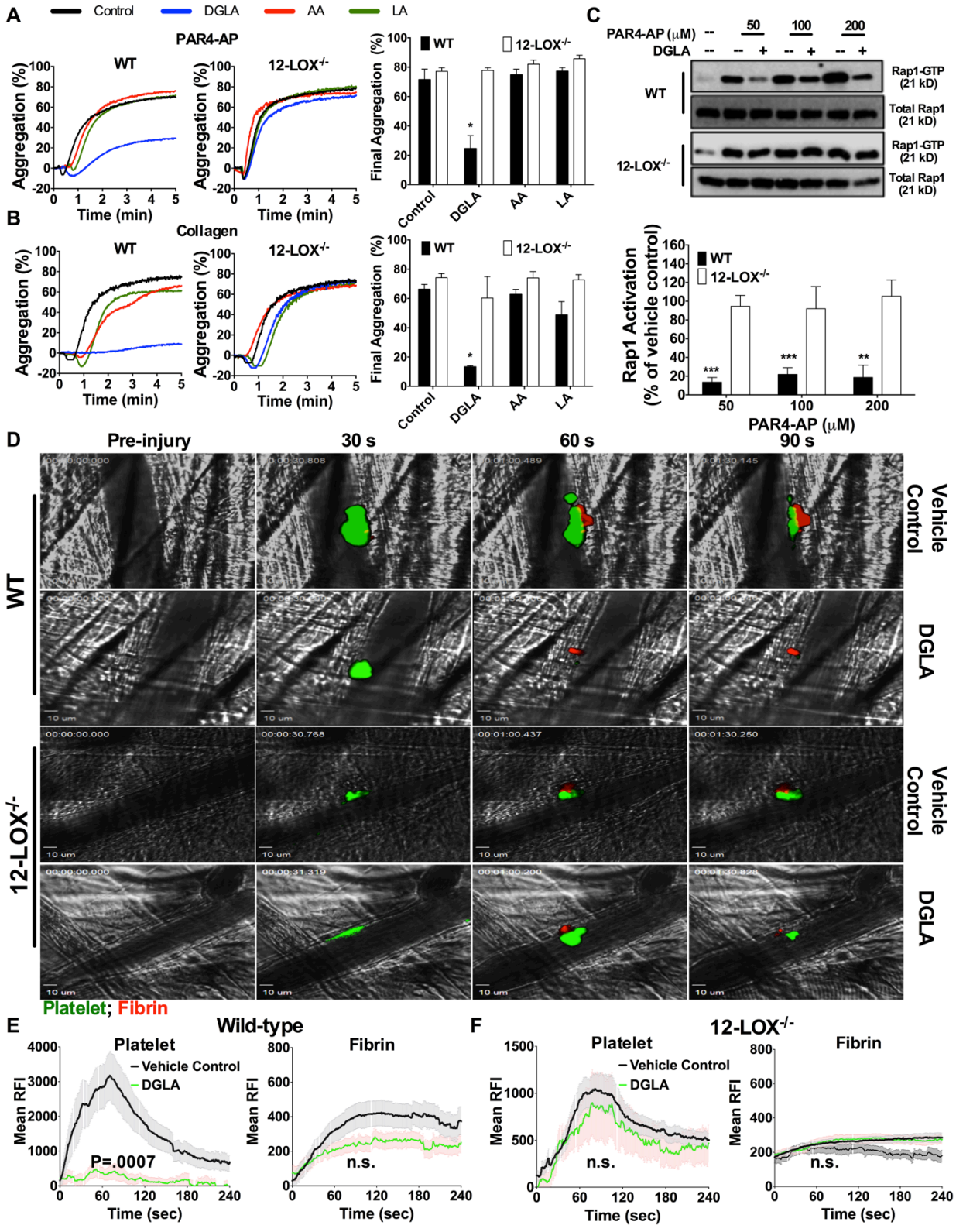


Figure 9.1 12-LOX is required for DGLA inhibition of platelet aggregation and thrombus formation.
Representative tracings and combined aggregation data of (A) WT (n=4) or (B) 12-LOX-/- (n=4) platelets stimulated with EC80 concentration of PAR4-AP (WT 100 μM; 12-LOX-/- 200 μM) or collagen (WT 5 μg/mL; 12-LOX-/- 2 or 5 μg/mL) in the

presence or absence of 10 μM of PUFAs (DGLA, AA, or LA). Aggregation was monitored for 10 minutes. Data represents mean ± SEM. *P<.05 two-tailed unpaired t-test. (C) Active Rap1 (Rap1-GTP) was selectively precipitated from the lysates of platelets isolated from WT or 12-LOX-/- mice incubated with vehicle control or 10 μM DGLA (n= 3 to 4 mice) prior to stimulation with increasing concentrations of PAR4-AP (50, 100, and 200 μM). Active Rap1 was normalized to the total amount of Rap1 in each sample, and each bar graph represents a percentage of vehicle control for each PAR4-AP concentration. Data represent mean ± SEM. **P<.01, ***P<.001 two-tailed unpaired t-test. (D) Representative images of laser-induced injury of the cremaster arterioles, platelet (green) and fibrin (red) accumulation monitored in real-time to assess thrombus growth in the WT vehicle control (n=3 mice, 10-15 thrombi per mouse), DGLA treated group (n=3 mice, 10-15 thrombi per mouse), 12-LOX-/- vehicle control (n=3 mice, 10-15 thrombi per mouse), and 12-LOX-/- treated with DGLA (n=3 mice, 10-15 thrombi per mouse). Scale bar: 40 μm. Mean fluorescence intensity (MFI) of platelet and fibrin accumulation at the site of injury were recorded over time in (E) WT and (F) 12-LOX-/- mice. Data represents mean ± SEM; two-way ANOVA. This figure was co-provided by Jennifer Yeung (aggregation and Rap1) and Dr. Reheman Adili (*in vivo* thrombosis).

To determine if DGLA inhibited platelet aggregation by impinging on intracellular signaling, the activation of Rap1, a common signaling effector required for integrin $\alpha_{IIb}\beta_3$ activation (Shattil et al., 2010; Shattil and Newman, 2004) was assessed in DGLA treated platelets stimulated with PAR4-AP [8,328,570]. In platelets isolated from WT mice, DGLA inhibited Rap1 activation at all concentrations of PAR4-AP tested (Figure 9.1C). Since DGLA was unable to inhibit platelet aggregation in 12-LOX$^{-/-}$ mice, we assessed if 12-LOX was also necessary for DGLA inhibition of Rap1 activation in platelets. Consistent with the platelet aggregation data, DGLA was unable to inhibit Rap1 activation in platelets from 12-LOX$^{-/-}$ mice at any of the concentrations of PAR4-AP tested (Figure 9.1C). Together, these data demonstrate that the antiplatelet effects mediated by DGLA require 12-LOX.

To determine whether the antiplatelet effects of DGLA observed *ex vivo* could contribute to the inhibition of platelet thrombus formation *in vivo*, a laser-induced cremaster arteriole thrombosis model was employed to examine thrombus formation (platelet and fibrin) in WT mice [571] (Figure 9.1D-F). Mice were intravenously injected with either vehicle control (DMSO) or 50 mg/kg of DGLA 10 minutes prior to the initiation of thrombosis by laser injury. Following vessel injury of vehicle control treated WT mice, fluorescently labeled platelets rapidly accumulated at the site of vascular injury then drastically diminished in size as the clot was resolved (Figure 9.1D and 9.1E; and Supplementary video 1). Simultaneously, fibrin

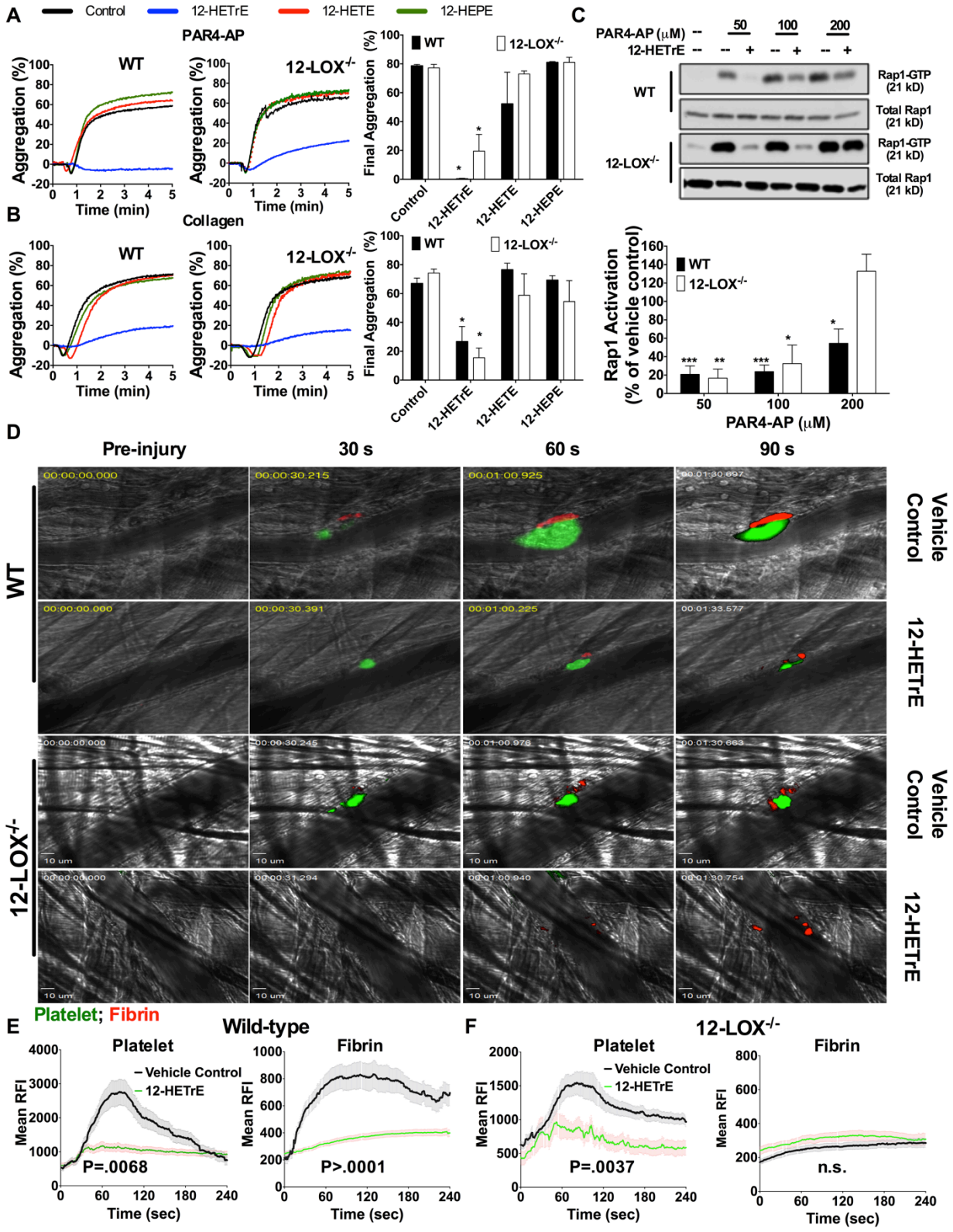


Figure 9.2 12-HETrE inhibits platelet aggregation and thrombus formation.
Representative tracings and combined aggregation data of washed platelets from (A) WT (n=4) or (B) 12-LOX$^{-/-}$ (n=4) mice pre-treated with 25 μM 12-LOX oxylipins (12-HETrE, 12-HETE, or 12-HEPE) for 10 minutes prior to stimulation with an EC_{80}

concentration of PAR4-AP (WT 100 μM; 12-LOX$^{-/-}$ 200 μM) or collagen (WT 5 μg/mL; 12-LOX$^{-/-}$ 2 or 5 μg/mL) in an aggregometer. Data represents mean ± SEM. *$P<.05$ two-tailed unpaired t-test. (C) Active Rap1 (Rap1-GTP) was selectively precipitated from the lysates of platelets isolated from WT or 12-LOX$^{-/-}$ mice incubated with vehicle control or 25 μM 12-HETrE (n= 3 to 4 mice) prior to stimulation with increasing concentrations of PAR4-AP (50, 100, and 200 μM). Active Rap1 was normalized to the total amount of Rap1 in each sample, and each bar graph represents a percentage of vehicle control for each PAR4-AP concentration. Data represent mean ± SEM. *$P<.05$, **$P<.01$, ***$P<.001$ two-tailed unpaired t-test. (D) Representative images of laser-induced injury of the cremaster arterioles, platelet (green) and fibrin (red) accumulation monitored in real-time to assess thrombi growth in the WT vehicle control (n=3 mice, 10-15 thrombi per mouse), 12-HETrE treated group (n=3 mice, 10-15 thrombi per mouse, 10-15 thrombi per mouse), 12-LOX$^{-/-}$ vehicle control (n=3-4 mice, 10-15 thrombi per mouse), and 12-LOX$^{-/-}$ treated with 12-HETrE (n=3 mice, 10-15 thrombi per mouse). 12-LOX$^{-/-}$ vehicle control data is the same set as 12-LOX$^{-/-}$ vehicle control used for 12-LOX$^{-/-}$ DGLA treated comparison in figure 1F. Scale bar: 40 μm. Mean fluorescence intensity (MFI) platelet and fibrin accumulation at the site of injury were recorded over time in (E) WT and (F) 12-LOX$^{-/-}$ mice. Data represents mean ± SEM; two-way ANOVA. This figure was co-provided by Jennifer Yeung (aggregation and Rap1) and Dr. Reheman Adili (*in vivo* thrombosis).

formation can be seen at the base of the developing thrombus of vehicle control treated WT mice (Figure 9.1D and 9.1E; and Supplementary video 1). WT mice treated with DGLA showed a significant reduction in platelet, but not fibrin accumulation (Figure 9.1D and 9.1E; and Supplementary video 2).

Figures 9.1A-C suggests a requirement for 12-LOX in DGLA-mediated inhibition of platelet activation *ex vivo*. To determine if this observation translates to an attenuation of platelet reactivity *in vivo*, thrombus formation was measured in 12-LOX$^{-/-}$ mice following laser-induced injury of the cremaster arteriole (Figure 9.1D and 9.1F). As previously reported, platelets from 12-LOX$^{-/-}$ mice exhibited a bleeding diathesis compared to WT mice as determined by the tail-bleeding assay [325]. Therefore, it would be expected that the 12-LOX$^{-/-}$ mice show a significant attenuation of thrombus following injury compared to the WT (Figure 9.1D and 9.1F). Interestingly, the accumulation of platelet and fibrin in thrombi between DGLA-treated 12-LOX$^{-/-}$ and vehicle control did not differ (Figure 9.1D and 9.1F; and Supplementary videos 3 and 4**)** supporting the *ex vivo* observation that 12-LOX is required to mediate DGLA-dependent inhibition of platelet function as well as thrombosis.

The 12-LOX oxylipin, 12-HETrE, inhibits platelet aggregation and thrombus growth

To confirm that 12-HETrE was the 12-LOX product of DGLA mediating the inhibitory effects observed in figure 9.1, washed platelets from either WT or 12-LOX$^{-/-}$ mice were treated

with 12-HETrE followed by stimulation with either PAR4-AP or collagen. Notably, 12-HETrE (25 μM) inhibited the aggregation of platelets from WT and 12-LOX$^{-/-}$ mice similarly in response to PAR4-AP or collagen (Figure 9.2A and 9.1B). As expected, no decrease in collagen- or PAR4-AP-mediated platelet aggregation was observed in either WT or 12-LOX$^{-/-}$ platelets pre-treated with 12-HETE, the pro-thrombotic 12-LOX-derived oxylipin of AA, compared to vehicle control. Additionally, incubation of platelets with 12-HEPE, a 12-LOX-derived oxylipin of eicosapentaenoic acid (EPA) with no known effects on aggregation [333,572,573], did not inhibit collagen- or PAR4-AP-induced aggregation in platelets from either WT or 12-LOX$^{-/-}$ mice.

To determine if 12-HETrE inhibited intracellular signaling, the activation of Rap1 was measured in PAR4-AP stimulated platelets in the presence of 12-HETrE or vehicle control. 12-HETrE suppressed Rap1 activation compared to vehicle control in platelets from either WT or 12-LOX$^{-/-}$ mice (Figure 9.2C). Thus, 12-HETrE was able to inhibit platelet aggregation and Rap1 activity independent of 12-LOX expression.

Although 12-HETrE significantly attenuated platelet activation, it remained unclear if 12-HETrE could directly inhibit platelet thrombus formation *in vivo*. To evaluate the effects of 12-HETrE on thrombus formation, the size and kinetics of the growing arterial thrombus were assessed following laser-induced injury of the cremaster muscle arterioles in WT and 12-LOX$^{-/-}$ mice treated with vehicle control or 6 mg/kg of 12-HETrE (Figure 9.1D and 9.2D). Following injury, platelets and fibrin were observed to rapidly accumulate at the injured arteriole wall in WT control mice (Figure 9.2D and Supplementary video 1). In contrast, WT mice treated with 12-HETrE had significantly smaller and less stable thrombi in response to laser injury as assessed by both platelet and fibrin accumulation (Figure 9.2D and 9.2E; Supplementary video 5).

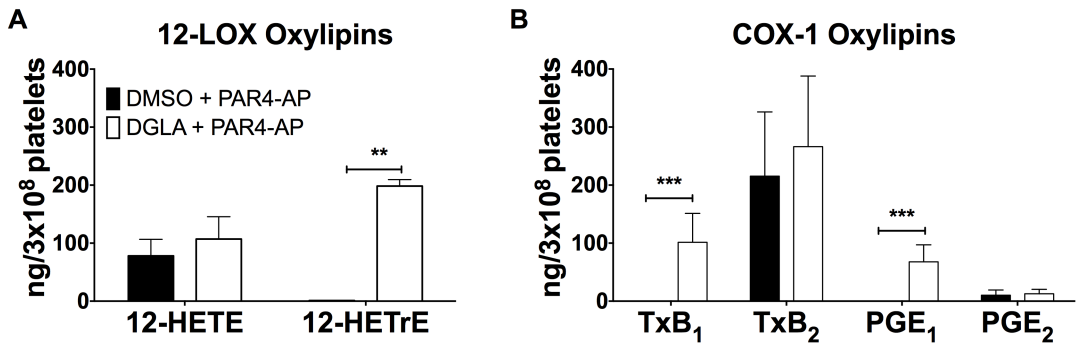


Figure 9.3 Exogenous DGLA enhances platelet production of metabolites.
12-LOX and COX-1 metabolites from washed human platelets (*n=7*) treated with DGLA (10 μM) or DMSO for 10 minutes prior to stimulation with PAR4-AP (200 μM) were detected using mass spectrometry. Data represents mean ± SEM; ***P<.01*, ****P<.001* two-tailed unpaired t-test. This figure co- provided by Jennifer Yeung and Cody Freedman (Dr. Theodore Holman's group).

12-LOX$^{-/-}$ mice treated with vehicle control exhibited a significant decrease in thrombus formation (platelet and fibrin accumulation) (Figure 9.2D and 9.2F; Supplementary video 3) compared to WT control mice following injury (Figure 9.2D and 9.2E; Supplementary video 1). Additionally, 12-LOX$^{-/-}$ mice treated with 12-HETrE exhibited significant inhibition of platelet accumulation compared to 12-LOX$^{-/-}$ alone (Figure 9.2F; Supplementary video 6). However, no difference in fibrin accumulation was observed between vehicle control and 12-HETrE treatment of 12-LOX$^{-/-}$.

DGLA-induced oxylipin production

Endogenously, only minute amounts of DGLA metabolites are produced by COX-1 (PGE_1 and TxB_1) or 12-LOX (12-HETrE) due to the low abundance of DGLA in the platelet plasma membrane [255]. To determine if the exogenous addition of DGLA (10 μM) increases the production of 12-LOX and COX-1 metabolites, the lipid releasate from platelets stimulated with PAR4-AP in the presence of vehicle control or DGLA was measured by LC/MS/MS. As expected, the amount of DGLA-dependent COX-1 and 12-LOX oxylipins was significantly potentiated in the DGLA-treated group compared to the DMSO control group (Figure 9.3A and 9.3B). The amount of AA-dependent metabolites from either 12-LOX (12-HETE) or COX-1

(TxB_2 or PGE_2) was unaltered in platelets incubated with DGLA (Figure 9.3A and 9.3B) supporting 12-LOX being in excess such that competition for the substrate is not necessary. This is a reasonable presumption based on previously published work showing that in the human platelet transcriptome, the mRNA for 12-LOX, ALOX12, is expressed in the top 8% of all transcripts in the platelet [574].

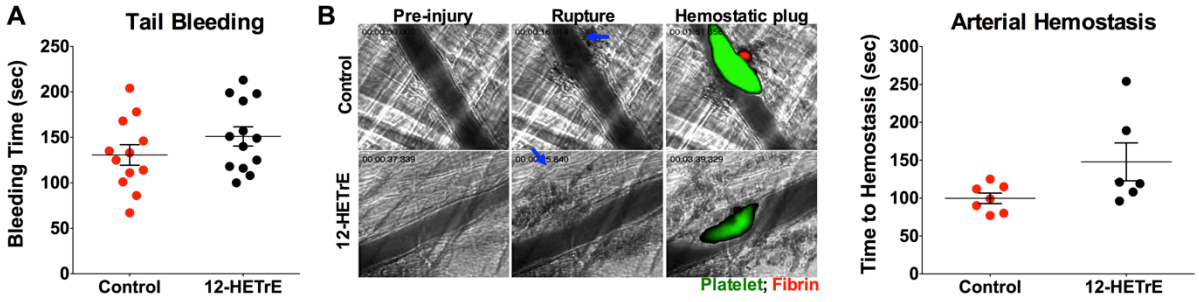


Figure 9.4 Hemostasis is not affected by 12-HETrE.
Mice were retro-orbitally injected with DMSO or 12-HETrE dissolved in saline prior to tail-bleeding. (A) Mean tail-bleeding time of control (n=12) or 12-HETrE (n=13) treated mice is denoted by the horizontal line. Arterial hemostasis induced by laser-induced puncturing of the cremaster muscle arterioles was performed to assess the kinetics of hemostatic plug formation. (B) Representative images of hemostatic plug formation, composed of platelet (green) and fibrin (red) were acquired over time. Blue arrows denote the site of vessel rupture and leakage of RBCs. (C) Time to form hemostatic plug in control (n=7) and 12-HETrE (n=6) mice as assessed by RBC leakage. Data represent mean ± SEM; two-tailed unpaired t-test. This figure co-provided by Jennifer Yeung (tail bleeding) and Dr. Reheman Adili (*in vivo* rupture).

12-HETrE does not disrupt hemostasis

Since 12-HETrE potently attenuated platelet accumulation in the laser-induced cremaster arteriole injury model of thrombosis, it is possible 12-HETrE also alters hemostasis resulting in increased bleeding. To determine if 12-HETrE treatment resulted in an increased bleeding diathesis, two hemostatic models were used to assess the impact of 12-HETrE on bleeding. First, the tail-bleeding time assay was utilized to determine the effects of 12-HETrE on primary hemostasis. 12-HETrE-treated mice showed no significant difference in tail bleeding time compared to the control mice following excision of the distal segment (5 mm) of the tail (Figure 9.4A). To confirm this assay was accurately reporting bleeding risk, heparin-treated mice were also assayed for bleeding time and observed to have a severe bleeding diathesis (data not shown).

A second hemostatic model was used to confirm hemostasis was not significantly altered following treatment with 12-HETrE. This model involved arteriole puncture of the cremaster muscle induced by severe laser injury [575] in order to monitor the cessation time of RBC leakage from the punctured arteriole wall (Figure 9.4B; Supplementary videos 7 and 8). No significant difference in the duration of RBC leakage was observed between 12-HETrE and control treated mice. In both the control and 12-HETrE-treated mice, a stable, non-occlusive clot formed in response to laser puncture of the vessel wall, resulting in cessation of RBC leakage from the vessel. Both distinct hemostatic models suggested 12-HETrE did not disrupt hemostasis.

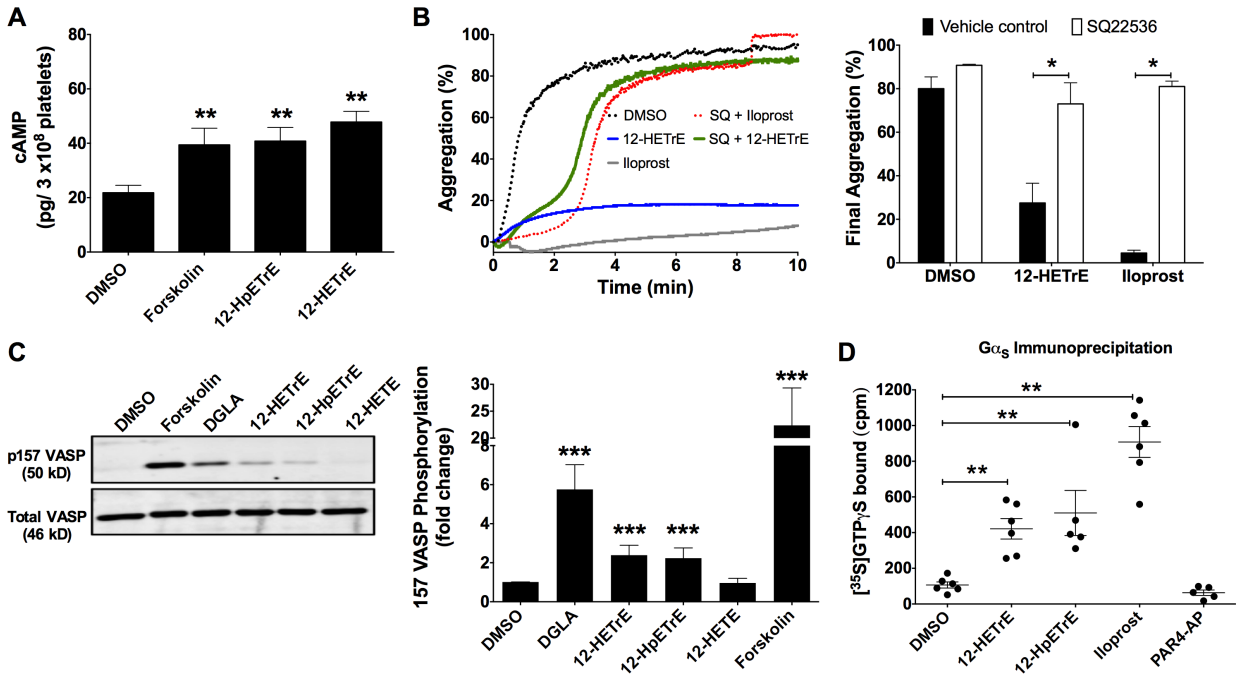


Figure 9.5 12-HETrE activates adenylyl cyclase-mediated signaling in platelets.
(A) Mass spectrometry quantification of cAMP was performed on lysed washed human platelets (*n*=5) incubated with DMSO, 12-HETrE (25 μM), 12-HpETrE (25 μM), or forskolin (.5 μM) for 1 minute. (B) Washed human platelets (*n*=4) were pre-treated with an adenylyl cyclase inhibitor, SQ22536 (25 μM), or DMSO for 20 minutes and then incubated with 12-HETrE (7.5 to 25 μM) or iloprost (.2 to .4 nM) for 1 minute prior to stimulation. Platelet aggregation induced by an EC_{80} concentration of PAR4-AP (35 to 50 μM) was measured for 10 minutes. Representative tracings of aggregation are shown on the left and bar graphs of the final aggregation of 4 independent experiments are shown on the right. (C) VASP phosphorylation was measured by western blot analysis on lysates from washed human platelets (*n*=8) incubated with DMSO, DGLA (10 μM), 12-HETrE (25 μM), 12-HpETrE (25 μM), or forskolin (.5 μM) for 1 minute using antibodies specific for phospho-VASP (p157 VASP) or total VASP. Phosphorylated VASP was normalized to total VASP and DMSO for fold change in 157 VASP phosphorylation. (D) $G\alpha_s$ was immunoprecipitated following incubation of human platelet membranes with DMSO, 12-HETrE (25 μM), 12-HpETrE (25 μM), Iloprost (10 μM) or PAR4-AP in the presence of [^{35}S]GTPγS. The immunoprecipitates (*n=6*) were then counted and background counts from normal IgG controls were subtracted. Data represent mean ± SEM. * *P<.05*, ***P<.01*, ****P<.001 two-tailed*

unpaired t-test (A-D*)*. This figure co-provided by by Jennifer Yeung (GTPγS binding and adenylyl cyclase inhibition rescue) and Dr. Benjamin Tourdot (VASP phosphorylation).

12-HETrE inhibits platelets in a Gα$_s$-linked GPCR-dependent manner

COX-derived oxylipins that inhibit platelet function primarily exert their inhibition through the activation of a GPCR coupled to Gα$_s$ resulting in adenylyl cyclase (AC) activation [576,577] and the generation of cAMP [578-582]. To determine if the DGLA-derived 12-LOX oxylipin 12-HETrE could be regulating platelet reactivity in a similar manner, cAMP formation was measured in washed human platelets stimulated with 12-HETrE or 12-HpETrE, a peroxidated, labile precursor of 12-HETrE. Following a 1 minute stimulation with 12-HETrE or 12-HpETrE [333], human platelets exhibited a significant increase in the level of intracellular cAMP compared to vehicle treated (DMSO) platelets (Figure 9.5.A). As expected, platelets stimulated with forskolin, a direct activator of AC, also showed an increase in cAMP levels. 12-HETrE-induced cAMP production was supportive of 12-HETrE inhibiting platelets through the activation of AC. To assess if 12-HETrE inhibited platelet aggregation in an AC-dependent manner, platelets were pre-treated with SQ 22536, an AC inhibitor [583] prior to incubation with 12-HETrE or iloprost, a prostacyclin receptor agonist known to signal through AC [584,585]. Iloprost and 12-HETrE were unable to inhibit PAR4-AP-mediated platelet aggregation in platelets pre-treated with SQ 22536 (Figure 9.5B), supporting an AC-dependent mechanism of platelet inhibition by 12-HETrE.

The cAMP activated kinase, protein kinase A (PKA), phosphorylates multiple proteins in platelets including vasodilator-stimulated phosphoprotein (VASP). Since serine 157 (S157) in VASP is a known PKA substrate [586], VASP phosphorylation was used as a surrogate readout for PKA activation. Washed human platelets treated with DGLA or its 12-LOX metabolites (12-HETrE, or 12-HpETrE) for 1 minute had enhanced VASP phosphorylation compared to DMSO treated platelets (Figure 9.5.C). As expected, forskolin treated platelets also had an increase in

VASP phosphorylation. This data demonstrates that the cAMP produced in platelets following exposure to 12-HETrE is capable of eliciting physiological effects.

The activation of a GPCR coupled to $G\alpha_s$ leads to the dissociation of GDP and the subsequent binding of GTP to $G\alpha_s$ initiating a well-established signaling cascade resulting in increases in cAMP levels through the activation of AC [587,588]. Since 12-HETrE was shown to induce cAMP formation and inhibit platelet activation in an AC-dependent manner, we sought to determine if 12-HETrE could activate $G\alpha_s$. Activation of $G\alpha_s$ was assessed by measuring the incorporation of the radiolabeled, non-hydrolyzable analog, [^{35}S]GTPγS, to $G\alpha_s$, immunoprecipitated from isolated human platelet membranes following treatment [589] with vehicle control (DMSO), 12-HETrE, 12-HpETrE, PAR4-AP, or iloprost. Treatment of human platelet membranes with 12-HETrE, 12-HpETrE, and iloprost elicited a significant increase in [^{35}S]GTPγS binding to immunoprecipitated $G\alpha_s$ compared to platelet membranes incubated with DMSO (Figure 9.5D). Activation of PAR4, a receptor that is known to selectively activate G_q and $G_{12/13}$, showed no [^{35}S]GTPγS binding confirming the selectivity for $G\alpha_s$ activation in the assay.

9.5 Discussion

Advances in antiplatelet therapy have significantly decreased the risk for morbidity and mortality due to thrombosis. However, even with the current standard-of-care antiplatelet therapies available, myocardial infarction and stroke due to occlusive thrombotic events remains one of the primary causes of morbidity and mortality globally. Therefore, identification of novel therapies remains an unmet clinical need. One potential approach to reduce thrombus formation is the dietary intake of DGLA, a naturally occurring ω-6 PUFA, which has been shown to attenuate platelet aggregation *ex vivo* [545,558,559]. However, to date the mechanism by which this

inhibition is regulated and the ability of DGLA to inhibit thrombus formation *in vivo* have not been elucidated. Recently, our lab identified a 12-LOX-derived oxylipin of DGLA, 12-HETrE, which inhibits human platelet activation [333]. In the current study, we sought to determine the relative contribution of 12-LOX-derived metabolites in DGLA-mediated inhibition of platelet function and thrombosis *in vivo*. In contrast to the previously reported dependence of DGLA-mediated inhibition of platelet function on COX-derived metabolites [545,558,559,562], we show here that DGLA, but not 12-HETrE, was unable to inhibit platelet aggregation in 12-LOX$^{-/-}$ mice suggesting that 12-LOX plays a key role in facilitating DGLA's antiplatelet effects.

In mice 12-LOX was required for DGLA to impinge on platelet activation (Figures 9.1 and 9.2), suggesting that 12-LOX metabolites are responsible for the predominance of DGLA inhibitory effects in mice. Interestingly, human platelets stimulated with DGLA had higher VASP phosphorylation than those treated with 12-HETrE (Figure 9.5C), indicating other DGLA metabolites, such as COX-1 derived oxylipins (Figure 9.3B), may also contribute to VASP phosphorylation. This observation is supported by previous data that COX-1 or 12-LOX inhibitors partially suppresses the ability of DGLA to inhibit human platelet aggregation [333].

The proposed inhibitory effect mediated through 12-LOX appears paradoxical based on previous work in our lab and others showing that 12-LOX is a positive mediator of platelet function [196,325,328,329]. However, due to the fact that 12-LOX is an enzyme whose function is to add an oxygen to a free fatty acid in order to produce a bioactive oxylipin, it is reasonable to conclude from the data presented here and elsewhere [540,545] that the substrate for 12-LOX is the determining factor in its effect on platelets and ultimately thrombosis. This conclusion is supported by work in COX which shows that oxidation of AA results in a pro-thrombotic milieu

of oxylipins [252,590] while other substrates such as DGLA can result in production of anti-thrombotic oxylipins [540,545,548,558,559].

The potent inhibition of thrombus formation by both DGLA and 12-HETrE, raises the potential that 12-HETrE will cause excessive bleeding similar to other antiplatelet agents [591-593]. Two hemostatic assays, the tail-bleeding assay and a second hemostatic model recently developed, the laser-induced cremaster arteriole puncture model, were used in this study to determine if the DGLA metabolite 12-HETrE prolonged bleeding following vascular injury. The mouse tail-bleeding assay for hemostasis is a physiological model, involving the measurement of cessation of bleeding following the excision of 5 mm of the distal tail, showed no prolonged bleed times in mice treated with 12-HETrE compared to the control. However, prolonged bleeding was observed in tail-bleeding following heparin administration (data not shown), supporting the sensitivity of the tail-bleeding assay approach. A second hemostatic assessment, the laser-induced cremaster arteriole puncture model, was performed by laser puncturing a hole through the cremaster arterial followed by the time measurement of fibrin and platelet plug formation in the mice. Similarly, no difference in bleeding times between control and 12-HETrE-treated mice was observed, supporting the hypothesis that 12-HETrE exerts an antithrombotic effect while at the same time maintaining primary hemostasis. These data support 12-HETrE either given directly or formed through ω-6 DGLA supplementation as a viable approach for prevention of thrombosis without creating a bleeding diathesis.

12-HETrE was shown here to directly activate a yet to be determined $G\alpha_s$-coupled GPCR. Direct addition of 12-HETrE to purified platelet membranes was shown to increase the binding of [^{35}S]GTPγS, the hydrolysis-resistant GTP analog, to the $G\alpha_s$-subunit resulting in cAMP formation, activation of PKA, and phosphorylation of VASP (Figure 9.6). Further studies

are required to identify if 12-HETrE is binding to a novel receptor or a previously characterized $G\alpha_s$-coupled GPCR on the human and mouse platelet. Identification of this receptor will be essential to determine if the mechanism of action elicited by 12-HETrE is mediated through binding the GPCR in an allosteric or orthosteric manner and how this binding compares to other previously identified oxylipins shown to signal the platelet in a $G\alpha_s$-dependent manner.

The discovery of 12-HETrE regulation of platelet function at both the *ex vivo* and *in vivo* levels and the delineation of the mechanism of action through the $G\alpha_s$-coupled GPCR establishes this oxylipin as an important eicosanoid in platelet biology. Beyond the platelet, it is also possible that 12-HETrE plays an important role in the regulatory function of other vascular cells similar to what is observed with other key oxylipins produced in the platelet, such as prostacyclin, PGE, PGD, and thromboxane. Further, this study describes for the first time how an omega-6 essential PUFA such as DGLA, can be used to alter the platelet signalosome in order to attenuate unwanted platelet activation and occlusive thrombus formation common in atherothrombotic diseases often leading to myocardial infarction and stroke. Future studies will seek to understand more fully how this newly discovered regulatory pathway limits platelet function and thrombotic risk while minimizing the risk of bleeding. This study fully supports future efforts to target the 12-HETrE pathway through the identification of the 12-HETrE receptor as a first-in-class antiplatelet therapeutic with minimal risk of bleeding.

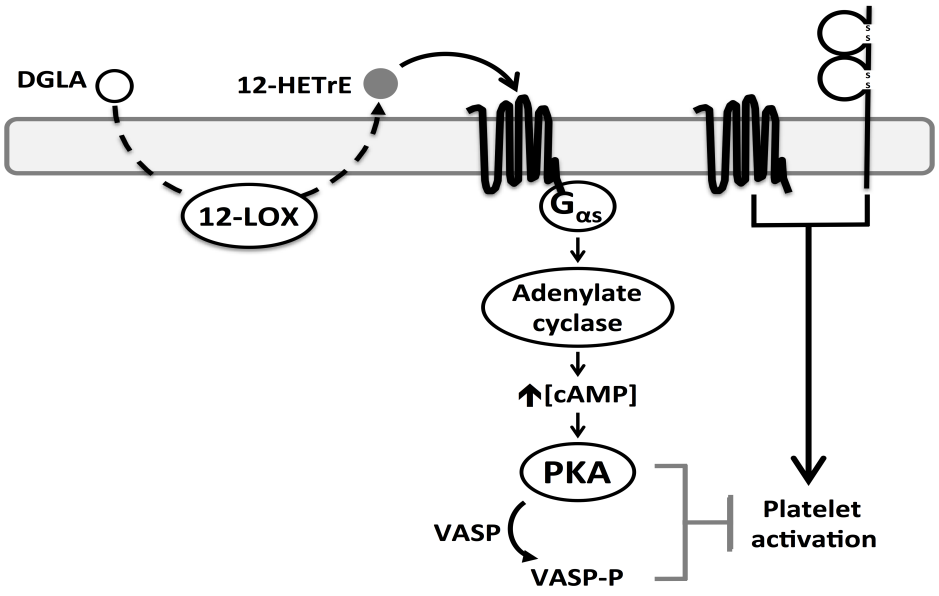


Figure 9.6 Proposed model of 12-HETrE inhibitory signaling in platelets.
Within platelets, 12-lipoxygenase (12-LOX) metabolizes free DGLA into the bioactive lipid, 12-HETrE. 12-HETrE can passively diffuse through the plasma membrane and presumably bind to an unidentified $G\alpha_s$-coupled receptor in a paracrine or autocrine manner. $G\alpha_s$ activates adenylyl cyclase, which increases the intracellular level of cyclic AMP (cAMP). Elevated cAMP activates protein kinase A (PKA), which phosphorylates a number of proteins, including vasodilator-stimulated phosphoprotein (VASP), leading to platelet inhibition in response to either GPCR- or ITAM-mediated platelet activation. This illustration was generated and kindly provided by Dr. Benjamin Tourdot.

CHAPTER 10

Discussion

Summary and significance

Regulation of platelet function is essential for the prevention and treatment of cardiovascular-related or immune-mediated thrombotic disorders, which can lead to stroke or myocardial infarction. Although current antiplatelet drugs effectively inhibit unwanted platelet activation and prevent thrombotic complications, these treatments are often associated with risk for bleeding. Therefore, identification of novel or alternative antiplatelet targets is warranted to prevent unwanted platelet activated as well as treat thrombotic disorders with minimal bleeding risk. In the context of my thesis studies described in chapters 5-9, 12-LOX has been demonstrated for the first time to play an important, but versatile role not only in modulating platelet activation involved in thrombotic pathologies, but also generating oxylipins from PUFAs that regulate platelet function. These studies established that targeting 12-LOX or utilizing its endogenous mechanism could represent potential avenues for antiplatelet therapy.

To support the claim that 12-LOX potentiates platelet activation, both pharmacological inhibition and genetic knockout of 12-LOX models were utilized to demonstrate 12-LOX activity was diminished or ablated resulting in attenuated platelet response. In chapter 5, one of the early generations of selective 12-LOX inhibitors, NCTT-956, attenuated human platelet activation (integrin αIIbβ3, calcium mobilization, PKC) in response to PAR4-AP and collagen agonists. 12-LOX inhibition could be overcome with the DAG mimetic, PMA, suggesting that PKC was upstream of 12-LOX activity[326]. Additionally, the work presented in chapter 6 demonstrated that

targeting platelet 12-LOX to prevent clot formation under shear stress may be superior than the current targeting strategy of inhibiting COX-1 with aspirin. I was also able to show that 12(S)-HETE directly enhanced dense granule secretion mediated by PAR1-AP compared to vehicle control treated platelets (chapter 6). Thus, this implicated the role of 12-HETE generated by 12-LOX in potentiating platelet activation. A modified and more potent 12-LOX inhibitor analog, ML355, described in chapter 7, also showed the benefits of using ML355 to attenuate ITT disorders, such as HIT. ML355 significantly attenuated human platelets response (aggregation, calcium flux, Rap1b activation, integrin αIIbβ3, dense and α-granules, and PKC) to immune complex crosslinking of the FcγRIIa receptor[328]. One of the proximal effectors of ITAM signaling, PLCγ2 phosphorylation, which is critical for its activation, was also demonstrated to be delayed when 12-LOX activity was inhibited. This suggested that 12-LOX activity was important for early PLCγ2 activation, affecting downstream effectors. In contrast, pharmacological inhibition of 12-LOX did not alter FcγRIIa phosphorylation, indicating 12-LOX does not directly regulate Src family kinases. Therefore, the data presented in chapter 8 suggest that 12-LOX may impinge on other early effectors preceding PLCγ2 activation, which includes the LAT signalosome components, Btk, Vav or Gads.

While 12-LOX predominantly oxidizes free AA into 12(S)-HETE in platelets, dietary supplementation can also modify the lipid content as well as eicosanoid generation. Although past studies have reported that higher intakes in animals and human of DGLA are cardioprotective, the mechanism by which dietary DGLA may provide cardioprotective benefits had not been fully elucidated. I had demonstrated (chapters 8, 9) that DGLA could not only be oxidized by COX-1 to PGE_1, but also by 12-LOX to 12(S)-HETrE. Wild-type and 12-$LOX^{-/-}$ mice were used to demonstrate that DGLA-mediated platelet inhibition required the presence of

12-LOX; however, the antiplatelet effects of 12(S)-HETrE did not depend on the expression of 12-LOX. These data strongly implicated the inhibitory role of DGLA was due to its oxidation of 12(S)-HETrE. 12(S)-HETrE was found to exert its anti-platelet effect through a $G\alpha_s$ signaling mechanism, involving the stimulation of adenylyl cyclase to generate cAMP and subsequent effectors activation. Eventually, 12(S)-HETrE was found to inhibit platelet reactivity through a G-protein coupled receptor (GPCR), prostacyclin (IP), using both pharmacological and genetic approaches to repress or ablate IP [594]. Importantly, these studies provided further understanding of the antithrombotic effects of DGLA as well as the rationale for the development of analogs derived from 12(S)-HETrE as a new approach for antithrombotic therapeutics.

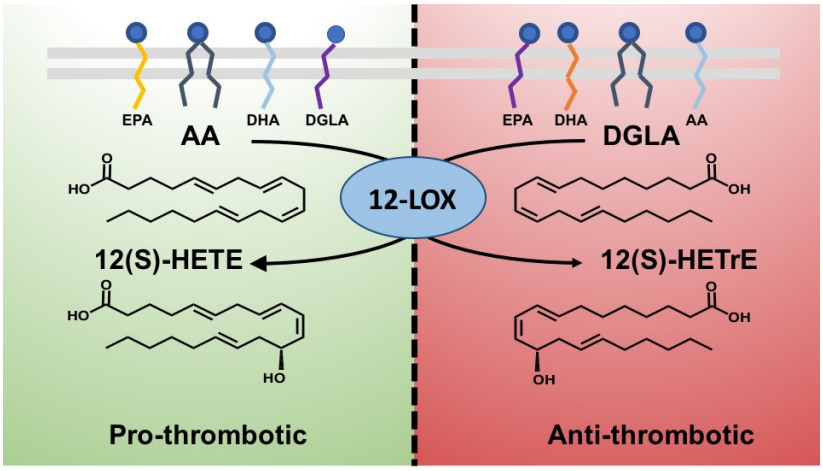


Figure 10.1 The pro- and anti-thrombotic oxylipins of 12-LOX.

Investigating the mechanisms of 12-LOX regulation in platelet activation

The work presented in chapters 5-8 primarily focused on the enzymatic function of 12-LOX, establishing the importance of selective oxylipin production in the regulation of platelet function; however, the underlying signaling mechanism by which 12-LOX and or its predominant AA-derived oxylipin, 12(S)-HETE, participates in platelet activation still remains to be fully elucidated. My results presented in chapter 7, by which 12-LOX could possibly regulate the ITAM pathway, especially the early activation of PLCγ2 activation, suggest that other upstream

effectors, LAT or BTK, may be regulated by 12-LOX independent of its oxylipin, 12(S)-HETE, activity or vice versa. In addition, other avenues regulation of or by 12-LOX in the platelets described below could be potential mechanisms that have yet to be explored.

12(S)-HETE has been reported to exert its action through multiple mechanisms: a) activation of high affinity GPCR, 12-HETER (GPR31); b) low affinity leukotriene B4 receptor (BLT_2); c) transcellular eicosanoid metabolism via lipoxygenase or cyclooxygenase pathways; or d) activation of the peroxisome proliferator-activated receptor γ (PPARγ). While these mechanisms of action for 12(S)-HETE have been implicated in cell lines; however, these pathways have not been confirmed nor assessed in platelets yet. It is much more likely that 12(S)-HETE potentiates platelet activation through 12-HETER, since BLT_2 has not been shown to be expressed in platelets to date. On the other hand, PPARγ is an intercellular receptor that is involved in platelet inhibition rather than activation[595]. 12(S)-HETE derived from cancer cells expressing platelet-type 12-LOX had been shown to enhance metastatic spread by inducing cancer cell and lymph endothelial cell motility (LEC). 12(S)-HETE was shown to directly enhance phosphorylation of myosin light chain (pMLC) and myosin phosphatase targeting subunit 1 (MYPT) in lymph endothelial cells, whereas cells pretreated with RHO-GTPase and ROCK inhibitors attenuated pMLC and MYPT[596]. Furthermore, 12(S)-HETE was shown to directly stimulate intracellular adhesion molecule-1 (ICAM-1) expression in human aortic endothelial cells through the RhoA/PKCα dependent activation of NF-κB. To corroborate the role of G protein-mediated signaling induced by 12(S)-HETE, G12 and G13 minigenes were used to competitively inhibit $G\alpha_{12/13}$ signaling in endothelial cells. Based on these data, 12-HETER appear to be coupled to $G\alpha_{12/13}$, which activates RhoA pathway [597]. These observations have yet to be assessed in platelets to confirm that 12(S)-HETE potentiates platelet activation through 12-HETER. If

verified, GPR31 could be a potential drug target used in therapy for prevention of thrombotic events.

The "shedding" of FcγRIIa and GPVI has been demonstrated as a potential mechanism for irreversible inactivation of both GPVI/FcRγ and FcγRIIa–mediated signaling [598]. Ligands binding to either GPVI or FcγRIIa induce both extracellular and intracellular cleavages mediated by either matrix metalloproteases (MMPs), ADAM10 and ADAM17, or intracellular calcium-dependent protease, calpain[599]. A key modulator of the MMPs facilitated shedding of the immunoglobulin receptors is the association of calmodulin with the cytoplasmic tail of GPVI and FcγRIIa; however, calmodulin inhibitor has been shown to induce calmodulin dissociation and prevent the shedding of the receptor ectodomain by MMPs. The removal of the receptors is thought to contribute to reduced platelet-activation[600]. Besides extracellular receptor shedding of the receptors, intracellular cysteine protease, calpain, has also been shown to remove the ITAM domain of the receptors. These findings suggest potential mechanisms for dampening clinical sequelae associated with ITAM-dependent signaling in patients with HIT. Thus, it is possible that the alternative non-enzymatic function 12-LOX is to prevent extracellular shedding of the receptors or cleavage of the ITAMs through its regulation of either calmodulin or calpain activity. 12-LOX may directly regulate the removal of calmodulin from the cytoplasmic tails or promote the recruitment and aid in the activation of calpain to remove the ITAM domains. As a consequence of receptors shedding or ITAM removal, ITAM-induced proximal signaling is attenuated concomitant with down-regulated platelet response.

The role of platelet-type 12-LOX is also strongly implicated in the aggressive metastatic nature and progression of human erythroleukemia, breast cancer, colon carcinoma, glioma, and epidermoid carcinoma[601]. Dilly et al. previously showed that 12-LOX was linked to the β4

cytoplasmic tail of α6β4 integrin (glycoprotein that plays a major role in promoting tumor cell invasion and metastases) following stimulation with extracellular matrix laminins. 12-LOX was found to be recruited to the cytoplasmic tail and then phosphorylated by Src family kinases in human epidermoid A431 cells in response to integrin stimulation. Pharmacological inhibition of Src kinase activity by PP2 or expression of dominant-negative mutant Src kinase reduced 12-LOX phosphorylation at Y19 and Y614 residues and 12(S)-HETE production in response to integrin stimulation in A431 cells[602]. It is possible that 12-LOX in platelets could be phosphorylated by specific Src kinases, and whether specific phosphorylated residues enhance oxylipin formation or dictate its substrate affinity has yet to be assessed. 12-LOX may also be regulating the cooperative integrin/ITAM pair, by which it facilitates the recruitment of proximal effectors Syk and assembly of SLP76/LAT/Btk/Vav and PLCγ2 with the integrin signaling complex in the $\alpha_{IIb}\beta_3$-mediated "outside-in" signaling described in chapter 1. In another scenario, 12-LOX could be inactivating and disrupting the constitutively bound C-terminal Src kinase (CSK) (an enzyme that keeps Src in inactive conformation) from β_3 and Src complex to shift the $\alpha_{IIb}\beta_3$ integrin from low to high affinity in an "inside-out" dependent signaling process[603].

ML355 mechanism of action, selectivity, and therapeutic use

Previous 12-LOX inhibitors had been categorized by their mechanism of action. For instance, baicalein, nordihydroguaiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid (ETYA), and BW755C are redox inhibitors, by which they blocked the oxidation of the nonheme ferrous (Fe^{2+}) state at the catalytic from transitioning to the active ferric (Fe^{3+}) state. In addition, OPC-29030 and L-655,238, block the translocation of 12-LOX to the peripheral membrane and subsequently prevented 12-HETE formation. Several of these 12-LOX inhibitors were deemed

non-selective, by which they directly inactivated $cPLA_2$, as well as inhibit cyclooxygenase-1 in platelets. The exact mechanism by which ML355 inactivates 12-LOX activity is not known and is currently being investigated. Although ML355 has been shown to be a potent inhibitor of human platelet 12-LOX at low concentrations without affecting other lipoxygenases (15-LOX-1, 15-LOX-2, 5-LOX, COX-1, and COX-2)[604] and no bleeding diathesis in mouse models [605], other potential or non-selective, and non-oxygenases targets have yet to be assessed. These are critical parameters that are currently being investigated before ML355 can transition into the clinical phase for use in the prevention and intervention of thrombotic complications.

12(S)-HETrE mechanism of action, and selectivity

Prostacyclin analogues had been developed over the years since the endogenous prostacyclin (PGI_2) is extremely labile with a half-life of approximately 40 seconds[606]. To date, only epoprostenol, treprostinil, and iloprost are FDA approved analogues for the treatment of pulmonary arterial hypertension. While epoprostenol and treprostinil are delivered through intravascular (IV) infusion, iloprost is administered via nebulization. Unfortunately, these analogues are labile and associated with side effects (hypotension, diarrhea, nausea, headache, jaw pain, flushing, and cough)[607]. Currently, their labeled use as an anti-platelet agent has not been approved. Previous clinical studies have shown infusions of iloprost for the treatment of peripheral vascular disease enhanced platelet reactivity and coagulation in some patients, which represented a risk for thromboembolism, especially in patients who were already in a prethrombotic condition[608]. In contrast to the clinical findings of iloprost, which were performed *in vitro*, the thrombosis mouse models we used to demonstrate the utility of 12(S)-HETrE, which also modulates platelet reactivity through the prostacyclin receptor (IP), does not enhance platelet reactivity or coagulation. 12(S)-HETrE prevented thrombus formation following injury

without affecting hemostasis. The side effects presented by prostacyclin analogues and other potential targets of 12(S)-HETrE are currently being evaluated before its transition into the clinics for treatment of platelet related disorders.

Targeting 12-LOX or its oxylipin, 12-HETE, for the treatment of HIT

Immune mediated heparin-induced thrombocytopenia (HIT) is an atypical immune disorder that afflicts 1 in 5000 hospitalized patients. HIT is characterized by low platelet count and thrombosis following heparin exposure (see chapter 7). Approximately 50% of these patients develop thromboembolic complications due to autoantibodies recognizing the complexes of heparin and platelet factor 4 (PF4). Risks for HIT includes the type of heparin (unfractionated or fractionated), and duration of heparin exposure. Besides heparin exposure, history of surgical interventions can lead to enhanced platelet reactivity with circulating PF4. Ultimately, these autoantibodies trigger platelet activation through the platelet FcγRIIa and consequently results in thrombocytopenia and thrombus formation. The only treatment for HIT is argatroban, a direct thrombin inhibitor (DTI); however, DTIs are associated with high bleeding risk. Previous studies had demonstrated the utility of regulating Syk (see section 1.4) to inhibit FcγRIIa-mediated platelet activation and thrombosis. Syk is not only expressed in platelets, but also in a variety of tissues, primarily in hematopoietic cells. Thereby, inhibiting Syk can adversely prevent critical lymphocytic function[609], lymphatic development[610], and increase the risk for tumorigenesis[611]. I had proposed in chapter 7 that targeting platelet 12-LOX with ML355 could be a potential therapy for immune-mediated thrombosis disorder, such as HIT. ML355 is much more selective in inhibiting 12-LOX without targeting the lipoxygenases, 15-LOX and 5-LOX, expressed in other hematopoietic cells. This would be much more viable than targeting 12-HETER, since 12-HETER may also be expressed in other myeloid cells as well as lymphatic tissues.

Conclusion

Antiplatelet therapies have decreased the incidence and mortality of thrombotic complications associated with myocardial infarction and stroke. Although the current antiplatelet therapies provide additional thrombotic protection, these have come at the expense of increases in bleeding. The goal of my thesis was to delineate a target in platelet function that is involved in pathological intravascular thrombosis; however dispensable in hemostasis. As indicated above, regulating the 12-LOX pathway in platelet activation serves a promising therapeutic approach for thrombotic related diseases, including immune-mediated thrombosis, such as HIT. The orally bioavailable 12-LOX inhibitor, ML355, has been demonstrated to inhibit thrombosis *in vivo* with minimal impact on hemostasis based on the presence of fibrin formation in murine models.

Besides directly targeting 12-LOX, the action of oxylipins, derived from the oxygenase, also demonstrate potential therapeutic avenue in the prevention of thrombotic events. 12(S)-HETE, the predominant oxylipin derived from 12-LOX oxidation, is deemed to be pro-thrombotic through its action on GPR31. Although GPR31 was discovered as the high affinity receptor for 12(S)-HETE, its functional role in platelets has received limited attention. Characterizing the mechanism of GPR31 function in platelets can aid in the development of small molecules inhibitor for the receptor to elucidate GPR31 function and a potential therapeutic target for treatment of thrombosis. Additionally, the anti-thrombotic oxylipin derived from DGLA, 12(S)-HETrE, also aids in acceleration of potent drug design that will also inhibit thrombosis without prolonging bleeding.

In general, elucidating the role of 12-LOX with selective inhibitors was essential to differentiate between the role of 12-LOX in pathophysiological thrombosis and hemostasis. These inhibitors provided insights that regulating 12-LOX activity is essential in preventing

thrombosis with minimal bleeding diathesis. In addition, these inhibitor studies established the activity of 12-LOX potentiated thrombosis; however, the role of its derived oxylipin, 12(S)-HETE, in platelet signaling is still unclear. Further understanding of comprehensive molecular and cellular mechanism of 12(S)-HETE would help delineate its significance in platelet biology and facility development of novel prophylactic treatment for thrombotic related diseases.

Appendix A

Protein Kinase Regulation of 12-Lipoxygenase-Mediated Human Platelet Activation[8]

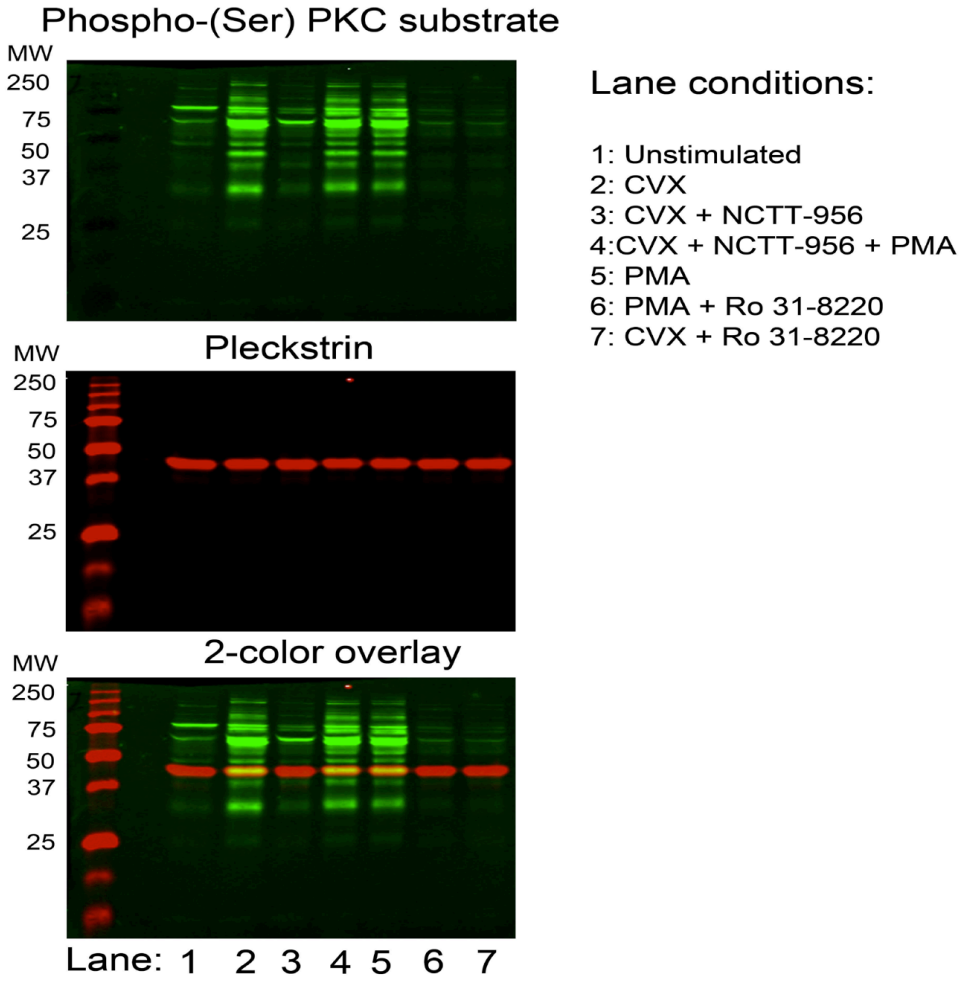


Figure A.1 Pleckstrin is a 47 kDa protein corresponding to one of the phospho-(Ser)-PKC substrate bands.
To identify which of the phospho-(Ser) PKC substrate bands is pleckstrin, platelet samples were treated as indicated in the lane conditions on the right. Green represents phospho-(Ser) PKC substrate antibody staining, red represents pleckstrin antibody staining and the bottom panel is an overlay of the two analyzed with the 2-laser Licor imaging system. Pleckstrin staining correlates to a 4 7 kDa protein band in the phospho-(Ser) PKC substrate. Staining of This band in the phospho-(Ser) PKC substrate staining is significantly diminished in the presence of NCTT-956 or Ro 31-8220, but it rescued in the presence of NCTT-956 + PMA

[8] This section has been published in Molecular Pharmacology. Yeung J, Apopa PL, Vesci J, Kenyon V, Rai G, Jadhav A, Simeonov A, Holman TR, Maloney DJ, Boutaud O, Holinstat M. Protein kinase C regulation of 12-lipoxygenase-mediated human platelet activation. Molecular pharmacology. 2012 Mar 1;81(3):420-30.

Appendix B

12-Lipoxygenase Activity Plays an Important Role in PAR4 and GPVI-Mediated Platelet[9] Reactivity

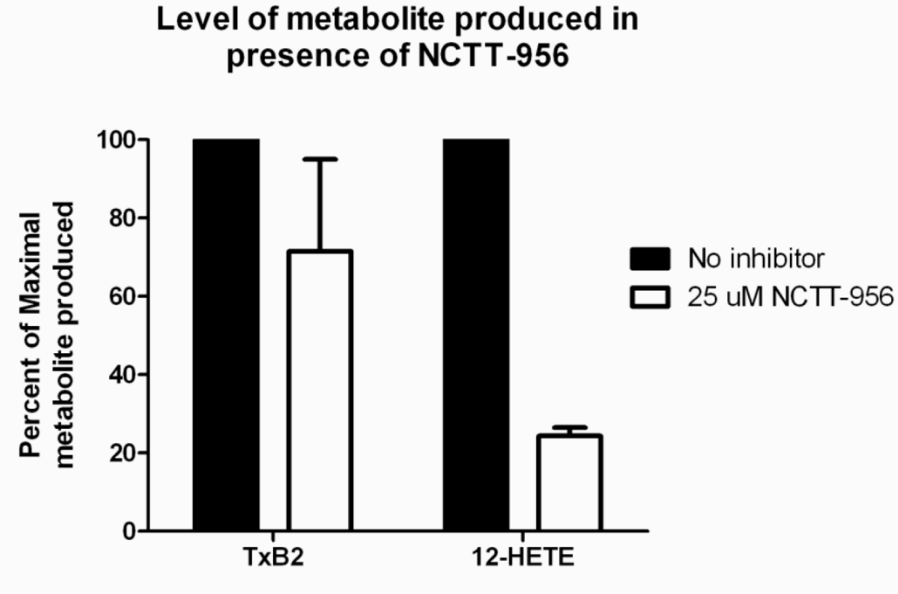


Figure B.1 12-LOX inhibitor selectivity towards 12-LOX.
To confirm substrate selectivity for NCTT-956, platelets were stimulated in the absence or presence of 25 μM NCTT-956 and the levels of TxB2 and 12-HETE formation were assessed by mass spectrometry (N=2-3). While NCTT-956 did not significantly affect TxB2 formation, the level of 12-HETE produced in the presence of 25 μM NCTT-596 was substantially reduced. This figure was provided by our collaborator Dr. Theodore Holman's group

[9] This section has been published in Thrombosis and Hemostasis. Yeung J, Apopa PL, Vesci J, Stolla M, Rai G, Simeonov A, Jadhav A, Fernandez-Perez P, Maloney DJ, Boutaud O, Holman TR. 12-lipoxygenase activity plays an important role in PAR4 and GPVI-mediated platelet reactivity. Thrombosis and haemostasis. 2013 Sep;110(3):569.

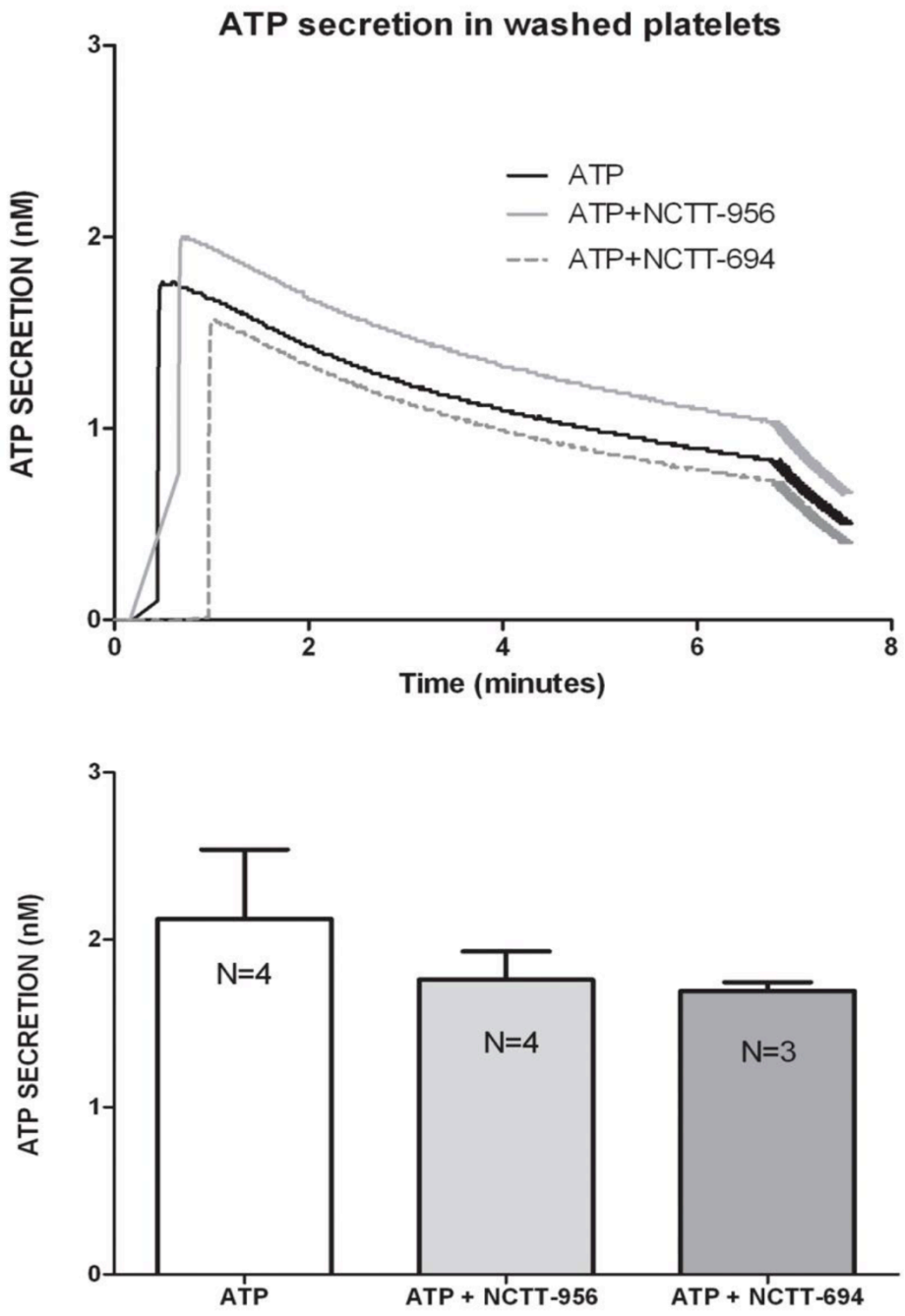


Figure B.2 12-LOX inhibitors do not interfere with ATP luciferase assay.
To confirm the positive and negative analogs for the 12-LOX inhibitors did not directly interact with the luciferase assay, washed platelets were incubated with chronolume for 2 minutes in the absence or presence of NCTT-956 or NCTT-694. Following incubation, the platelets were spiked with 2 nM ATP and the level of ATP was confirmed in the lumi-aggregometer. Neither NCTT-956 nor NCTT-694 significantly inhibited the interaction of the chronolume reagent with ATP, confirming the observed inhibition of ATP secretion in Figure 2 is not due to inhibition of the reaction due to the addition of the small molecule inhibitors.

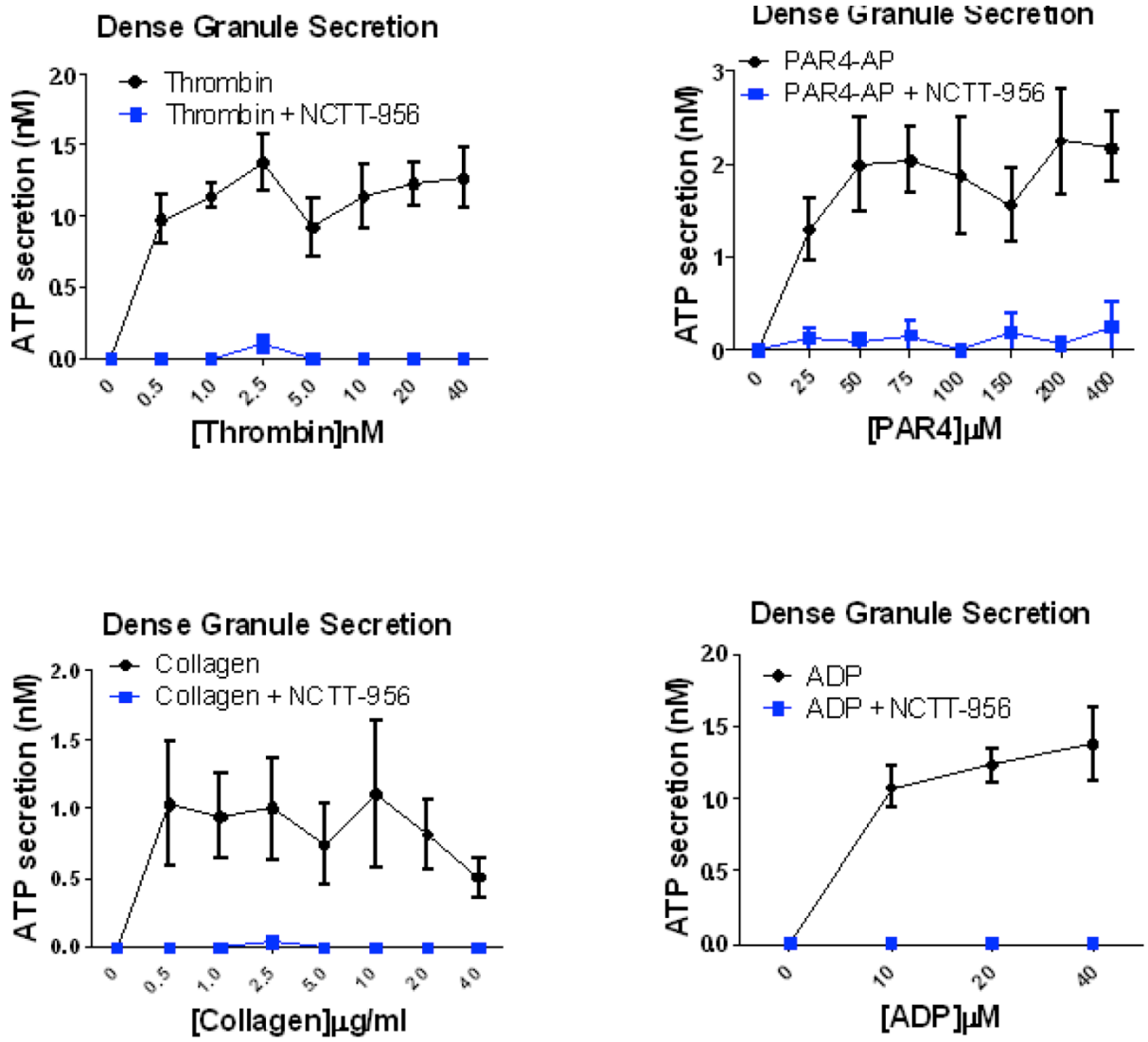


Figure B.3 NCTT-956 inhibition of dense granule secretion.
The presence of 25 µM NCTT-956 inhibits ATP secretion (dense granule secretion) following stimulation with a number of agonists including thrombin, PAR4-AP, collagen, and ADP. This figure was co- provided by Jennifer Yeung and Dr. Patrick Apopa.

Appendix C

Platelet 12-LOX is Essential for FcγRIIa-Mediated Platelet Activation[10]

	White Blood Cells						Red Blood Cells				Platelets	
	WBC (K/μL)	NE (K/μL)	LY (K/μL)	MO (K/μL)	EO (K/μL)	BA (K/μL)	RBC (M/uL)	Hb (g/dL)	HCT(%)	MCV (fL)	PLT (K/μL)	MPV (fL)
hFcR/ALOX12$^{-/-}$	6.55 ± 1.84	0.96 ± 0.14	5.2 ± 1.68	0.33 ± 0.033	0.045 ± 0.017	0.01 ± 0.0058	8.29 ± 0.43	12.63 ± 0.39	39 ± 1.32	47.18 ± 1.248	985 ± 79	5.08 ± 0.048
hFcR/ALOX12$^{+/+}$	9.43 ± 0.65	1.45 ± 0.12	7.69 ± 0.67	0.26 ± 0.10	0.02 ± 0.0058	0.013 ± .013	9.44 ± 0.39	13.9 ± 0.85	41.73 ± 2.15	44.17 ± 0.636	870.7 ± 152	4.97 ± 0.12

Table C.1 Complete blood count was performed on hFcR/ALOX12-/- (n=4) and hFcR/ALOX12+/+ (n=3) mice.
Data represents the mean ± SEM. An unpaired, two-tailed t-test was performed and no significant differences were observed. This figure was generated by Dr. Benjamin Tourdot.

[10] This has been published in Blood. Yeung J, Tourdot BE, Fernandez-Perez P, Vesci J, Ren J, Smyrniotis CJ, Luci DK, Jadhav A, Simeonov A, Maloney DJ, Holman TR. Platelet 12-LOX is essential for FcγRIIa-mediated platelet activation. Blood. 2014 Oct 2;124(14):2271-9.

Appendix D

Investigations of Human Platelet-Type 12-Lipoxygenase: Role of Lipoxygenase Products in Platelet Activation[11]

Figure D.1 Charge remote allylic fragmentation.
This figure was provided by our collaborator Dr. Theodore Holman's group.

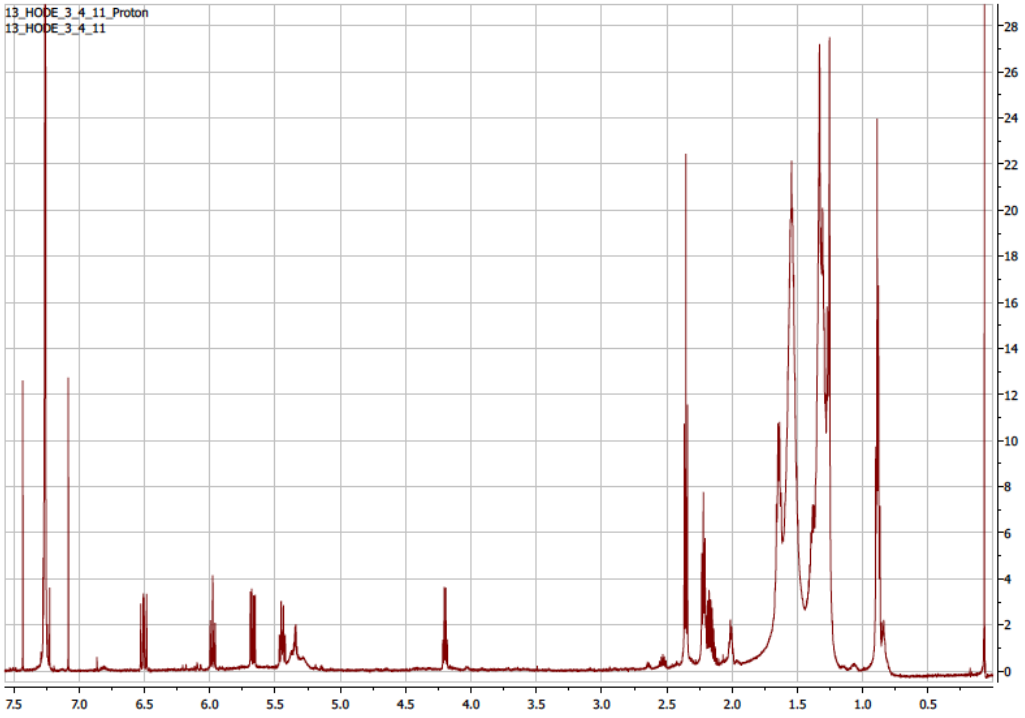


Figure D.2 1D proton NMR of 13-HODE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

[11] This section has been published in Journal of Lipids Research. Ikei KN, Yeung J, Apopa PL, Ceja J, Vesci J, Holman TR, Holinstat M. Investigations of human platelet-type 12-lipoxygenase: role of lipoxygenase products in platelet activation. Journal of lipid research. 2012 Dec 1;53(12):2546-59.

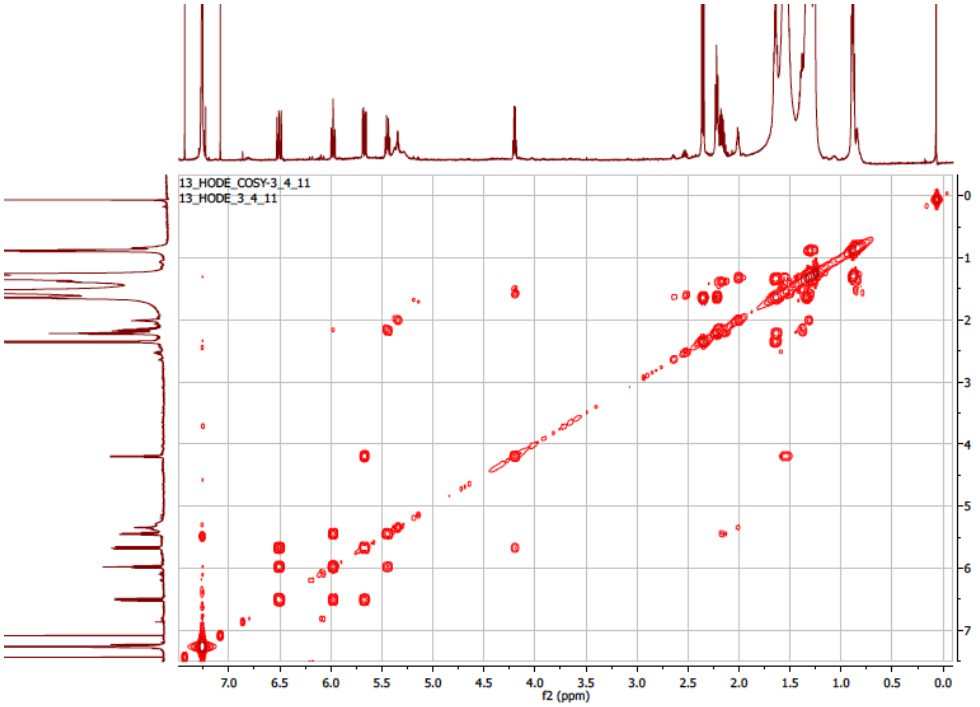


Figure D.3 2D COSY NMR of 13-HODE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

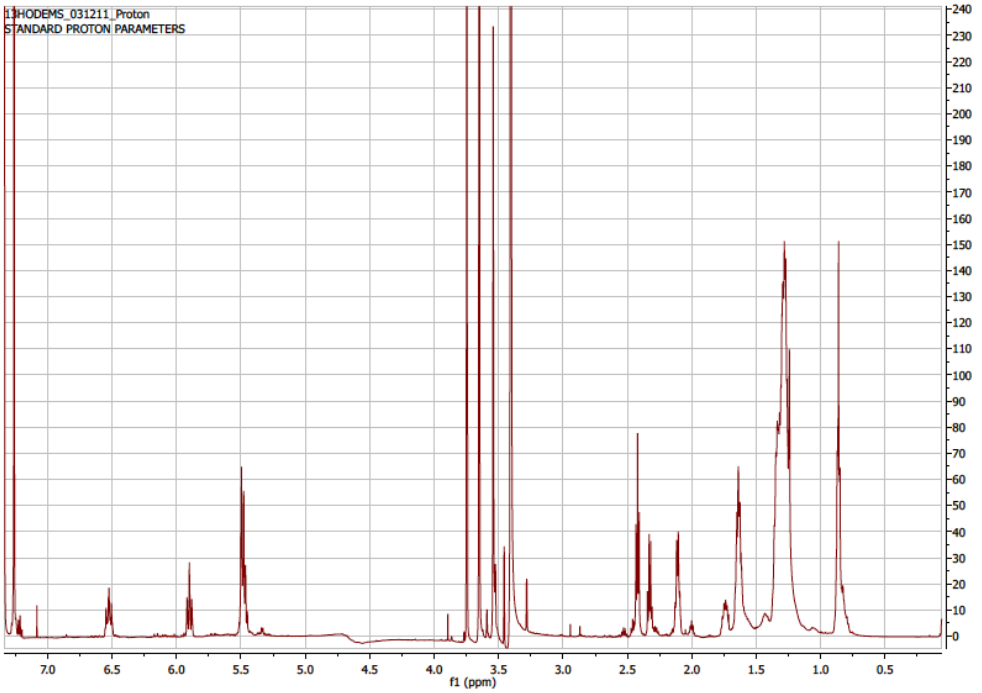


Figure D.4 1D Proton NMR of 13-HODE ester with Mosher (S).
This figure was provided by our collaborator Dr. Theodore Holman's group.

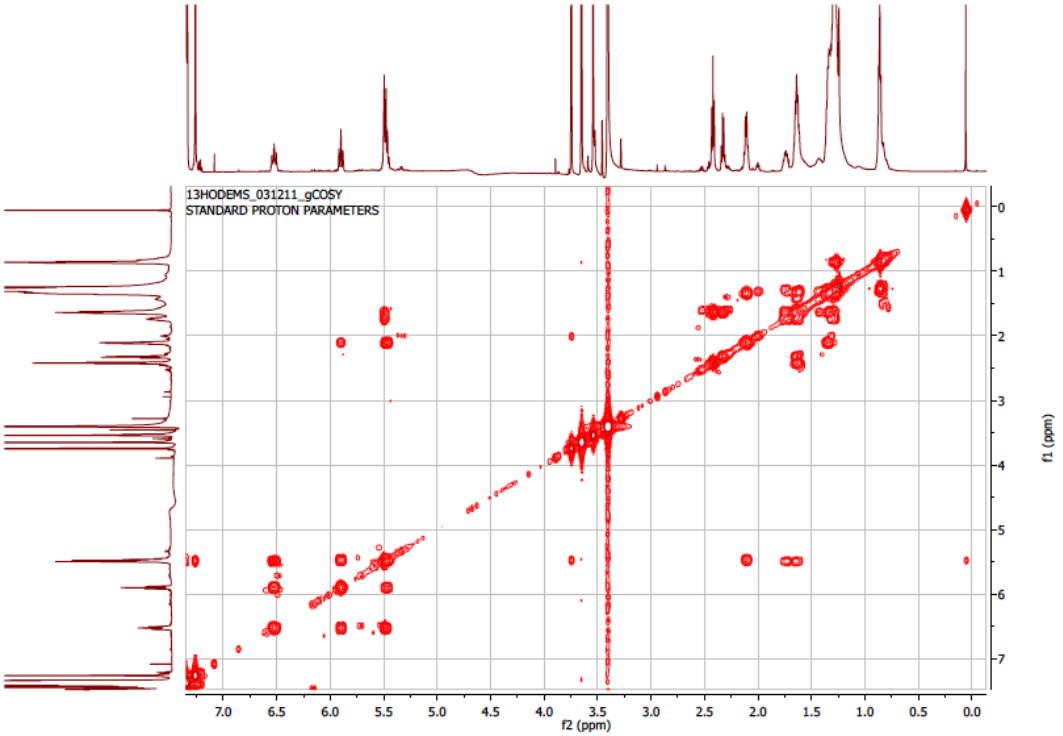


Figure D.5 2D COSY NMR of 13-HODE ester with Mosher (S).
This figure was provided by our collaborator Dr. Theodore Holman's group.

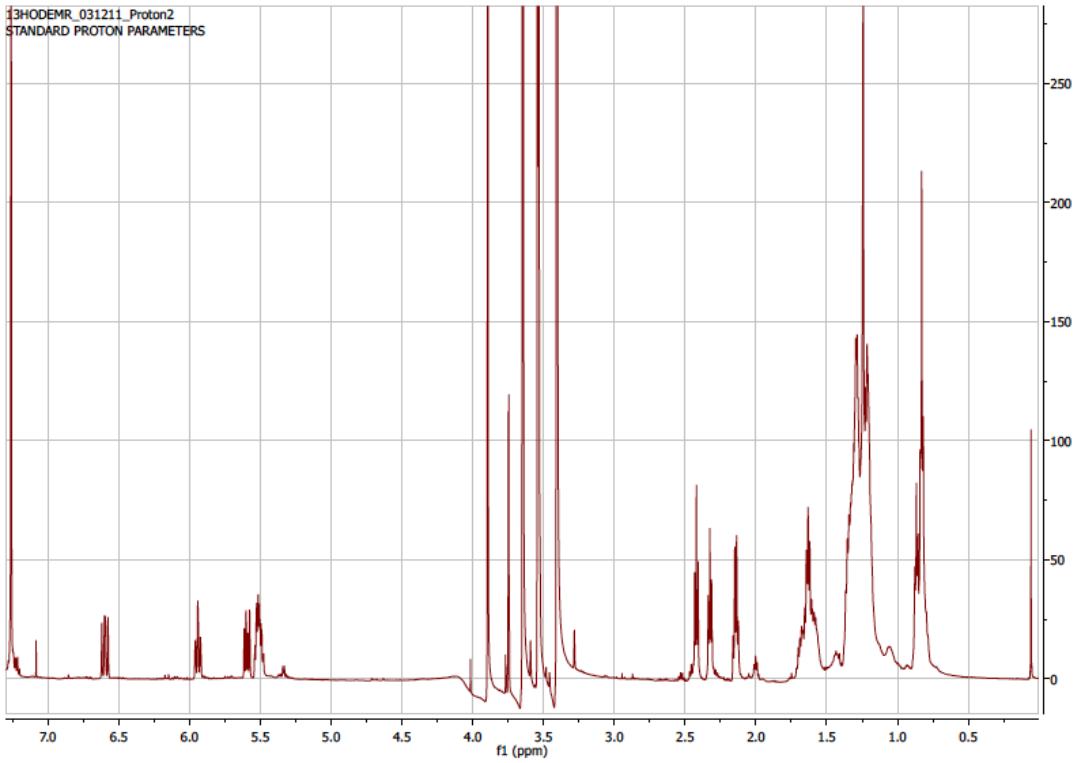


Figure D.6 1D Proton NMR of 13-HODE ester with Mosher (R).
This figure was provided by our collaborator Dr. Theodore Holman's group.

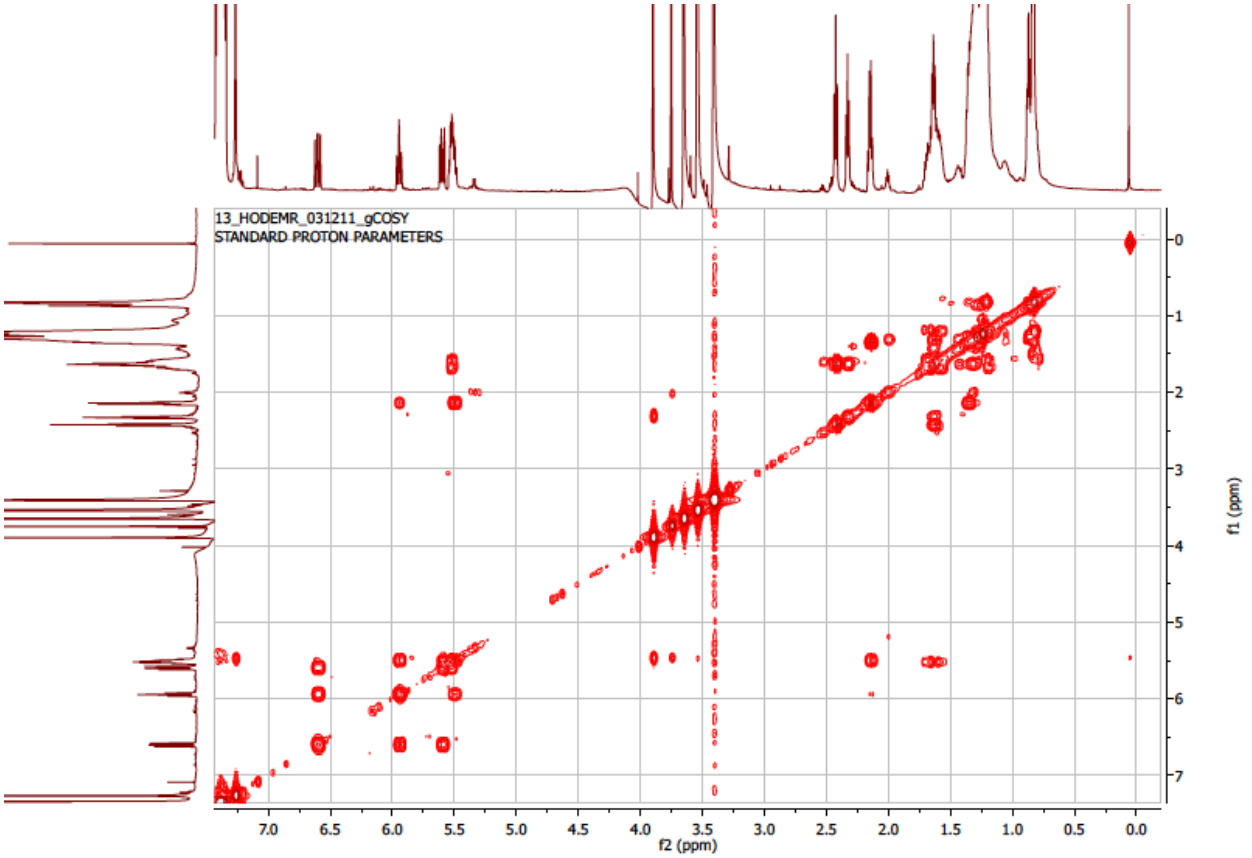


Figure D.7 2D COSY NMR of 13-HODE ester with Mosher (R).
This figure was provided by our collaborator Dr. Theodore Holman's group.

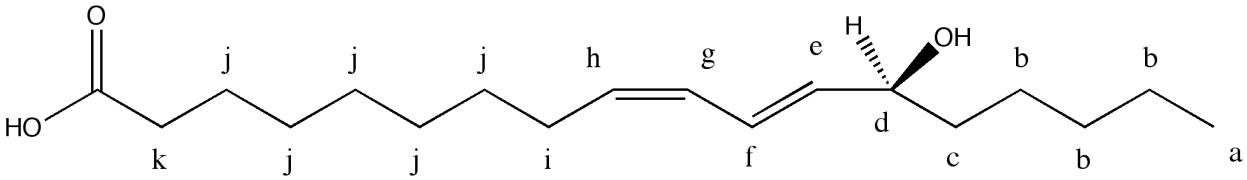


Figure D.8 13-HODE with labeled protons.
This figure was provided by our collaborator Dr. Theodore Holman's group.

H Position	ppm Fatty Alcohol	ppm Mosher (S)	ppm Mosher (R)	(S)ppm-(R)ppm
a	0.88, m	0.86, m	0.82, m	0.04
b	1.32, m	1.26, m	1.21, m	0.05
c	1.62, m	1.62, m	1.58, m	0.04
d	4.19, q	5.48, m	5.53, m	-0.05
e	5.68, dd	5.5, m	5.59, dd	-0.4
f	6.51, q	6.52, m	6.60, q	-0.08
g	5.98, t	5.90, t	5.94, t	-0.04
h	5.45, m	5.47, m	5.50, m	-0.03
i	2.16, m	2.1	2.13, m	-0.03
j	2.20, m	1.35, m	1.35, m	0
k	2.34, t	1.63, m	1.63, m	0

Splitting: s; singlet, d; doublet, t; triplet, qa; quartet, qi; quintet, m; multiplet, dd; doublet of doublets.

Figure D.9 Chemical Shifts of 13-HOTrE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

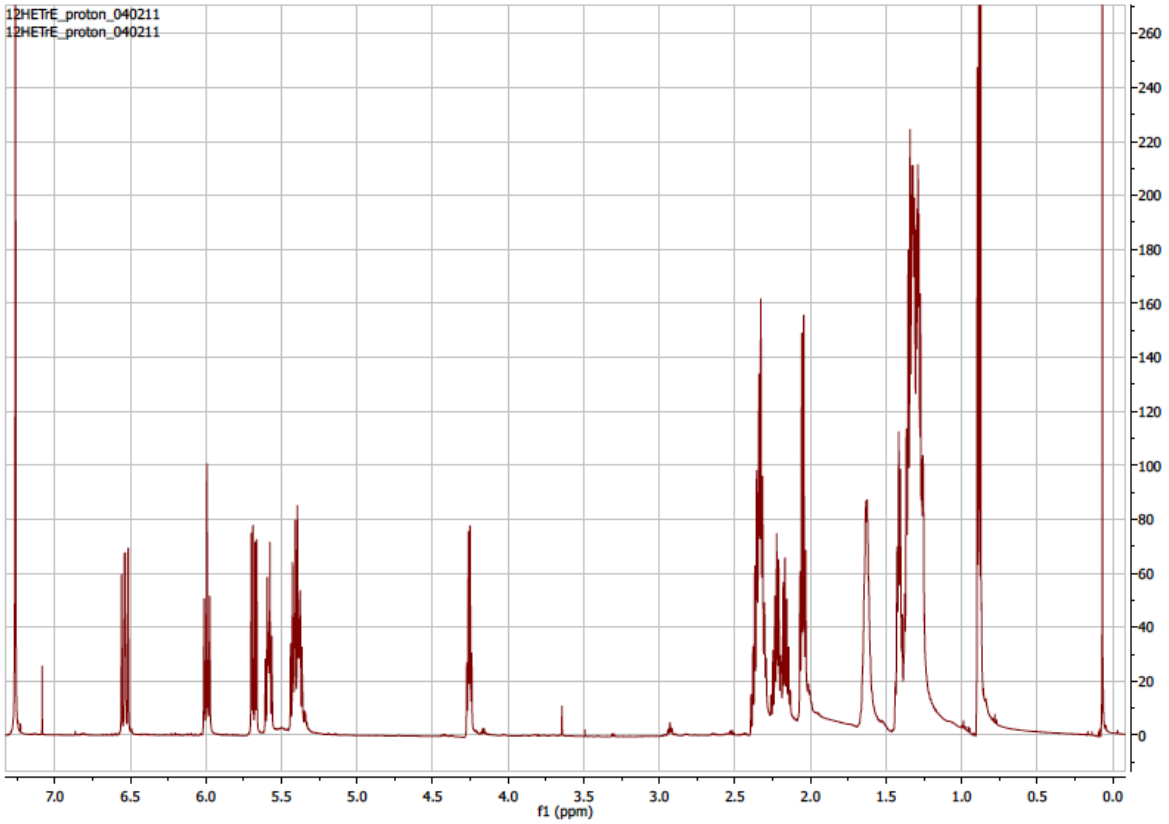


Figure D.10 1D Proton NMR of 12-HETrE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

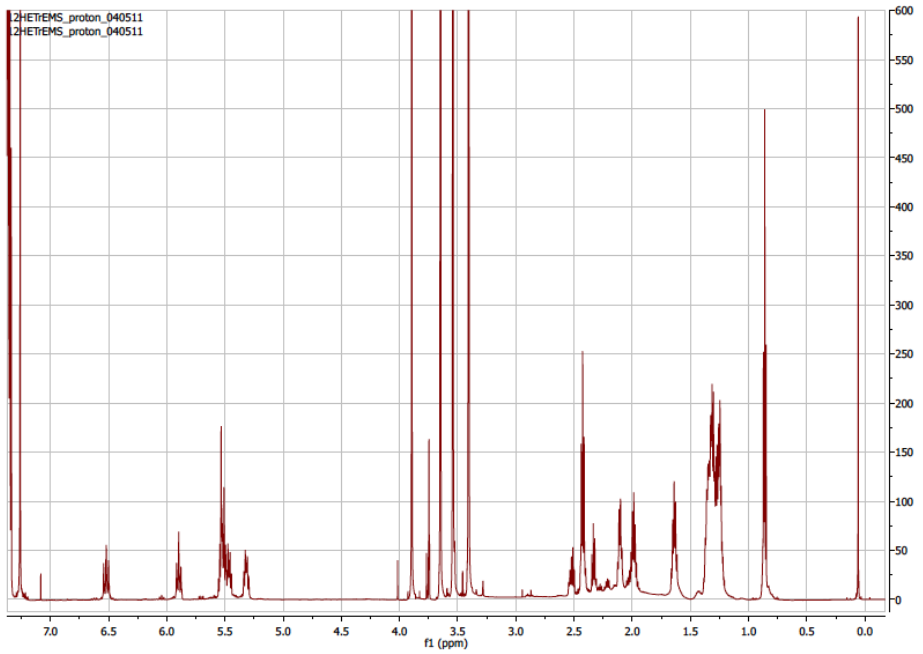


Figure D.11 1D proton NMR of 12-HETrE ester with Mosher (S).

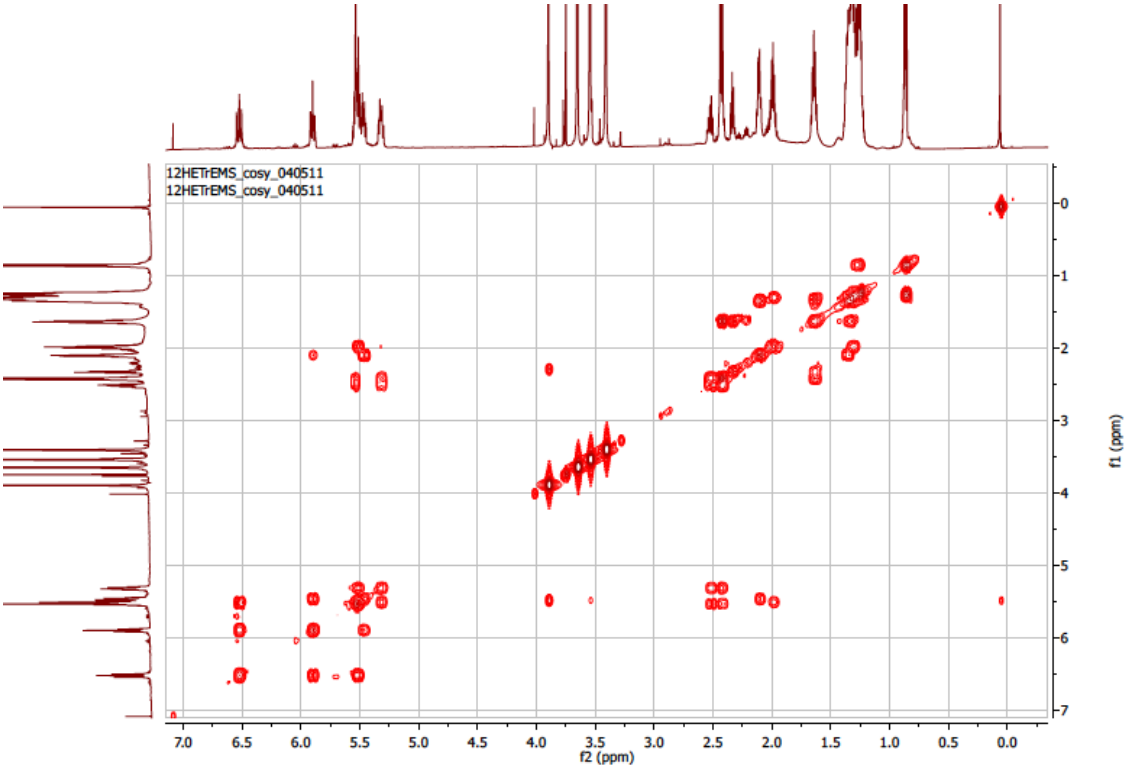


Figure D.12 2D COSY NMR of 12-HETrE ester with Mosher (S).
This figure was provided by our collaborator Dr. Theodore Holman's group.

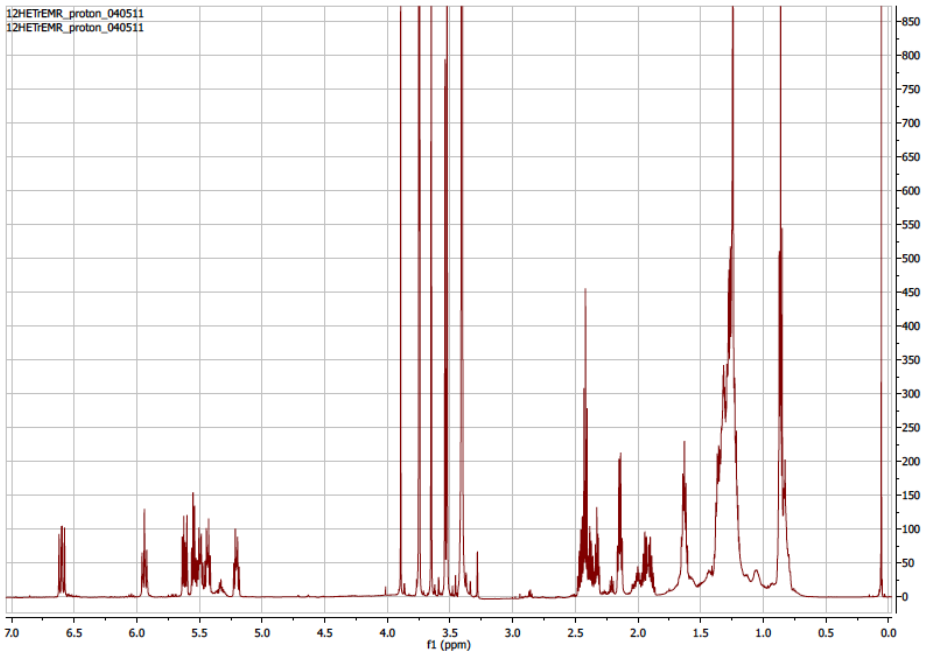


Figure D.13 1D Proton NMR of 12-HETrE ester with Mosher (R)

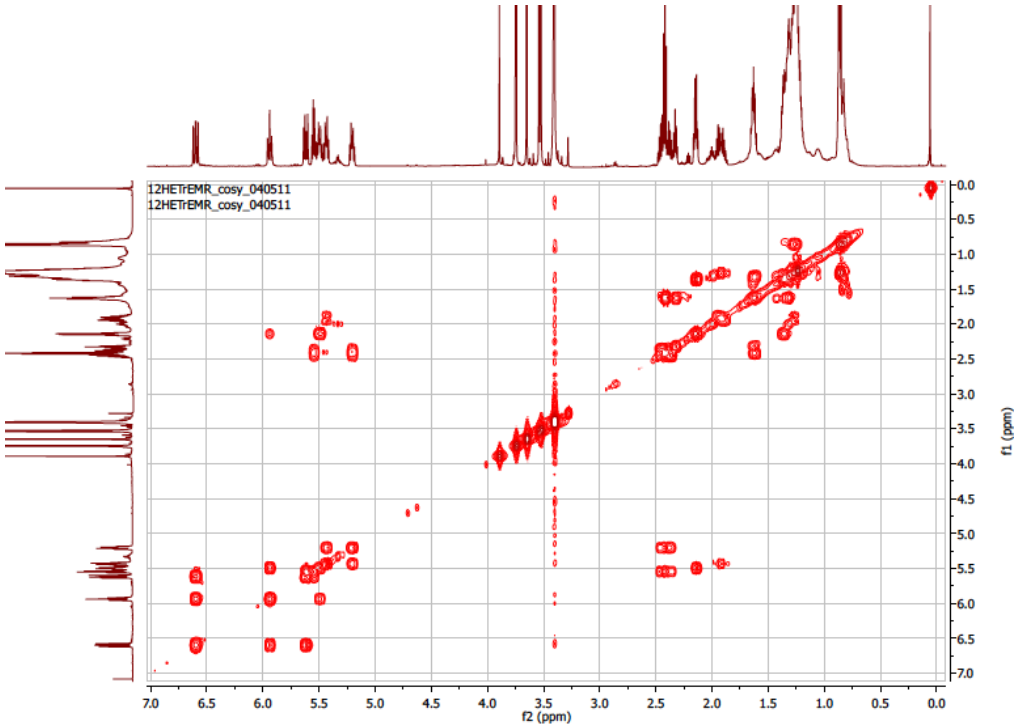


Figure D.14 2D COSY NMR of 12-HETrE ester with Mosher (R).
This figure was provided by our collaborator Dr. Theodore Holman's group.

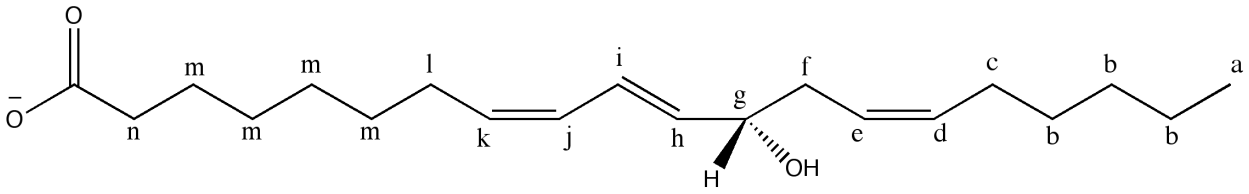


Figure D.15 12-HETrE with labeled protons.
This figure was provided by our collaborator Dr. Theodore Holman's group.

H Position	ppm Fatty Alcohol	ppm Mosher (S)	ppm Mosher (R)	(S)ppm-(R)ppm
a	0.89, t	0.86, t	0.86, t	0.00
b	1.31, m	1.27, m	1.26, m	0.01
c	2.05, qa	1.98, m	1.92, m	0.06
d	5.58, m	5.51, m	5.44, m	0.07
e	5.42, m	5.31, m	5.21, m	0.10
f	2.34, m	2.47, m	2.42, m	0.05
g	4.26, qa	5.53, m	5.55, m	-0.02
h	5.68, dd	5.52, m	5.62, dd	-0.10
i	6.53, qa	6.52, m	6.59, qa	-0.07
j	6.00, t	5.90, t	5.94, t	-0.04
k	5.41, m	5.47, m	5.50, m	-0.03
l	2.17, m	2.11, m	2.14, qa	-0.03
m	1.42, m	1.35, m	1.35, m	0.00
n	1.63, m	1.63, m	1.63, m	0.00

Splitting: s; singlet, d; doublet, t; triplet, qa; quartet, qi; quintet, m; multiplet, dd; doublet of doublets.

Figure D.16 Chemical Shifts of 12-HETrE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

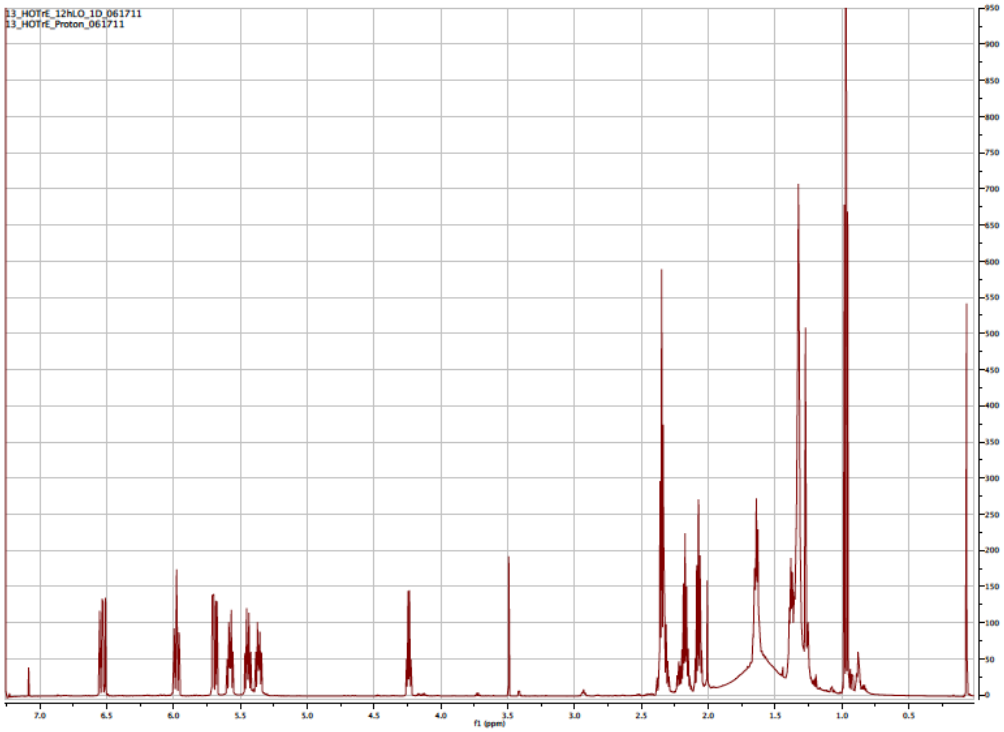


Figure D.17 1D Proton NMR of 13-HOTrE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

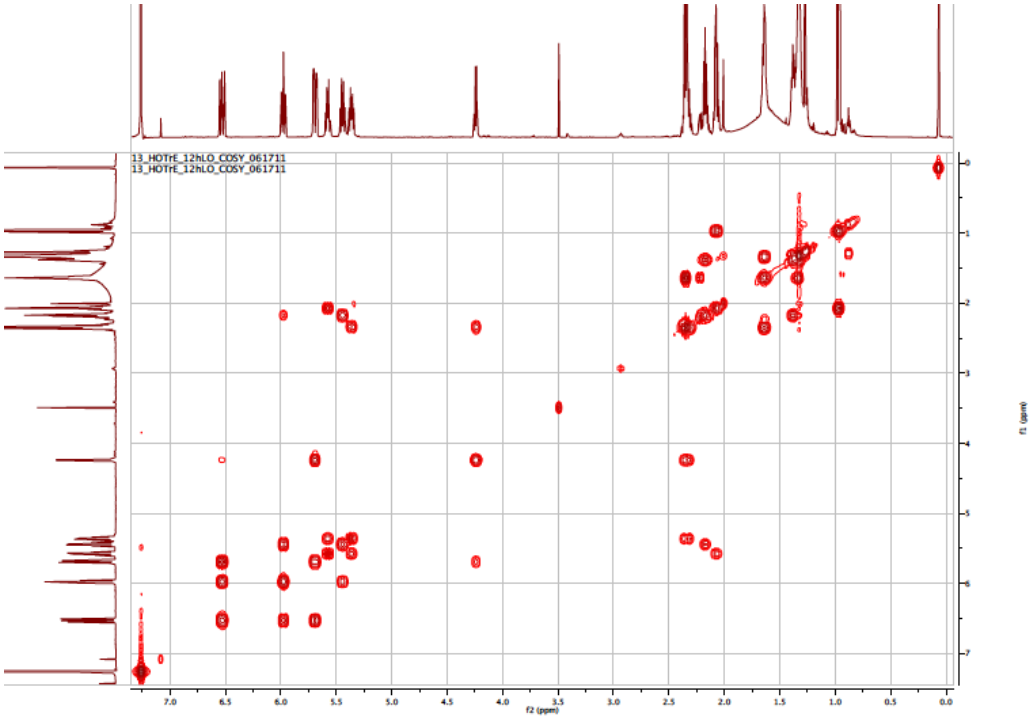


Figure D.18 2D COSY NMR of 13-HOTrE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

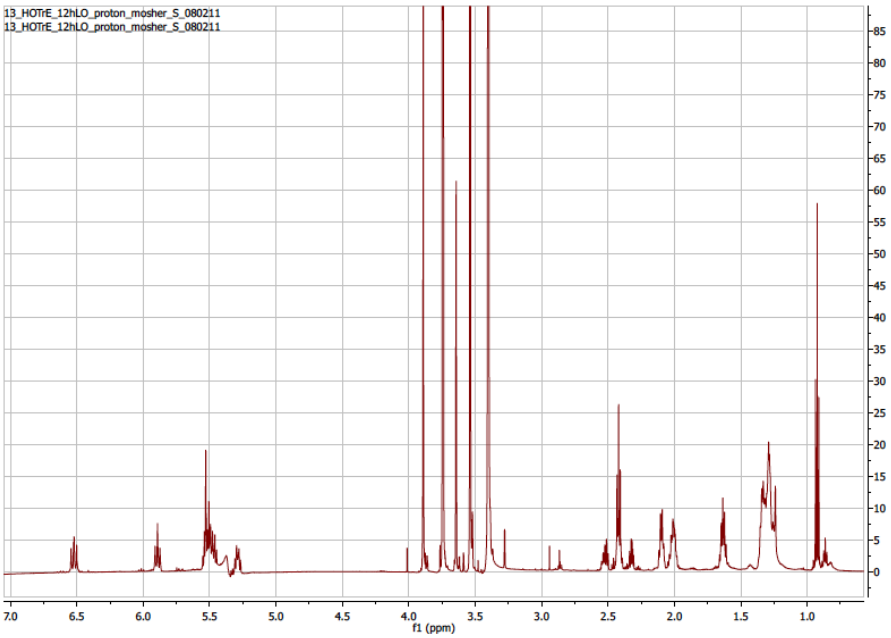


Figure D.19 1D Proton NMR of 13-HOTrE ester with Mosher (S). This figure was provided by our collaborator Dr. Theodore Holman's group.

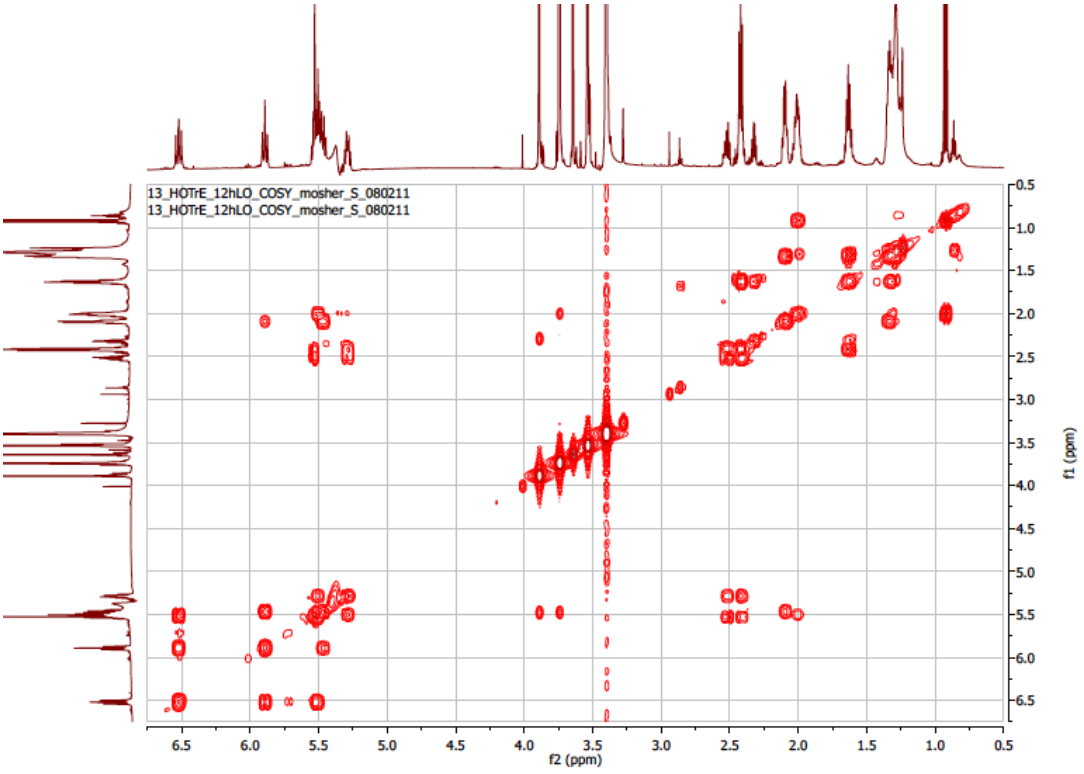


Figure D.20 2D COSY NMR of 13-HOTrE ester with Mosher (S). This figure was provided by our collaborator Dr. Theodore Holman's group.

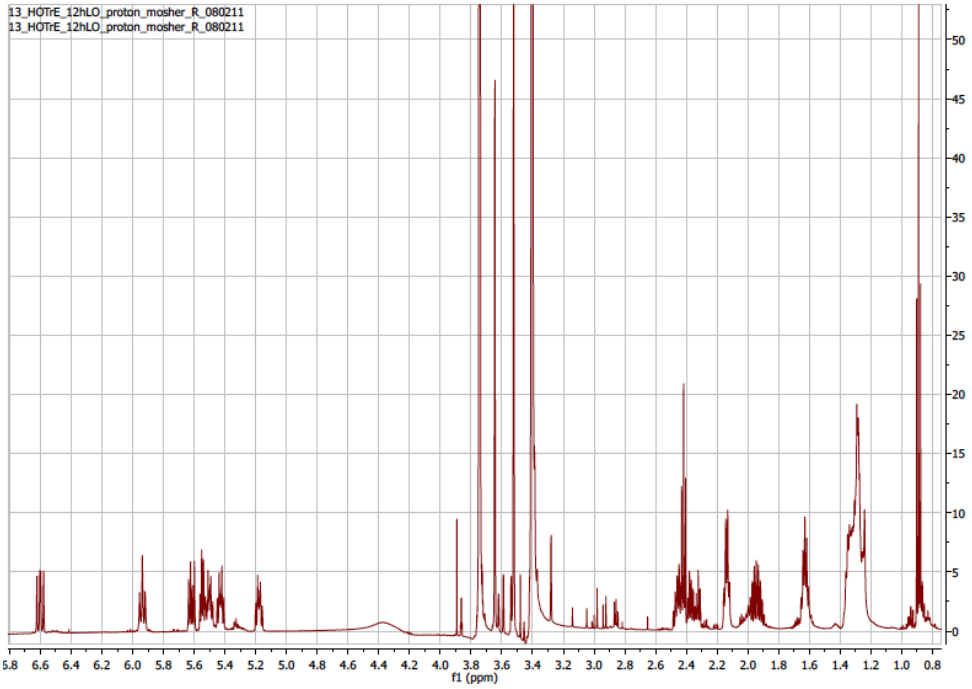


Figure D.21 1D Proton NMR or 13-HOTrE ester with Mosher (R). This figure was provided by our collaborator Dr. Theodore Holman's group.

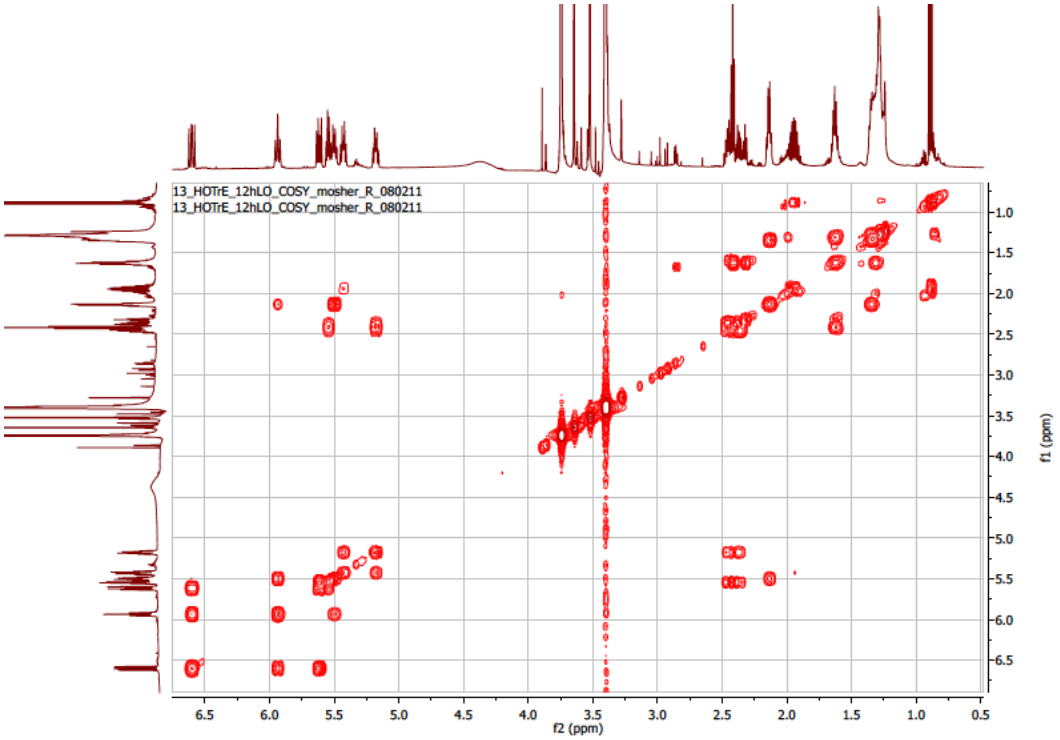


Figure D.22 2D COSY NMR of 13-HOTrE ester with Mosher (R). This figure was provided by our collaborator Dr. Theodore Holman's group.

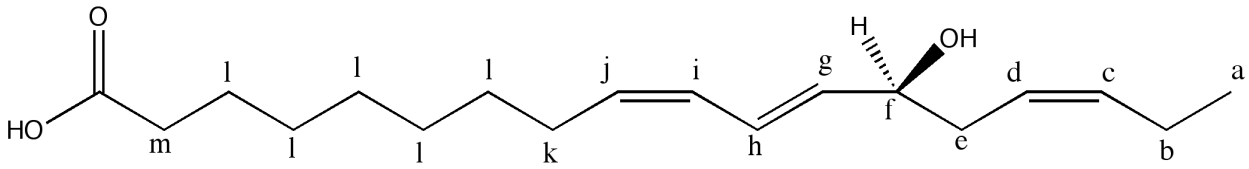


Figure D.23 13-HOTrE with labeled protons.
This figure was provided by our collaborator Dr. Theodore Holman's group.

H Position	ppm Fatty Alcohol	ppm Mosher (S)	ppm Mosher (R)	(S)ppm-(R)ppm
a	0.97, t	0.92, t	0.88, t	0.04
b	2.07, qi	2, m	1.94, m	0.06
c	5.57, m	5.5, m	5.42, m	0.08
d	5.36, m	5.28, m	5.18, m	0.10
e	2.35, m	2.42, t	2.42, m	0.00
f	4.24, qa	5.52, m	5.54, m	-0.02
g	5.69, dd	5.53, m	5.61, dd	-0.08
h	6.53, qa	6.52, qa	6.6, qa	-0.08
i	5.97, t,	5.89, t	5.93, t	-0.04
j	5.44, qa	5.48, m	5.5, m	-0.02
k	2.17, m	2.09, m	2.13, m	-0.04
l	1.37, m	1.35, m	1.32, m	0.03
m	1.64, qi	1.63, qi	1.63, qi	0.00

Splitting: s; singlet, d; doublet, t; triplet, qa; quartet, qi; quintet, m; multiplet, dd; doublet of doublets.

Figure D.24 Chemical Shifts of 13-HOTrE.
This figure was provided by our collaborator Dr. Theodore Holman's group.

Bibliography

1. Brewer DB. Max Schultze (1865), G. Bizzozero (1882) and the discovery of the platelet. *Br J Haematol*. 2006;133(3):251-258.

2. White JG, Escolar G. Current concepts of platelet membrane response to surface activation. *Platelets*. 1993;4(4):175-189.

3. White JG, Conard WJ. The fine structure of freeze-fractured blood platelets. *Am J Pathol*. 1973;70(1):45-56.

4. Escolar G, Krumwiede M, White JG. Organization of the actin cytoskeleton of resting and activated platelets in suspension. *Am J Pathol*. 1986;123(1):86-94.

5. White JG. Fine structural alterations induced in platelets by adenosine diphosphate. *Blood*. 1968;31(5):604-622.

6. White JG, Krumwiede M. Further studies of the secretory pathway in thrombin-stimulated human platelets. *Blood*. 1987;69(4):1196-1203.

7. Coller BS, Shattil SJ. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood*. 2008;112(8):3011-3025.

8. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*. 2010;11(4):288-300.

9. Tadokoro S, Shattil SJ, Eto K, et al. Talin binding to integrin beta tails: a final common step in integrin activation. *Science*. 2003;302(5642):103-106.

10. Moser M, Nieswandt B, Ussar S, Pozgajova M, Fassler R. Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med*. 2008;14(3):325-330.

11. Tourdot BE, Holinstat M. Targeting 12-Lipoxygenase as a Potential Novel Antiplatelet Therapy. *Trends Pharmacol Sci*. 2017;38(11):1006-1015.

12. Ulrichts H, Udvardy M, Lenting PJ, et al. Shielding of the A1 domain by the D'D3 domains of von Willebrand factor modulates its interaction with platelet glycoprotein Ib-IX-V. *J Biol Chem*. 2006;281(8):4699-4707.

13. Lisman T, Raynal N, Groeneveld D, et al. A single high-affinity binding site for von Willebrand factor in collagen III, identified using synthetic triple-helical peptides. *Blood*. 2006;108(12):3753-3756.

14. Luo SZ, Mo X, Afshar-Kharghan V, Srinivasan S, Lopez JA, Li R. Glycoprotein Ibalpha forms disulfide bonds with 2 glycoprotein Ibbeta subunits in the resting platelet. *Blood*. 2007;109(2):603-609.

15. Du X. Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. *Curr Opin Hematol*. 2007;14(3):262-269.

16. Wu Y, Suzuki-Inoue K, Satoh K, et al. Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling. *Blood*. 2001;97(12):3836-3845.

17. Ozaki Y, Satoh K, Yatomi Y, Miura S, Fujimura Y, Kume S. Protein tyrosine phosphorylation in human platelets induced by interaction between glycoprotein Ib and von Willebrand factor. *Biochim Biophys Acta*. 1995;1243(3):482-488.

18. Kroll MH, Harris TS, Moake JL, Handin RI, Schafer AI. von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. *J Clin Invest*. 1991;88(5):1568-1573.

19. Francesconi M, Casonato A, Pontara E, Dalla Via L, Girolami A, Deana R. Type IIB von Willebrand factor induces phospholipase A2 activation and cytosolic Ca2+ increase in platelets. *Biochem Biophys Res Commun*. 1995;214(1):102-109.

20. Arthur JF, Gardiner EE, Matzaris M, et al. Glycoprotein VI is associated with GPIb-IX-V on the membrane of resting and activated platelets. *Thromb Haemost*. 2005;93(4):716-723.

21. Liu J, Pestina TI, Berndt MC, Jackson CW, Gartner TK. Botrocetin/VWF-induced signaling through GPIb-IX-V produces TxA2 in an alphaIIbbeta3- and aggregation-independent manner. *Blood*. 2005;106(8):2750-2756.

22. Kasirer-Friede A, Cozzi MR, Mazzucato M, De Marco L, Ruggeri ZM, Shattil SJ. Signaling through GP Ib-IX-V activates alpha IIb beta 3 independently of other receptors. *Blood*. 2004;103(9):3403-3411.

23. Li Z, Xi X, Du X. A mitogen-activated protein kinase-dependent signaling pathway in the activation of platelet integrin alpha IIbbeta3. *J Biol Chem*. 2001;276(45):42226-42232.

24. Li Z, Xi X, Gu M, et al. A stimulatory role for cGMP-dependent protein kinase in platelet activation. *Cell*. 2003;112(1):77-86.

25. Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? *Blood*. 2003;102(2):449-461.

26. Moroi M, Jung SM. Platelet glycoprotein VI: its structure and function. *Thromb Res*. 2004;114(4):221-233.

27. Nieswandt B, Bergmeier W, Schulte V, Rackebrandt K, Gessner JE, Zirngibl H. Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRgamma chain. *J Biol Chem*. 2000;275(31):23998-24002.

28. Knight CG, Morton LF, Onley DJ, et al. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc Res*. 1999;41(2):450-457.

29. Locke D, Chen H, Liu Y, Liu C, Kahn ML. Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI. *J Biol Chem*. 2002;277(21):18801-18809.

30. Suzuki-Inoue K, Tulasne D, Shen Y, et al. Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling. *J Biol Chem*. 2002;277(24):21561-21566.

31. Quek LS, Pasquet JM, Hers I, et al. Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. *Blood*. 2000;96(13):4246-4253.

32. Pasquet JM, Gross B, Quek L, et al. LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. *Mol Cell Biol*. 1999;19(12):8326-8334.

33. Hughes CE, Auger JM, McGlade J, Eble JA, Pearce AC, Watson SP. Differential roles for the adapters Gads and LAT in platelet activation by GPVI and CLEC-2. *J Thromb Haemost*. 2008;6(12):2152-2159.

34. Judd BA, Myung PS, Obergfell A, et al. Differential requirement for LAT and SLP-76 in GPVI versus T cell receptor signaling. *J Exp Med*. 2002;195(6):705-717.

35. Quek LS, Bolen J, Watson SP. A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. *Curr Biol*. 1998;8(20):1137-1140.

36. Pearce AC, Senis YA, Billadeau DD, Turner M, Watson SP, Vigorito E. Vav1 and vav3 have critical but redundant roles in mediating platelet activation by collagen. *J Biol Chem*. 2004;279(52):53955-53962.

37. Pearce AC, Wilde JI, Doody GM, et al. Vav1, but not Vav2, contributes to platelet aggregation by CRP and thrombin, but neither is required for regulation of phospholipase C. *Blood*. 2002;100(10):3561-3569.

38. Nieuwenhuis HK, Sakariassen KS, Houdijk WP, Nievelstein PF, Sixma JJ. Deficiency of platelet membrane glycoprotein Ia associated with a decreased platelet adhesion to subendothelium: a defect in platelet spreading. *Blood*. 1986;68(3):692-695.

39. Emsley J, King SL, Bergelson JM, Liddington RC. Crystal structure of the I domain from integrin alpha2beta1. *J Biol Chem*. 1997;272(45):28512-28517.

40. Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin alpha2beta1. *Cell*. 2000;101(1):47-56.

41. Calderwood DA, Tuckwell DS, Humphries MJ. Specificity of integrin I-domain-ligand binding. *Biochem Soc Trans*. 1995;23(4):504S.

42. Tuckwell D, Calderwood DA, Green LJ, Humphries MJ. Integrin alpha 2 I-domain is a binding site for collagens. *J Cell Sci*. 1995;108 (Pt 4):1629-1637.

43. Jung SM, Moroi M. Platelets interact with soluble and insoluble collagens through characteristically different reactions. *J Biol Chem*. 1998;273(24):14827-14837.

44. Inoue O, Suzuki-Inoue K, Dean WL, Frampton J, Watson SP. Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2. *J Cell Biol*. 2003;160(5):769-780.

45. Arman M, Krauel K, Tilley DO, et al. Amplification of bacteria-induced platelet activation is triggered by FcgammaRIIA, integrin alphaIIbbeta3, and platelet factor 4. *Blood*. 2014;123(20):3166-3174.

46. Pampolina C, McNicol A. Streptococcus sanguis-induced platelet activation involves two waves of tyrosine phosphorylation mediated by FcgammaRIIA and alphaIIbbeta3. *Thromb Haemost*. 2005;93(5):932-939.

47. Fitzgerald JR, Loughman A, Keane F, et al. Fibronectin-binding proteins of Staphylococcus aureus mediate activation of human platelets via fibrinogen and fibronectin bridges to integrin GPIIb/IIIa and IgG binding to the FcgammaRIIa receptor. *Mol Microbiol*. 2006;59(1):212-230.

48. Sullam PM, Jarvis GA, Valone FH. Role of immunoglobulin G in platelet aggregation by viridans group streptococci. *Infect Immun*. 1988;56(11):2907-2911.

49. Boilard E, Pare G, Rousseau M, et al. Influenza virus H1N1 activates platelets through FcgammaRIIA signaling and thrombin generation. *Blood*. 2014;123(18):2854-2863.

50. Rosenfeld SI, Looney RJ, Leddy JP, Phipps DC, Abraham GN, Anderson CL. Human platelet Fc receptor for immunoglobulin G. Identification as a 40,000-molecular-weight membrane protein shared by monocytes. *J Clin Invest*. 1985;76(6):2317-2322.

51. Maxwell KF, Powell MS, Hulett MD, et al. Crystal structure of the human leukocyte Fc receptor, Fc gammaRIIa. *Nat Struct Biol*. 1999;6(5):437-442.

52. McKenzie SE, Taylor SM, Malladi P, et al. The role of the human Fc receptor Fc gamma RIIA in the immune clearance of platelets: a transgenic mouse model. *J Immunol*. 1999;162(7):4311-4318.

53. Tomiyama Y, Kunicki TJ, Zipf TF, Ford SB, Aster RH. Response of human platelets to activating monoclonal antibodies: importance of Fc gamma RII (CD32) phenotype and level of expression. *Blood*. 1992;80(9):2261-2268.

54. Karas SP, Rosse WF, Kurlander RJ. Characterization of the IgG-Fc receptor on human platelets. *Blood*. 1982;60(6):1277-1282.

55. Warmerdam PA, van de Winkel JG, Vlug A, Westerdaal NA, Capel PJ. A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding. *J Immunol*. 1991;147(4):1338-1343.

56. Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, Phillips DR. Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function. *Nature*. 1999;401(6755):808-811.

57. Zhi H, Rauova L, Hayes V, et al. Cooperative integrin/ITAM signaling in platelets enhances thrombus formation in vitro and in vivo. *Blood*. 2013;121(10):1858-1867.

58. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. 1991;64(6):1057-1068.

59. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. *Nature*. 1997;389(6647):183-186.

60. Soulet C, Hechler B, Gratacap MP, et al. A differential role of the platelet ADP receptors P2Y1 and P2Y12 in Rac activation. *J Thromb Haemost*. 2005;3(10):2296-2306.

61. Foster CJ, Prosser DM, Agans JM, et al. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest*. 2001;107(12):1591-1598.

62. Cohen S, Braiman A, Shubinsky G, Isakov N. Protein kinase C-theta in platelet activation. *FEBS letters*. 2011;585(20):3208-3215.

63. Trumel C, Payrastre B, Plantavid M, et al. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. *Blood*. 1999;94(12):4156-4165.

64. Jung SM, Ohnuma M, Watanabe N, Sonoda M, Handa M, Moroi M. Analyzing the mechanism of Rap1 activation in platelets: Rap1 activation is related to the release reaction mediated through the collagen receptor GPVI. *Thrombosis research*. 2006;118(4):509-521.

65. Kim S, Kunapuli SP. P2Y12 receptor in platelet activation. *Platelets*. 2011;22(1):54-58.

66. Schror K. The basic pharmacology of ticlopidine and clopidogrel. *Platelets*. 1993;4(5):252-261.

67. McTavish D, Faulds D, Goa KL. Ticlopidine. An updated review of its pharmacology and therapeutic use in platelet-dependent disorders. *Drugs*. 1990;40(2):238-259.

68. Albers GW. Role of ticlopidine for prevention of stroke. *Stroke*. 1992;23(6):912-916.

69. Derian CK, Friedman PA. Effect of ticlopidine ex vivo on platelet intracellular calcium mobilization. *Thromb Res*. 1988;50(1):65-76.

70. Cattaneo M. New P2Y(12) inhibitors. *Circulation*. 2010;121(1):171-179.

71. Gent M, Blakely JA, Easton JD, et al. The Canadian American Ticlopidine Study (CATS) in thromboembolic stroke. *Lancet*. 1989;1(8649):1215-1220.

72. Hass WK, Easton JD, Adams HP, Jr., et al. A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. Ticlopidine Aspirin Stroke Study Group. *The New England journal of medicine*. 1989;321(8):501-507.

73. Quinn MJ, Fitzgerald DJ. Ticlopidine and clopidogrel. *Circulation*. 1999;100(15):1667-1672.

74. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *The New England journal of medicine*. 2001;345(7):494-502.

75. Mehta SR, Yusuf S, Peters RJ, et al. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *Lancet*. 2001;358(9281):527-533.

76. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet*. 1996;348(9038):1329-1339.

77. Mega JL, Close SL, Wiviott SD, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. *The New England journal of medicine*. 2009;360(4):354-362.

78. Simon T, Verstuyft C, Mary-Krause M, et al. Genetic determinants of response to clopidogrel and cardiovascular events. *The New England journal of medicine*. 2009;360(4):363-375.

79. Sugidachi A, Asai F, Ogawa T, Inoue T, Koike H. The in vivo pharmacological profile of CS-747, a novel antiplatelet agent with platelet ADP receptor antagonist properties. *British journal of pharmacology*. 2000;129(7):1439-1446.

80. Sugidachi A, Asai F, Yoneda K, et al. Antiplatelet action of R-99224, an active metabolite of a novel thienopyridine-type G(i)-linked P2T antagonist, CS-747. *British journal of pharmacology*. 2001;132(1):47-54.

81. Hasegawa M, Sugidachi A, Ogawa T, Isobe T, Jakubowski JA, Asai F. Stereoselective inhibition of human platelet aggregation by R-138727, the active metabolite of CS-747 (prasugrel, LY640315), a novel P2Y12 receptor inhibitor. *Thrombosis and haemostasis*. 2005;94(3):593-598.

82. Savi P, Pereillo JM, Uzabiaga MF, et al. Identification and biological activity of the active metabolite of clopidogrel. *Thrombosis and haemostasis*. 2000;84(5):891-896.

83. Farid NA, Payne CD, Small DS, et al. Cytochrome P450 3A inhibition by ketoconazole affects prasugrel and clopidogrel pharmacokinetics and pharmacodynamics differently. *Clinical pharmacology and therapeutics*. 2007;81(5):735-741.

84. Brandt JT, Payne CD, Wiviott SD, et al. A comparison of prasugrel and clopidogrel loading doses on platelet function: magnitude of platelet inhibition is related to active metabolite formation. *American heart journal*. 2007;153(1):66 e69-16.

85. Montalescot G, Wiviott SD, Braunwald E, et al. Prasugrel compared with clopidogrel in patients undergoing percutaneous coronary intervention for ST-elevation myocardial infarction (TRITON-TIMI 38): double-blind, randomised controlled trial. *Lancet*. 2009;373(9665):723-731.

86. Birkeland K, Parra D, Rosenstein R. Antiplatelet therapy in acute coronary syndromes: focus on ticagrelor. *Journal of blood medicine*. 2010;1:197-219.

87. Anderson SD, Shah NK, Yim J, Epstein BJ. Efficacy and safety of ticagrelor: a reversible P2Y12 receptor antagonist. *The Annals of pharmacotherapy*. 2010;44(3):524-537.

88. Mahaffey KW, Wojdyla DM, Carroll K, et al. Ticagrelor compared with clopidogrel by geographic region in the Platelet Inhibition and Patient Outcomes (PLATO) trial. *Circulation*. 2011;124(5):544-554.

89. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2009;361(11):1045-1057.

90. Cannon CP, Husted S, Harrington RA, et al. Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non-ST-segment elevation acute coronary syndrome: primary results of the DISPERSE-2 trial. *Journal of the American College of Cardiology*. 2007;50(19):1844-1851.

91. Leonardi S, Rao SV, Harrington RA, et al. Rationale and design of the randomized, double-blind trial testing INtraveNous and Oral administration of elinogrel, a selective and reversible P2Y(12)-receptor inhibitor, versus clopidogrel to eVAluate Tolerability and Efficacy in nonurgent Percutaneous Coronary Interventions patients (INNOVATE-PCI). *American heart journal*. 2010;160(1):65-72.

92. Gurbel PA, Bliden KP, Antonino MJ, et al. The effect of elinogrel on high platelet reactivity during dual antiplatelet therapy and the relation to CYP2C19*2 genotype: first experience in patients. *J Thromb Haemost*. 2010;8(1):43-53.

93. Harrington RA, Stone GW, McNulty S, et al. Platelet inhibition with cangrelor in patients undergoing PCI. *N Engl J Med*. 2009;361(24):2318-2329.

94. Bhatt DL, Lincoff AM, Gibson CM, et al. Intravenous platelet blockade with cangrelor during PCI. *N Engl J Med*. 2009;361(24):2330-2341.

95. Wang YX, Vincelette J, da Cunha V, et al. A novel P2Y(12) adenosine diphosphate receptor antagonist that inhibits platelet aggregation and thrombus formation in rat and dog models. *Thrombosis and haemostasis*. 2007;97(5):847-855.

96. Post JM, Alexander S, Wang YX, et al. Novel P2Y12 adenosine diphosphate receptor antagonists for inhibition of platelet aggregation (II): pharmacodynamic and pharmacokinetic characterization. *Thrombosis research*. 2008;122(4):533-540.

97. Gogstad GO, Brosstad F, Krutnes MB, Hagen I, Solum NO. Fibrinogen-binding properties of the human platelet glycoprotein IIb-=IIIa complex: a study using crossed-radioimmunoelectrophoresis. *Blood*. 1982;60(3):663-671.

98. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30(12):2341-2349.

99. Kristensen SD, Wurtz M, Grove EL, et al. Contemporary use of glycoprotein IIb/IIIa inhibitors. *Thrombosis and haemostasis*. 2012;107(2):215-224.

100. Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Coller BS, Jordan RE. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood*. 1996;88(3):907-914.

101. Tam SH, Sassoli PM, Jordan RE, Nakada MT. Abciximab (ReoPro, chimeric 7E3 Fab) demonstrates equivalent affinity and functional blockade of glycoprotein IIb/IIIa and alpha(v)beta3 integrins. *Circulation*. 1998;98(11):1085-1091.

102. Simon DI, Xu H, Ortlepp S, Rogers C, Rao NK. 7E3 monoclonal antibody directed against the platelet glycoprotein IIb/IIIa cross-reacts with the leukocyte integrin Mac-1 and blocks adhesion to fibrinogen and ICAM-1. *Arteriosclerosis, thrombosis, and vascular biology*. 1997;17(3):528-535.

103. Kleiman NS, Raizner AE, Jordan R, et al. Differential inhibition of platelet aggregation induced by adenosine diphosphate or a thrombin receptor-activating peptide in patients treated with bolus chimeric 7E3 Fab: implications for inhibition of the internal pool of GPIIb/IIIa receptors. *Journal of the American College of Cardiology*. 1995;26(7):1665-1671.

104. Kastrati A, Mehilli J, Schuhlen H, et al. A clinical trial of abciximab in elective percutaneous coronary intervention after pretreatment with clopidogrel. *N Engl J Med*. 2004;350(3):232-238.

105. Lim GB. Antiplatelet therapy: Intracoronary abciximab during PCI for STEMI. *Nature reviews Cardiology*. 2011;9(5):258.

106. Van de Werf F, Ardissino D, Bueno H, et al. Acute coronary syndromes: considerations for improved acceptance and implementation of management guidelines. *Expert review of cardiovascular therapy*. 2012;10(4):489-503.

107. Schomig A, Schmitt C, Dibra A, et al. One year outcomes with abciximab vs. placebo during percutaneous coronary intervention after pre-treatment with clopidogrel. *European heart journal*. 2005;26(14):1379-1384.

108. Kastrati A, Mehilli J, Neumann FJ, et al. Abciximab in patients with acute coronary syndromes undergoing percutaneous coronary intervention after clopidogrel pretreatment: the ISAR-REACT 2 randomized trial. *JAMA*. 2006;295(13):1531-1538.

109. Bhatt DL, Topol EJ. Current role of platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes. *JAMA*. 2000;284(12):1549-1558.

110. Coller BS. Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *The Journal of clinical investigation*. 1997;100(11 Suppl):S57-60.

111. Ammar T, Scudder LE, Coller BS. In vitro effects of the platelet glycoprotein IIb/IIIa receptor antagonist c7E3 Fab on the activated clotting time. *Circulation*. 1997;95(3):614-617.

112. Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nat Rev Drug Discov*. 2010;9(2):154-169.

113. Scarborough RM, Rose JW, Hsu MA, et al. Barbourin. A GPIIb-IIIa-specific integrin antagonist from the venom of Sistrurus m. barbouri. *J Biol Chem*. 1991;266(15):9359-9362.

114. Randomised placebo-controlled trial of effect of eptifibatide on complications of percutaneous coronary intervention: IMPACT-II. Integrilin to Minimise Platelet Aggregation and Coronary Thrombosis-II. *Lancet*. 1997;349(9063):1422-1428.

115. Gilchrist IC, O'Shea JC, Kosoglou T, et al. Pharmacodynamics and pharmacokinetics of higher-dose, double-bolus eptifibatide in percutaneous coronary intervention. *Circulation*. 2001;104(4):406-411.

116. Novel dosing regimen of eptifibatide in planned coronary stent implantation (ESPRIT): a randomised, placebo-controlled trial. *Lancet*. 2000;356(9247):2037-2044.

117. Kleiman NS, Lincoff AM, Flaker GC, et al. Early percutaneous coronary intervention, platelet inhibition with eptifibatide, and clinical outcomes in patients with acute coronary syndromes. PURSUIT Investigators. *Circulation*. 2000;101(7):751-757.

118. Stone GW, Bertrand ME, Moses JW, et al. Routine upstream initiation vs deferred selective use of glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: the ACUITY Timing trial. *JAMA : the journal of the American Medical Association*. 2007;297(6):591-602.

119. Giugliano RP, White JA, Bode C, et al. Early versus delayed, provisional eptifibatide in acute coronary syndromes. *The New England journal of medicine*. 2009;360(21):2176-2190.

120. Cannon CP, Weintraub WS, Demopoulos LA, et al. Comparison of early invasive and conservative strategies in patients with unstable coronary syndromes treated with the glycoprotein IIb/IIIa inhibitor tirofiban. *N Engl J Med*. 2001;344(25):1879-1887.

121. Topol EJ, Moliterno DJ, Herrmann HC, et al. Comparison of two platelet glycoprotein IIb/IIIa inhibitors, tirofiban and abciximab, for the prevention of ischemic events with percutaneous coronary revascularization. *N Engl J Med*. 2001;344(25):1888-1894.

122. Jing BB, Li YX, Zhang H, et al. Antithrombotic activity of Z4A5, a new platelet glycoprotein IIb/IIIa receptor antagonist evaluated in a rabbit arteriovenous shunt thrombosis model. *Thrombosis research*. 2011;128(5):463-469.

123. Hoylaerts MF. Platelet-vessel wall interactions in thrombosis and restenosis role of von Willebrand factor. *Verhandelingen - Koninklijke Academie voor Geneeskunde van Belgie*. 1997;59(3):161-183.

124. Pareti FI, Fujimura Y, Dent JA, Holland LZ, Zimmerman TS, Ruggeri ZM. Isolation and characterization of a collagen binding domain in human von Willebrand factor. *The Journal of biological chemistry*. 1986;261(32):15310-15315.

125. Nichols WL, Hultin MB, James AH, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia : the official journal of the World Federation of Hemophilia*. 2008;14(2):171-232.

126. Gibbins JM, Okuma M, Farndale R, Barnes M, Watson SP. Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. *FEBS Lett*. 1997;413(2):255-259.

127. Kageyama S, Yamamoto H, Nakazawa H, et al. Pharmacokinetics and pharmacodynamics of AJW200, a humanized monoclonal antibody to von Willebrand factor, in monkeys. *Arteriosclerosis, thrombosis, and vascular biology*. 2002;22(1):187-192.

128. Gilbert JC, DeFeo-Fraulini T, Hutabarat RM, et al. First-in-human evaluation of anti von Willebrand factor therapeutic aptamer ARC1779 in healthy volunteers. *Circulation*. 2007;116(23):2678-2686.

129. Knobl P, Jilma B, Gilbert JC, Hutabarat RM, Wagner PG, Jilma-Stohlawetz P. Anti-von Willebrand factor aptamer ARC1779 for refractory thrombotic thrombocytopenic purpura. *Transfusion*. 2009;49(10):2181-2185.

130. Siller-Matula JM, Merhi Y, Tanguay JF, et al. ARC15105 Is a Potent Antagonist of Von Willebrand Factor Mediated Platelet Activation and Adhesion. *Arteriosclerosis, thrombosis, and vascular biology*. 2012;32(4):902-909.

131. Vanhoorelbeke K, Depraetere H, Romijn RA, Huizinga EG, De Maeyer M, Deckmyn H. A consensus tetrapeptide selected by phage display adopts the conformation of a dominant discontinuous epitope of a monoclonal anti-VWF antibody that inhibits the von Willebrand factor-collagen interaction. *The Journal of biological chemistry*. 2003;278(39):37815-37821.

132. De Meyer SF, Staelens S, Badenhorst PN, et al. Coronary artery in-stent stenosis persists despite inhibition of the von Willebrand factor--collagen interaction in baboons. *Thrombosis and haemostasis*. 2007;98(6):1343-1349.

133. Massberg S, Konrad I, Bultmann A, et al. Soluble glycoprotein VI dimer inhibits platelet adhesion and aggregation to the injured vessel wall in vivo. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2004;18(2):397-399.

134. Schulz C, Penz S, Hoffmann C, et al. Platelet GPVI binds to collagenous structures in the core region of human atheromatous plaque and is critical for atheroprogression in vivo. *Basic Res Cardiol*. 2008;103(4):356-367.

135. Ungerer M, Rosport K, Bultmann A, et al. Novel antiplatelet drug revacept (Dimeric Glycoprotein VI-Fc) specifically and efficiently inhibited collagen-induced platelet aggregation without affecting general hemostasis in humans. *Circulation*. 2011;123(17):1891-1899.

136. Zafar MU, Ibanez B, Choi BG, et al. A new oral antiplatelet agent with potent antithrombotic properties: comparison of DZ-697b with clopidogrel a randomised phase I study. *Thrombosis and haemostasis*. 2010;103(1):205-212.

137. Huizinga EG, Tsuji S, Romijn RA, et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. *Science*. 2002;297(5584):1176-1179.

138. Uff S, Clemetson JM, Harrison T, Clemetson KJ, Emsley J. Crystal structure of the platelet glycoprotein Ib(alpha) N-terminal domain reveals an unmasking mechanism for receptor activation. *The Journal of biological chemistry*. 2002;277(38):35657-35663.

139. Wu D, Vanhoorelbeke K, Cauwenberghs N, et al. Inhibition of the von Willebrand (VWF)-collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons. *Blood*. 2002;99(10):3623-3628.

140. Hennan JK, Swillo RE, Morgan GA, et al. Pharmacologic inhibition of platelet vWF-GPIb alpha interaction prevents coronary artery thrombosis. *Thrombosis and haemostasis*. 2006;95(3):469-475.

141. Yang J, Ji S, Dong N, Zhao Y, Ruan C. Engineering and characterization of a chimeric anti-platelet glycoprotein Ibalpha monoclonal antibody and preparation of its Fab fragment. *Hybridoma*. 2010;29(2):125-132.

142. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacological reviews*. 2006;58(3):488-520.

143. Schror K. The pharmacology of cilostazol. *Diabetes, obesity & metabolism*. 2002;4 Suppl 2:S14-19.

144. Liu Y, Fong M, Cone J, Wang S, Yoshitake M, Kambayashi J. Inhibition of adenosine uptake and augmentation of ischemia-induced increase of interstitial adenosine by cilostazol, an

agent to treat intermittent claudication. *Journal of cardiovascular pharmacology*. 2000;36(3):351-360.

145. Uehara S, Hirayama A. Effects of cilostazol on platelet function. *Arzneimittelforschung*. 1989;39(12):1531-1534.

146. Suri A, Forbes WP, Bramer SL. Effects of CYP3A inhibition on the metabolism of cilostazol. *Clinical pharmacokinetics*. 1999;37 Suppl 2:61-68.

147. Suh JW, Lee SP, Park KW, et al. Multicenter randomized trial evaluating the efficacy of cilostazol on ischemic vascular complications after drug-eluting stent implantation for coronary heart disease: results of the CILON-T (influence of CILostazol-based triple antiplatelet therapy ON ischemic complication after drug-eluting stenT implantation) trial. *Journal of the American College of Cardiology*. 2011;57(3):280-289.

148. Angiolillo DJ, Capranzano P, Goto S, et al. A randomized study assessing the impact of cilostazol on platelet function profiles in patients with diabetes mellitus and coronary artery disease on dual antiplatelet therapy: results of the OPTIMUS-2 study. *European heart journal*. 2008;29(18):2202-2211.

149. Aktas B, Utz A, Hoenig-Liedl P, Walter U, Geiger J. Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. *Stroke; a journal of cerebral circulation*. 2003;34(3):764-769.

150. Gamboa A, Abraham R, Diedrich A, et al. Role of adenosine and nitric oxide on the mechanisms of action of dipyridamole. *Stroke; a journal of cerebral circulation*. 2005;36(10):2170-2175.

151. Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A. European Stroke Prevention Study. 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. *Journal of the neurological sciences*. 1996;143(1-2):1-13.

152. Group ES, Halkes PH, van Gijn J, Kappelle LJ, Koudstaal PJ, Algra A. Aspirin plus dipyridamole versus aspirin alone after cerebral ischaemia of arterial origin (ESPRIT): randomised controlled trial. *Lancet*. 2006;367(9523):1665-1673.

153. Giannarelli C, Zafar MU, Badimon JJ. Prostanoid and TP-receptors in atherothrombosis: is there a role for their antagonism? *Thrombosis and haemostasis*. 2010;104(5):949-954.

154. Hong TT, Huang J, Driscoll E, Lucchesi BR. Preclinical evaluation of S18886 in an experimental model of coronary arterial thrombosis. *Journal of cardiovascular pharmacology*. 2006;48(5):239-248.

155. Bousser MG, Amarenco P, Chamorro A, et al. Terutroban versus aspirin in patients with cerebral ischaemic events (PERFORM): a randomised, double-blind, parallel-group trial. *Lancet*. 2011;377(9782):2013-2022.

156. Tanaka T, Fukuta Y, Higashino R, et al. Antiplatelet effect of Z-335, a new orally active and long-lasting thromboxane receptor antagonist. *Eur J Pharmacol*. 1998;357(1):53-60.

157. Matsuno H, Uematsu T, Niwa M, et al. Pharmacokinetic and pharmacodynamic properties of a new thromboxane receptor antagonist (Z-335) after single and multiple oral administrations to healthy volunteers. *J Clin Pharmacol*. 2002;42(7):782-790.

158. Cyrus T, Yao Y, Ding T, Dogne JM, Pratico D. A novel thromboxane receptor antagonist and synthase inhibitor, BM-573, reduces development and progression of atherosclerosis in LDL receptor deficient mice. *European journal of pharmacology*. 2007;561(1-3):105-111.

159. Tchana-Sato V, Dogne JM, Lambermont B, et al. Effects of BM-573, a thromboxane A2 modulator on systemic hemodynamics perturbations induced by U-46619 in the pig. *Prostaglandins & other lipid mediators*. 2005;78(1-4):82-95.

160. Chackalamannil S, Wang Y, Greenlee WJ, et al. Discovery of a novel, orally active himbacine-based thrombin receptor antagonist (SCH 530348) with potent antiplatelet activity. *J Med Chem*. 2008;51(11):3061-3064.

161. Chintala M, Strony J, Yang B, Kurowski S, Li Q. SCH 602539, a protease-activated receptor-1 antagonist, inhibits thrombosis alone and in combination with cangrelor in a Folts model of arterial thrombosis in cynomolgus monkeys. *Arterioscler Thromb Vasc Biol*. 2010;30(11):2143-2149.

162. Becker RC, Moliterno DJ, Jennings LK, et al. Safety and tolerability of SCH 530348 in patients undergoing non-urgent percutaneous coronary intervention: a randomised, double-blind, placebo-controlled phase II study. *Lancet*. 2009;373(9667):919-928.

163. Morrow DA, Scirica BM, Fox KA, et al. Evaluation of a novel antiplatelet agent for secondary prevention in patients with a history of atherosclerotic disease: design and rationale for the Thrombin-Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2 degrees P)-TIMI 50 trial. *American heart journal*. 2009;158(3):335-341 e333.

164. Kogushi M, Matsuoka T, Kawata T, et al. The novel and orally active thrombin receptor antagonist E5555 (Atopaxar) inhibits arterial thrombosis without affecting bleeding time in guinea pigs. *Eur J Pharmacol*. 2011;657(1-3):131-137.

165. Serebruany VL, Kogushi M, Dastros-Pitei D, Flather M, Bhatt DL. The in-vitro effects of E5555, a protease-activated receptor (PAR)-1 antagonist, on platelet biomarkers in healthy volunteers and patients with coronary artery disease. *Thromb Haemost*. 2009;102(1):111-119.

166. Goto S, Ogawa H, Takeuchi M, Flather MD, Bhatt DL, Investigators JL. Double-blind, placebo-controlled Phase II studies of the protease-activated receptor 1 antagonist E5555 (atopaxar) in Japanese patients with acute coronary syndrome or high-risk coronary artery disease. *European heart journal*. 2010;31(21):2601-2613.

167. Chintala M, Shimizu K, Ogawa M, Yamaguchi H, Doi M, Jensen P. Basic and translational research on proteinase-activated receptors: antagonism of the proteinase-activated

receptor 1 for thrombin, a novel approach to antiplatelet therapy for atherothrombotic disease. *Journal of pharmacological sciences*. 2008;108(4):433-438.

168. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation*. 2011;123(4):e18-e209.

169. Mendis S, Lindholm LH, Anderson SG, et al. Total cardiovascular risk approach to improve efficiency of cardiovascular prevention in resource constrain settings. *Journal of clinical epidemiology*. 2011.

170. Capone ML, Tacconelli S, Rodriguez LG, Patrignani P. NSAIDs and cardiovascular disease: transducing human pharmacology results into clinical read-outs in the general population. *Pharmacological reports : PR*. 2010;62(3):530-535.

171. Nawarskas JJ, Clark SM. Ticagrelor: a novel reversible oral antiplatelet agent. *Cardiology in review*. 2011;19(2):95-100.

172. Trelle S, Reichenbach S, Wandel S, et al. Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ*. 2011;342:c7086.

173. Rafferty M, Walters MR, Dawson J. Anti-platelet therapy and aspirin resistance - clinically and chemically relevant? *Curr Med Chem*. 2010;17(36):4578-4586.

174. Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. *The New England journal of medicine*. 2005;353(22):2373-2383.

175. De Schryver EL, Algra A. Secondary stroke prevention with antithrombotic drugs. *Current vascular pharmacology*. 2010;8(1):129-133.

176. Iyu D, Glenn JR, White AE, Fox SC, Dovlatova N, Heptinstall S. P2Y(12) and EP3 antagonists promote the inhibitory effects of natural modulators of platelet aggregation that act via cAMP. *Platelets*. 2011.

177. Riva L, Di Pasquale G, Casella G, Calabrese D, Zagnoni S, Pallotti MG. [Antiplatelet therapy in acute coronary syndromes: state of the art and new perspectives]. *Giornale italiano di cardiologia*. 2010;11(12 Suppl 3):27S-33S.

178. Leclerc JR. Platelet glycoprotein IIb/IIIa antagonists: lessons learned from clinical trials and future directions. *Critical care medicine*. 2002;30(5 Suppl):S332-340.

179. Kimmelstiel C, Phang R, Rehman A, et al. Short-term comparative outcomes associated with the use of GP IIb/IIIa antagonists in patients undergoing coronary intervention. *Journal of thrombosis and thrombolysis*. 2001;11(3):203-209.

180. Zhao XQ, Theroux P, Snapinn SM, Sax FL. Intracoronary thrombus and platelet glycoprotein IIb/IIIa receptor blockade with tirofiban in unstable angina or non-Q-wave myocardial infarction. Angiographic results from the PRISM-PLUS trial (Platelet receptor

inhibition for ischemic syndrome management in patients limited by unstable signs and symptoms). PRISM-PLUS Investigators. *Circulation*. 1999;100(15):1609-1615.

181. Hennekens CH, Schneider WR, Hebert PR, Tantry US, Gurbel PA. Hypothesis formulation from subgroup analyses: nonadherence or nonsteroidal anti-inflammatory drug use explains the lack of clinical benefit of aspirin on first myocardial infarction attributed to "aspirin resistance". *American heart journal*. 2010;159(5):744-748.

182. Bergstrom S, Holman RT. Total conjugation of linoleic acid in oxidation with lipoxidase. *Nature*. 1948;161(4080):55.

183. Theorell H, Holman RT, Akeson A. Crystalline lipoxidase. *Acta chemica Scandinavica*. 1947;1(6):571-576.

184. Morgan LT, Thomas CP, Kuhn H, O'Donnell VB. Thrombin-activated human platelets acutely generate oxidized docosahexaenoic-acid-containing phospholipids via 12-lipoxygenase. *The Biochemical journal*. 2010;431(1):141-148.

185. Brash AR. Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *The Journal of biological chemistry*. 1999;274(34):23679-23682.

186. Yoshimoto T, Takahashi Y. Arachidonate 12-lipoxygenases. *Prostaglandins & other lipid mediators*. 2002;68-69:245-262.

187. Kragballe K, Fallon JD. Increased aggregation and arachidonic acid transformation by psoriatic platelets: evidence that platelet-derived 12-hydroxy-eicosatetraenoic acid increases keratinocyte DNA synthesis in vitro. *Archives of dermatological research*. 1986;278(6):449-453.

188. McDonald CJ, Calabresi P. Psoriasis and occlusive vascular disease. *The British journal of dermatology*. 1978;99(5):469-475.

189. Yoshimura R, Matsuyama M, Tsuchida K, Kawahito Y, Sano H, Nakatani T. Expression of lipoxygenase in human bladder carcinoma and growth inhibition by its inhibitors. *The Journal of urology*. 2003;170(5):1994-1999.

190. James MJ, Gibson RA, Cleland LG. Dietary polyunsaturated fatty acids and inflammatory mediator production. *Am J Clin Nutr*. 2000;71(1 Suppl):343S-348S.

191. Willis AL. Prostaglandins and the future of medicine (an overview of currently evolving data and ideas). 1. Introductory comments: outline and scope of the series. *Prostaglandins*. 1974;5(6):506-511.

192. Holinstat M, Boutaud O, Apopa PL, et al. Protease-activated receptor signaling in platelets activates cytosolic phospholipase A2alpha differently for cyclooxygenase-1 and 12-lipoxygenase catalysis. *Arteriosclerosis, thrombosis, and vascular biology*. 2011;31(2):435-442.

193. Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM. Platelet-activating factor and related lipid mediators. *Annu Rev Biochem*. 2000;69:419-445.

194. Kroll MH, Schafer AI. Biochemical mechanisms of platelet activation. *Blood*. 1989;74(4):1181-1195.

195. Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. *J Thromb Haemost*. 2003;1(7):1602-1612.

196. Nyby MD, Sasaki M, Ideguchi Y, et al. Platelet lipoxygenase inhibitors attenuate thrombin- and thromboxane mimetic-induced intracellular calcium mobilization and platelet aggregation. *J Pharmacol Exp Ther*. 1996;278(2):503-509.

197. Fiore S, Ryeom SW, Weller PF, Serhan CN. Lipoxin recognition sites. Specific binding of labeled lipoxin A4 with human neutrophils. *The Journal of biological chemistry*. 1992;267(23):16168-16176.

198. Romano M, Chen XS, Takahashi Y, Yamamoto S, Funk CD, Serhan CN. Lipoxin synthase activity of human platelet 12-lipoxygenase. *The Biochemical journal*. 1993;296 (Pt 1):127-133.

199. Serhan CN, Sheppard KA. Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro. *The Journal of clinical investigation*. 1990;85(3):772-780.

200. Weber PC, Fischer S. Arachidonic acid and eicosapentaenoic acid metabolism in platelets and vessel walls. *Medical biology*. 1984;62(2):129.

201. Chakrabarti SK, Cole BK, Wen Y, Keller SR, Nadler JL. 12/15-lipoxygenase products induce inflammation and impair insulin signaling in 3T3-L1 adipocytes. *Obesity*. 2009;17(9):1657-1663.

202. Dyerberg J, Bang HO. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *Lancet*. 1979;2(8140):433-435.

203. Knauss HJ, Sheffner AL. Effect of unsaturated fatty acid supplements upon mortality and clotting parameters in rats fed thrombogenic diets. *J Nutr*. 1967;93(3):393-400.

204. Tamura Y, Hirai A, Terano T, et al. Clinical and epidemiological studies of eicosapentaenoic acid (EPA) in Japan. *Progress in lipid research*. 1986;25(1-4):461-466.

205. Takenaga M, Hirai A, Terano T, Tamura Y, Kitagawa H, Yoshida S. Comparison of the in vitro effect of eicosapentaenoic acid (EPA)-derived lipoxygenase metabolites on human platelet function with those of arachidonic acid. *Thrombosis research*. 1986;41(3):373-384.

206. Lorenz R, Spengler U, Fischer S, Duhm J, Weber PC. Platelet function, thromboxane formation and blood pressure control during supplementation of the Western diet with cod liver oil. *Circulation*. 1983;67(3):504-511.

207. Vericel E, Calzada C, Chapuy P, Lagarde M. The influence of low intake of n-3 fatty acids on platelets in elderly people. *Atherosclerosis*. 1999;147(1):187-192.

208. Vericel E, Polette A, Bacot S, Calzada C, Lagarde M. Pro- and antioxidant activities of docosahexaenoic acid on human blood platelets. *Journal of thrombosis and haemostasis : JTH*. 2003;1(3):566-572.

209. Siess W, Roth P, Scherer B, Kurzmann I, Bohlig B, Weber PC. Platelet-membrane fatty acids, platelet aggregation, and thromboxane formation during a mackerel diet. *Lancet*. 1980;1(8166):441-444.

210. Larson MK, Shearer GC, Ashmore JH, et al. Omega-3 fatty acids modulate collagen signaling in human platelets. *Prostaglandins, leukotrienes, and essential fatty acids*. 2011;84(3-4):93-98.

211. Morita I, Takahashi R, Saito Y, Murota S. Stimulation of eicosapentaenoic acid metabolism in washed human platelets by 12-hydroperoxyeicosatetraenoic acid. *The Journal of biological chemistry*. 1983;258(17):10197-10199.

212. Calzada C, Vericel E, Mitel B, Coulon L, Lagarde M. 12(S)-Hydroperoxy-eicosatetraenoic acid increases arachidonic acid availability in collagen-primed platelets. *Journal of lipid research*. 2001;42(9):1467-1473.

213. Dadaian M, Westlund P. Eicosanoid metabolism in human platelets is modified by albumin. *Advances in experimental medicine and biology*. 1999;469:23-27.

214. Westlund P, Palmblad J, Falck JR, Lumin S. Synthesis, structural identification and biological activity of 11,12-dihydroxyeicosatetraenoic acids formed in human platelets. *Biochimica et biophysica acta*. 1991;1081(3):301-307.

215. Gomolka B, Siegert E, Blossey K, Schunck WH, Rothe M, Weylandt KH. Analysis of omega-3 and omega-6 fatty acid-derived lipid metabolite formation in human and mouse blood samples. *Prostaglandins & other lipid mediators*. 2011;94(3-4):81-87.

216. von Schacky C, Siess W, Fischer S, Weber PC. A comparative study of eicosapentaenoic acid metabolism by human platelets in vivo and in vitro. *Journal of lipid research*. 1985;26(4):457-464.

217. Morita I, Takahashi R, Saito Y, Murota S. Effects of eicosapentaenoic acid on arachidonic acid metabolism in cultured vascular cells and platelets: species difference. *Thrombosis research*. 1983;31(2):211-217.

218. Schwartzman ML, Balazy M, Masferrer J, Abraham NG, McGiff JC, Murphy RC. 12(R)-hydroxyicosatetraenoic acid: a cytochrome-P450-dependent arachidonate metabolite that inhibits Na+,K+-ATPase in the cornea. *Proceedings of the National Academy of Sciences of the United States of America*. 1987;84(22):8125-8129.

219. Capdevila J, Yadagiri P, Manna S, Falck JR. Absolute configuration of the hydroxyeicosatetraenoic acids (HETEs) formed during catalytic oxygenation of arachidonic acid by microsomal cytochrome P-450. *Biochemical and biophysical research communications*. 1986;141(3):1007-1011.

220. Sekiya F, Takagi J, Usui T, et al. 12S-hydroxyeicosatetraenoic acid plays a central role in the regulation of platelet activation. *Biochem Biophys Res Commun*. 1991;179(1):345-351.

221. Setty BN, Werner MH, Hannun YA, Stuart MJ. 15-Hydroxyeicosatetraenoic acid-mediated potentiation of thrombin-induced platelet functions occurs via enhanced production of phosphoinositide-derived second messengers--sn-1,2-diacylglycerol and inositol-1,4,5-trisphosphate. *Blood*. 1992;80(11):2765-2773.

222. Siegel MI, McConnell RT, Porter NA, Cuatrecasas P. Arachidonate metabolism via lipoxygenase and 12L-hydroperoxy-5,8,10,14-icosatetraenoic acid peroxidase sensitive to anti-inflammatory drugs. *Proceedings of the National Academy of Sciences of the United States of America*. 1980;77(1):308-312.

223. Chang J, Blazek E, Kreft AF, Lewis AJ. Inhibition of platelet and neutrophil phospholipase A2 by hydroxyeicosatetraenoic acids (HETES). A novel pharmacological mechanism for regulating free fatty acid release. *Biochemical pharmacology*. 1985;34(9):1571-1575.

224. Vanderhoek JY, Bryant RW, Bailey JM. 15-hydroxy-5,8,11,13-eicosatetraenoic acid: A potent and selective inhibitor of platelet lipoxygenase. *The Journal of biological chemistry*. 1980;255(13):5996-5998.

225. Takenaga M, Kitagawa H, Hirai A, Tamura Y, Yoshida S. Mechanism of anti-platelet aggregating action of dilazep. *Journal of pharmacobio-dynamics*. 1985;8(2):77-83.

226. Lagarde M, Calzada C, Vericel E. Pathophysiologic role of redox status in blood platelet activation. Influence of docosahexaenoic acid. *Lipids*. 2003;38(4):465-468.

227. Kuhn H, Romisch I, Belkner J. The role of lipoxygenase-isoforms in atherogenesis. *Molecular nutrition & food research*. 2005;49(11):1014-1029.

228. Gonzalez-Nunez D, Claria J, Rivera F, Poch E. Increased levels of 12(S)-HETE in patients with essential hypertension. *Hypertension*. 2001;37(2):334-338.

229. Quintana LF, Guzman B, Collado S, Claria J, Poch E. A coding polymorphism in the 12-lipoxygenase gene is associated to essential hypertension and urinary 12(S)-HETE. *Kidney international*. 2006;69(3):526-530.

230. Bern MM. Platelet functions in diabetes mellitus. *Diabetes*. 1978;27(3):342-350.

231. Vericel E, Croset M, Sedivy P, Courpron P, Dechavanne M, Lagarde M. Platelets and aging. I--Aggregation, arachidonate metabolism and antioxidant status. *Thrombosis research*. 1988;49(3):331-342.

232. Lagarde M, Vericel E, Guichardant M, Dechavanne M. Inhibition of thrombin-induced platelet arachidonic acid release by 15-hydroperoxy-arachidonic acid. *Biochemical and biophysical research communications*. 1981;99(4):1398-1402.

233. Tohjima T, Honda N, Mochizuki K, et al. Decreased activity of arachidonate 12-lipoxygenase in platelets of Japanese patients with non-insulin-dependent diabetes mellitus. *Metabolism: clinical and experimental*. 1998;47(3):257-263.

234. Antonipillai I, Nadler J, Vu EJ, Bughi S, Natarajan R, Horton R. A 12-lipoxygenase product, 12-hydroxyeicosatetraenoic acid, is increased in diabetics with incipient and early renal disease. *The Journal of clinical endocrinology and metabolism*. 1996;81(5):1940-1945.

235. Sun FF, McGuire JC, Morton DR, Pike JE, Sprecher H, Kunau WH. Inhibition of platelet arachidonic acid 12-lipoxygenase by acetylenic acid compounds. *Prostaglandins*. 1981;21(2):333-343.

236. Sekiya K, Okuda H, Arichi S. Selective inhibition of platelet lipoxygenase by esculetin. *Biochimica et biophysica acta*. 1982;713(1):68-72.

237. Morita I, Saito Y, Chang WC, Murota S. Effects of purified eicosapentaenoic acid on arachidonic acid metabolism in cultured murine aortic smooth muscle cells, vessel walls and platelets. *Lipids*. 1983;18(1):42-49.

238. Sekiya K, Okuda H. Selective inhibition of platelet lipoxygenase by baicalein. *Biochemical and biophysical research communications*. 1982;105(3):1090-1095.

239. Daret D, Blin P, Larrue J. Synthesis of hydroxy fatty acids from linoleic acid by human blood platelets. *Prostaglandins*. 1989;38(2):203-214.

240. You KM, Jong HG, Kim HP. Inhibition of cyclooxygenase/lipoxygenase from human platelets by polyhydroxylated/methoxylated flavonoids isolated from medicinal plants. *Archives of pharmacal research*. 1999;22(1):18-24.

241. Kalvegren H, Andersson J, Grenegard M, Bengtsson T. Platelet activation triggered by Chlamydia pneumoniae is antagonized by 12-lipoxygenase inhibitors but not cyclooxygenase inhibitors. *European journal of pharmacology*. 2007;566(1-3):20-27.

242. Si D, Wang Y, Zhou YH, et al. Mechanism of CYP2C9 inhibition by flavones and flavonols. *Drug metabolism and disposition: the biological fate of chemicals*. 2009;37(3):629-634.

243. Deschamps JD, Kenyon VA, Holman TR. Baicalein is a potent in vitro inhibitor against both reticulocyte 15-human and platelet 12-human lipoxygenases. *Bioorg Med Chem*. 2006;14(12):4295-4301.

244. Berger ME, Golub MS, Chang CT, et al. Flavonoid potentiation of contractile responses in rat blood vessels. *The Journal of pharmacology and experimental therapeutics*. 1992;263(1):78-83.

245. Guichardant M, Michel M, Borel C, et al. Effects of 9,12,15-octadecatrien-6-ynoic acid on the metabolism of arachidonic acid in platelets and on the platelet aggregation. *Agents and actions Supplements*. 1992;37:215-221.

246. Wube AA, Bucar F, Asres K, et al. Knipholone, a selective inhibitor of leukotriene metabolism. *Phytomedicine : international journal of phytotherapy and phytopharmacology*. 2006;13(6):452-456.

247. Ozeki Y, Nagamura Y, Ito H, et al. An anti-platelet agent, OPC-29030, inhibits translocation of 12-lipoxygenase and 12-hydroxyeicosatetraenoic acid production in human platelets. *British journal of pharmacology*. 1999;128(8):1699-1704.

248. Suzuki H, Ueda T, Juranek I, et al. Hinokitiol, a selective inhibitor of the platelet-type isozyme of arachidonate 12-lipoxygenase. *Biochemical and biophysical research communications*. 2000;275(3):885-889.

249. Inamori Y, Tsujibo H, Ohishi H, et al. Cytotoxic effect of hinokitiol and tropolone on the growth of mammalian cells and on blastogenesis of mouse splenic T cells. *Biological & pharmaceutical bulletin*. 1993;16(5):521-523.

250. Deschamps JD, Gautschi JT, Whitman S, et al. Discovery of platelet-type 12-human lipoxygenase selective inhibitors by high-throughput screening of structurally diverse libraries. *Bioorg Med Chem*. 2007;15(22):6900-6908.

251. Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. The PURSUIT Trial Investigators. Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy. *The New England journal of medicine*. 1998;339(7):436-443.

252. Hamberg M, Samuelsson B. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proceedings of the National Academy of Sciences of the United States of America*. 1974;71(9):3400-3404.

253. Mozaffarian D, Benjamin EJ, Go AS, et al. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation*. 2015;131(4):e29-322.

254. Jackson SP. Arterial thrombosis--insidious, unpredictable and deadly. *Nat Med*. 2011;17(11):1423-1436.

255. Tourdot BE, Ahmed I, Holinstat M. The emerging role of oxylipins in thrombosis and diabetes. *Front Pharmacol*. 2014;4:176.

256. Rouzer CA, Marnett LJ. Cyclooxygenases: structural and functional insights. *J Lipid Res*. 2009;50 Suppl:S29-34.

257. Chandrasekharan JA, Marginean A, Sharma-Walia N. An insight into the role of arachidonic acid derived lipid mediators in virus associated pathogenesis and malignancies. *Prostaglandins Other Lipid Mediat*. 2016;126:46-54.

258. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol*. 2011;31(5):986-1000.

259. Patterson E, Wall R, Fitzgerald GF, Ross RP, Stanton C. Health implications of high dietary omega-6 polyunsaturated Fatty acids. *J Nutr Metab*. 2012;2012:539426.

260. Nakahata N. Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacol Ther*. 2008;118(1):18-35.

261. Oelz O, Oelz R, Knapp HR, Sweetman BJ, Oates JA. Biosynthesis of prostaglandin D2. 1. Formation of prostaglandin D2 by human platelets. *Prostaglandins*. 1977;13(2):225-234.

262. Whittle BJ, Moncada S, Vane JR. Comparison of the effects of prostacyclin (PGI2), prostaglandin E1 and D2 on platelet aggregation in different species. *Prostaglandins*. 1978;16(3):373-388.

263. Song WL, Stubbe J, Ricciotti E, et al. Niacin and biosynthesis of PGD(2)by platelet COX-1 in mice and humans. *J Clin Invest*. 2012;122(4):1459-1468.

264. Bushfield M, McNicol A, MacIntyre DE. Inhibition of platelet-activating-factor-induced human platelet activation by prostaglandin D2. Differential sensitivity of platelet transduction processes and functional responses to inhibition by cyclic AMP. *Biochem J*. 1985;232(1):267-271.

265. Pettipher R. The roles of the prostaglandin D(2) receptors DP(1) and CRTH2 in promoting allergic responses. *Br J Pharmacol*. 2008;153 Suppl 1:S191-199.

266. Spik I, Brenuchon C, Angeli V, et al. Activation of the prostaglandin D2 receptor DP2/CRTH2 increases allergic inflammation in mouse. *J Immunol*. 2005;174(6):3703-3708.

267. Harris SG, Phipps RP. Prostaglandin D(2), its metabolite 15-d-PGJ(2), and peroxisome proliferator activated receptor-gamma agonists induce apoptosis in transformed, but not normal, human T lineage cells. *Immunology*. 2002;105(1):23-34.

268. Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacol Ther*. 2004;103(2):147-166.

269. Bundy GL, Morton DR, Peterson DC, Nishizawa EE, Miller WL. Synthesis and platelet aggregation inhibiting activity of prostaglandin D analogues. *J Med Chem*. 1983;26(6):790-799.

270. Mahmud I, Smith DL, Whyte MA, et al. On the identification and biological properties of prostaglandin J2. *Prostaglandins Leukot Med*. 1984;16(2):131-146.

271. Cheng Y, Austin SC, Rocca B, et al. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science*. 2002;296(5567):539-541.

272. Haslam RJ, Dickinson NT, Jang EK. Cyclic nucleotides and phosphodiesterases in platelets. *Thromb Haemost*. 1999;82(2):412-423.

273. Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circ Res*. 2006;99(12):1293-1304.

274. Hui Y, Ricciotti E, Crichton I, et al. Targeted deletions of cyclooxygenase-2 and atherogenesis in mice. *Circulation*. 2010;121(24):2654-2660.

275. Sergeant S, Rahbar E, Chilton FH. Gamma-linolenic acid, Dihommo-gamma linolenic, Eicosanoids and Inflammatory Processes. *Eur J Pharmacol*. 2016;785:77-86.

276. Lagarde M, Bernoud-Hubac N, Calzada C, Vericel E, Guichardant M. Lipidomics of essential fatty acids and oxygenated metabolites. *Mol Nutr Food Res*. 2013;57(8):1347-1358.

277. Needleman P, Whitaker MO, Wyche A, Watters K, Sprecher H, Raz A. Manipulation of platelet aggregation by prostaglandins and their fatty acid precursors: pharmacological basis for a therapeutic approach. *Prostaglandins*. 1980;19(1):165-181.

278. Negishi M, Sugimoto Y, Ichikawa A. Prostanoid receptors and their biological actions. *Prog Lipid Res*. 1993;32(4):417-434.

279. Kramer HJ, Stevens J, Grimminger F, Seeger W. Fish oil fatty acids and human platelets: dose-dependent decrease in dienoic and increase in trienoic thromboxane generation. *Biochem Pharmacol*. 1996;52(8):1211-1217.

280. Fischer S, Weber PC. Thromboxane (TX)A3 and prostaglandin (PG)I3 are formed in man after dietary eicosapentaenoic acid: identification and quantification by capillary gas chromatography-electron impact mass spectrometry. *Biomed Mass Spectrom*. 1985;12(9):470-476.

281. Wada M, DeLong CJ, Hong YH, et al. Enzymes and receptors of prostaglandin pathways with arachidonic acid-derived versus eicosapentaenoic acid-derived substrates and products. *J Biol Chem*. 2007;282(31):22254-22266.

282. Iyu D, Glenn JR, White AE, Johnson A, Heptinstall S, Fox SC. The role of prostanoid receptors in mediating the effects of PGE3 on human platelet function. *Thromb Haemost*. 2012;107(4):797-799.

283. Needleman P, Raz A, Minkes MS, Ferrendelli JA, Sprecher H. Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. *Proc Natl Acad Sci U S A*. 1979;76(2):944-948.

284. Kobzar G, Mardla V, Jarving I, Samel N. Comparison of anti-aggregatory effects of PGI2, PGI3 and iloprost on human and rabbit platelets. *Cell Physiol Biochem*. 2001;11(5):279-284.

285. Hegde S, Kaushal N, Ravindra KC, et al. Delta12-prostaglandin J3, an omega-3 fatty acid-derived metabolite, selectively ablates leukemia stem cells in mice. *Blood*. 2011;118(26):6909-6919.

286. Lefils-Lacourtablaise J, Socorro M, Geloen A, et al. The eicosapentaenoic acid metabolite 15-deoxy-delta(12,14)-prostaglandin J3 increases adiponectin secretion by adipocytes partly via a PPARgamma-dependent mechanism. *PLoS One*. 2013;8(5):e63997.

287. Jin L, Lin S, Rong H, et al. Structural basis for iloprost as a dual peroxisome proliferator-activated receptor alpha/delta agonist. *J Biol Chem*. 2011;286(36):31473-31479.

288. Radmark O, Samuelsson B. 5-Lipoxygenase: mechanisms of regulation. *J Lipid Res*. 2009;50 Suppl:S40-45.

289. Singh RK, Gupta S, Dastidar S, Ray A. Cysteinyl leukotrienes and their receptors: molecular and functional characteristics. *Pharmacology*. 2010;85(6):336-349.

290. Bednar M, Smith B, Pinto A, Mullane KM. Neutrophil depletion suppresses 111In-labeled platelet accumulation in infarcted myocardium. *J Cardiovasc Pharmacol*. 1985;7(5):906-912.

291. Knauer KA, Fish JE, Adkinson NF, Jr., Lichtenstein LM, Peters SP, Newball HH. Platelet activation in antigen-induced bronchoconstriction. *N Engl J Med*. 1981;305(15):892-893.

292. Clark JD, Lin LL, Kriz RW, et al. A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell*. 1991;65(6):1043-1051.

293. Woods JW, Evans JF, Ethier D, et al. 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. *J Exp Med*. 1993;178(6):1935-1946.

294. Brock TG, Paine R, 3rd, Peters-Golden M. Localization of 5-lipoxygenase to the nucleus of unstimulated rat basophilic leukemia cells. *J Biol Chem*. 1994;269(35):22059-22066.

295. Dixon RA, Diehl RE, Opas E, et al. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature*. 1990;343(6255):282-284.

296. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science*. 1987;237(4819):1171-1176.

297. Sjolinder M, Tornhamre S, Claesson HE, Hydman J, Lindgren J. Characterization of a leukotriene C4 export mechanism in human platelets: possible involvement of multidrug resistance-associated protein 1. *J Lipid Res*. 1999;40(3):439-446.

298. Pace-Asciak CR, Klein J, Spielberg SP. Metabolism of leukotriene A4 into C4 by human platelets. *Biochim Biophys Acta*. 1986;877(1):68-74.

299. Sala A, Zarini S, Folco G, Murphy RC, Henson PM. Differential metabolism of exogenous and endogenous arachidonic acid in human neutrophils. *J Biol Chem*. 1999;274(40):28264-28269.

300. Penrose JF, Spector J, Lam BK, et al. Purification of human lung leukotriene C4 synthase and preparation of a polyclonal antibody. *Am J Respir Crit Care Med*. 1995;152(1):283-289.

301. Maugeri N, Evangelista V, Celardo A, et al. Polymorphonuclear leukocyte-platelet interaction: role of P-selectin in thromboxane B2 and leukotriene C4 cooperative synthesis. *Thromb Haemost*. 1994;72(3):450-456.

302. Maclouf J, Antoine C, Henson PM, Murphy RC. Leukotriene C4 formation by transcellular biosynthesis. *Ann N Y Acad Sci*. 1994;714:143-150.

303. Bigby TD, Meslier N. Transcellular lipoxygenase metabolism between monocytes and platelets. *J Immunol*. 1989;143(6):1948-1954.

304. Laidlaw TM, Kidder MS, Bhattacharyya N, et al. Cysteinyl leukotriene overproduction in aspirin-exacerbated respiratory disease is driven by platelet-adherent leukocytes. *Blood*. 2012;119(16):3790-3798.

305. Laidlaw TM, Boyce JA. Platelets in patients with aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol*. 2015;135(6):1407-1414; quiz 1415.

306. Powell WS, Gravel S, Khanapure SP, Rokach J. Biological inactivation of 5-oxo-6,8,11,14-eicosatetraenoic acid by human platelets. *Blood*. 1999;93(3):1086-1096.

307. Hasegawa S, Ichiyama T, Hashimoto K, et al. Functional expression of cysteinyl leukotriene receptors on human platelets. *Platelets*. 2010;21(4):253-259.

308. Mause SF, von Hundelshausen P, Zernecke A, Koenen RR, Weber C. Platelet microparticles: a transcellular delivery system for RANTES promoting monocyte recruitment on endothelium. *Arterioscler Thromb Vasc Biol*. 2005;25(7):1512-1518.

309. von Hundelshausen P, Koenen RR, Sack M, et al. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood*. 2005;105(3):924-930.

310. Koenen RR, von Hundelshausen P, Nesmelova IV, et al. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat Med*. 2009;15(1):97-103.

311. von Hundelshausen P, Koenen RR, Weber C. Platelet-mediated enhancement of leukocyte adhesion. *Microcirculation*. 2009;16(1):84-96.

312. Cummings HE, Liu T, Feng C, et al. Cutting edge: Leukotriene C4 activates mouse platelets in plasma exclusively through the type 2 cysteinyl leukotriene receptor. *J Immunol*. 2013;191(12):5807-5810.

313. Mais DE, Saussy DL, Jr., Magee DE, Bowling NL. Interaction of 5-HETE, 12-HETE, 15-HETE and 5,12-diHETE at the human platelet thromboxane A2/prostaglandin H2 receptor. *Eicosanoids*. 1990;3(2):121-124.

314. Mehta P, Mehta J, Lawson D, Krop I, Letts LG. Leukotrienes potentiate the effects of epinephrine and thrombin on human platelet aggregation. *Thromb Res*. 1986;41(5):731-738.

315. Funk CD, Furci L, FitzGerald GA. Molecular cloning, primary structure, and expression of the human platelet/erythroleukemia cell 12-lipoxygenase. *Proc Natl Acad Sci U S A*. 1990;87(15):5638-5642.

316. Funk CD, Furci L, Fitzgerald GA. Molecular cloning of the human platelet 12-lipoxygenase. *Trans Assoc Am Physicians*. 1990;103:180-186.

317. Yamamoto S. Mammalian lipoxygenases: molecular structures and functions. *Biochim Biophys Acta*. 1992;1128(2-3):117-131.

318. Chang J, Blazek E, Kreft AF, Lewis AJ. Inhibition of platelet and neutrophil phospholipase A2 by hydroxyeicosatetraenoic acids (HETES). A novel pharmacological mechanism for regulating free fatty acid release. *Biochem Pharmacol*. 1985;34(9):1571-1575.

319. Sekiya F, Takagi J, Sasaki K, et al. Feedback regulation of platelet function by 12S-hydroxyeicosatetraenoic acid: inhibition of arachidonic acid liberation from phospholipids. *Biochim Biophys Acta*. 1990;1044(1):165-168.

320. Johnson EN, Brass LF, Funk CD. Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. *Proc Natl Acad Sci U S A*. 1998;95(6):3100-3105.

321. Takenaga M, Hirai A, Terano T, Tamura Y, Kitagawa H, Yoshida S. Comparison of the in vitro effect of eicosapentaenoic acid (EPA)-derived lipoxygenase metabolites on human platelet function with those of arachidonic acid. *Thromb Res*. 1986;41(3):373-384.

322. Fonlupt P, Croset M, Lagarde M. 12-HETE inhibits the binding of PGH2/TXA2 receptor ligands in human platelets. *Thromb Res*. 1991;63(2):239-248.

323. Calzada C, Vericel E, Lagarde M. Low concentrations of lipid hydroperoxides prime human platelet aggregation specifically via cyclo-oxygenase activation. *Biochem J*. 1997;325 (Pt 2):495-500.

324. Coulon L, Calzada C, Moulin P, Vericel E, Lagarde M. Activation of p38 mitogen-activated protein kinase/cytosolic phospholipase A2 cascade in hydroperoxide-stressed platelets. *Free Radic Biol Med*. 2003;35(6):616-625.

325. Yeung J, Apopa PL, Vesci J, et al. 12-lipoxygenase activity plays an important role in PAR4 and GPVI-mediated platelet reactivity. *Thromb Haemost*. 2013;110(3):569-581.

326. Yeung J, Apopa PL, Vesci J, et al. Protein kinase C regulation of 12-lipoxygenase-mediated human platelet activation. *Mol Pharmacol*. 2012;81(3):420-430.

327. Yeung J, Tourdot BE, Adili R, et al. 12(S)-HETrE, a 12-Lipoxygenase Oxylipin of Dihomo-gamma-Linolenic Acid, Inhibits Thrombosis via Galphas Signaling in Platelets. *Arterioscler Thromb Vasc Biol*. 2016;36(10):2068-2077.

328. Yeung J, Tourdot BE, Fernandez-Perez P, et al. Platelet 12-LOX is essential for FcgammaRIIa-mediated platelet activation. *Blood*. 2014;124(14):2271-2279.

329. Thomas CP, Morgan LT, Maskrey BH, et al. Phospholipid-esterified eicosanoids are generated in agonist-activated human platelets and enhance tissue factor-dependent thrombin generation. *J Biol Chem*. 2010;285(10):6891-6903.

330. Guo Y, Zhang W, Giroux C, et al. Identification of the orphan G protein-coupled receptor GPR31 as a receptor for 12-(S)-hydroxyeicosatetraenoic acid. *J Biol Chem*. 2011;286(39):33832-33840.

331. Hampson AJ, Grimaldi M. 12-hydroxyeicosatetrenoate (12-HETE) attenuates AMPA receptor-mediated neurotoxicity: evidence for a G-protein-coupled HETE receptor. *J Neurosci*. 2002;22(1):257-264.

332. Sun L, Xu YW, Han J, Liang H, Wang N, Cheng Y. 12/15-Lipoxygenase metabolites of arachidonic acid activate PPARgamma: a possible neuroprotective effect in ischemic brain. *J Lipid Res*. 2015;56(3):502-514.

333. Ikei KN, Yeung J, Apopa PL, et al. Investigations of human platelet-type 12-lipoxygenase: role of lipoxygenase products in platelet activation. *J Lipid Res*. 2012;53(12):2546-2559.

334. Fischer S, von Schacky C, Siess W, Strasser T, Weber PC. Uptake, release and metabolism of docosahexaenoic acid (DHA, c22:6 omega 3) in human platelets and neutrophils. *Biochem Biophys Res Commun*. 1984;120(3):907-918.

335. Akiba S, Murata T, Kitatani K, Sato T. Involvement of lipoxygenase pathway in docosapentaenoic acid-induced inhibition of platelet aggregation. *Biol Pharm Bull*. 2000;23(11):1293-1297.

336. Careaga MM, Sprecher H. Synthesis of two hydroxy fatty acids from 7,10,13,16,19-docosapentaenoic acid by human platelets. *J Biol Chem*. 1984;259(23):14413-14417.

337. Yeung J, Tourdot BE, Adili R, et al. 12-HETrE, a 12-Lipoxygenase Oxylipin of Dihomo-gamma-Linolenic Acid, Inhibits Thrombosis via Galphas Signaling in Platelets. *Arterioscler Thromb Vasc Biol*. 2016.

338. Brash AR, Boeglin WE, Chang MS. Discovery of a second 15S-lipoxygenase in humans. *Proc Natl Acad Sci U S A*. 1997;94(12):6148-6152.

339. Shappell SB, Boeglin WE, Olson SJ, Kasper S, Brash AR. 15-lipoxygenase-2 (15-LOX-2) is expressed in benign prostatic epithelium and reduced in prostate adenocarcinoma. *Am J Pathol*. 1999;155(1):235-245.

340. Ivanov I, Kuhn H, Heydeck D. Structural and functional biology of arachidonic acid 15-lipoxygenase-1 (ALOX15). *Gene*. 2015;573(1):1-32.

341. Jiang WG, Watkins G, Douglas-Jones A, Mansel RE. Reduction of isoforms of 15-lipoxygenase (15-LOX)-1 and 15-LOX-2 in human breast cancer. *Prostaglandins Leukot Essent Fatty Acids*. 2006;74(4):235-245.

342. Shureiqi I, Wu Y, Chen D, et al. The critical role of 15-lipoxygenase-1 in colorectal epithelial cell terminal differentiation and tumorigenesis. *Cancer Res*. 2005;65(24):11486-11492.

343. Wong PY, Westlund P, Hamberg M, Granstrom E, Chao PH, Samuelsson B. 15-Lipoxygenase in human platelets. *J Biol Chem*. 1985;260(16):9162-9165.

344. Kim HY, Karanian JW, Salem N, Jr. Formation of 15-lipoxygenase product from docosahexaenoic acid (22:6w3) by human platelets. *Prostaglandins*. 1990;40(5):539-549.

345. Vericel E, Lagarde M. 15-Hydroperoxyeicosatetraenoic acid inhibits human platelet aggregation. *Lipids*. 1980;15(6):472-474.

346. Vedelago HR, Mahadevappa VG. Differential effects of 15-HPETE on arachidonic acid metabolism in collagen-stimulated human platelets. *Biochem Biophys Res Commun*. 1988;150(1):177-184.

347. Bild G, Bhat S, Axelrod B, Iatridis P. Inhibition of aggregation of human platelets by 8, 15-dihydroperoxides of 5, 9, 11, 13-eicosatetraenoic and 9, 11, 13-eicosatrienoic acids. *Prostaglandins*. 1978;16(5):795-801.

348. Vijil C, Hermansson C, Jeppsson A, Bergstrom G, Hulten LM. Arachidonate 15-lipoxygenase enzyme products increase platelet aggregation and thrombin generation. *PLoS One*. 2014;9(2):e88546.

349. Lannan KL, Spinelli SL, Blumberg N, Phipps RP. Maresin 1 induces a novel pro-resolving phenotype in human platelets. *J Thromb Haemost*. 2017.

350. Yamaja Setty BN, Berger M, Stuart MJ. 13-Hydroxyoctadeca-9,11-dienoic acid (13-HODE) inhibits thromboxane A2 synthesis, and stimulates 12-HETE production in human platelets. *Biochem Biophys Res Commun*. 1987;148(2):528-533.

351. Guichardant M, Naltachayan-Durbin S, Lagarde M. Occurrence of the 15-hydroxy derivative of dihomogammalinolenic acid in human platelets and its biological effect. *Biochim Biophys Acta*. 1988;962(1):149-154.

352. Tloti MA, Moon DG, Weston LK, Kaplan JE. Effect of 13-hydroxyoctadeca-9,11-dienoic acid (13-HODE) on thrombin induced platelet adherence to endothelial cells in vitro. *Thromb Res*. 1991;62(4):305-317.

353. Chen P, Vericel E, Lagarde M, Guichardant M. Poxytrins, a class of oxygenated products from polyunsaturated fatty acids, potently inhibit blood platelet aggregation. *FASEB J*. 2011;25(1):382-388.

354. Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics*. 2004;14(1):1-18.

355. Wu S, Moomaw CR, Tomer KB, Falck JR, Zeldin DC. Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. *J Biol Chem*. 1996;271(7):3460-3468.

356. Capdevila JH, Falck JR, Harris RC. Cytochrome P450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res*. 2000;41(2):163-181.

357. Zhu Y, Schieber EB, McGiff JC, Balazy M. Identification of arachidonate P-450 metabolites in human platelet phospholipids. *Hypertension*. 1995;25(4 Pt 2):854-859.

358. Fitzpatrick FA, Ennis MD, Baze ME, Wynalda MA, McGee JE, Liggett WF. Inhibition of cyclooxygenase activity and platelet aggregation by epoxyeicosatrienoic acids. Influence of stereochemistry. *J Biol Chem*. 1986;261(32):15334-15338.

359. Balazy M. Metabolism of 5,6-epoxyeicosatrienoic acid by the human platelet. Formation of novel thromboxane analogs. *J Biol Chem*. 1991;266(35):23561-23567.

360. VanRollins M. Epoxygenase metabolites of docosahexaenoic and eicosapentaenoic acids inhibit platelet aggregation at concentrations below those affecting thromboxane synthesis. *J Pharmacol Exp Ther*. 1995;274(2):798-804.

361. Krotz F, Riexinger T, Buerkle MA, et al. Membrane-potential-dependent inhibition of platelet adhesion to endothelial cells by epoxyeicosatrienoic acids. *Arterioscler Thromb Vasc Biol*. 2004;24(3):595-600.

362. Krotz F, Hellwig N, Burkle MA, et al. A sulfaphenazole-sensitive EDHF opposes platelet-endothelium interactions in vitro and in the hamster microcirculation in vivo. *Cardiovasc Res*. 2010;85(3):542-550.

363. Tunaru S, Chennupati R, Nusing RM, Offermanns S. Arachidonic Acid Metabolite 19(S)-HETE Induces Vasorelaxation and Platelet Inhibition by Activating Prostacyclin (IP) Receptor. *PLoS One*. 2016;11(9):e0163633.

364. Hill E, Fitzpatrick F, Murphy RC. Biological activity and metabolism of 20-hydroxyeicosatetraenoic acid in the human platelet. *Br J Pharmacol*. 1992;106(2):267-274.

365. Schwartzman ML, Falck JR, Yadagiri P, Escalante B. Metabolism of 20-hydroxyeicosatetraenoic acid by cyclooxygenase. Formation and identification of novel endothelium-dependent vasoconstrictor metabolites. *J Biol Chem*. 1989;264(20):11658-11662.

366. Tsai IJ, Croft KD, Puddey IB, Beilin LJ, Barden A. 20-Hydroxyeicosatetraenoic acid synthesis is increased in human neutrophils and platelets by angiotensin II and endothelin-1. *Am J Physiol Heart Circ Physiol*. 2011;300(4):H1194-1200.

367. Knapp HR, Miller AJ, Lawson JA. Urinary excretion of diols derived from eicosapentaenoic acid during n-3 fatty acid ingestion by man. *Prostaglandins*. 1991;42(1):47-54.

368. Fleming I. Cytochrome p450 and vascular homeostasis. *Circ Res*. 2001;89(9):753-762.

369. VanRollins M, Kaduce TL, Fang X, Knapp HR, Spector AA. Arachidonic acid diols produced by cytochrome P-450 monooxygenases are incorporated into phospholipids of vascular endothelial cells. *J Biol Chem*. 1996;271(24):14001-14009.

370. Kim DH, Puri N, Sodhi K, et al. Cyclooxygenase-2 dependent metabolism of 20-HETE increases adiposity and adipocyte enlargement in mesenchymal stem cell-derived adipocytes. *J Lipid Res*. 2013;54(3):786-793.

371. Pratt PF, Rosolowsky M, Campbell WB. Effects of epoxyeicosatrienoic acids on polymorphonuclear leukocyte function. *Life Sci*. 2002;70(21):2521-2533.

372. Recchiuti A, Serhan CN. Pro-Resolving Lipid Mediators (SPMs) and Their Actions in Regulating miRNA in Novel Resolution Circuits in Inflammation. *Front Immunol*. 2012;3:298.

373. Levy BD, Romano M, Chapman HA, Reilly JJ, Drazen J, Serhan CN. Human alveolar macrophages have 15-lipoxygenase and generate 15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid and lipoxins. *J Clin Invest*. 1993;92(3):1572-1579.

374. Chavis C, Vachier I, Chanez P, Bousquet J, Godard P. 5(S),15(S)-dihydroxyeicosatetraenoic acid and lipoxin generation in human polymorphonuclear cells: dual specificity of 5-lipoxygenase towards endogenous and exogenous precursors. *J Exp Med*. 1996;183(4):1633-1643.

375. Serhan CN, Sheppard KA. Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro. *J Clin Invest*. 1990;85(3):772-780.

376. Edenius C, Stenke L, Lindgren JA. On the mechanism of transcellular lipoxin formation in human platelets and granulocytes. *Eur J Biochem*. 1991;199(2):401-409.

377. Borgeson E, Docherty NG, Murphy M, et al. Lipoxin A(4) and benzo-lipoxin A(4) attenuate experimental renal fibrosis. *FASEB J*. 2011;25(9):2967-2979.

378. Vital SA, Becker F, Holloway PM, et al. Formyl-Peptide Receptor 2/3/Lipoxin A4 Receptor Regulates Neutrophil-Platelet Aggregation and Attenuates Cerebral Inflammation: Impact for Therapy in Cardiovascular Disease. *Circulation*. 2016;133(22):2169-2179.

379. Dona M, Fredman G, Schwab JM, et al. Resolvin E1, an EPA-derived mediator in whole blood, selectively counterregulates leukocytes and platelets. *Blood*. 2008;112(3):848-855.

380. Fredman G, Van Dyke TE, Serhan CN. Resolvin E1 regulates adenosine diphosphate activation of human platelets. *Arterioscler Thromb Vasc Biol*. 2010;30(10):2005-2013.

381. Abdulnour RE, Dalli J, Colby JK, et al. Maresin 1 biosynthesis during platelet-neutrophil interactions is organ-protective. *Proc Natl Acad Sci U S A*. 2014;111(46):16526-16531.

382. Lagarde M, Vericel E, Liu M, Chen P, Guichardant M. Structure-function relationships of non-cyclic dioxygenase products from polyunsaturated fatty acids: poxytrins as a class of bioactive derivatives. *Biochimie*. 2014;107 Pt A:91-94.

383. Balas L, Guichardant M, Durand T, Lagarde M. Confusion between protectin D1 (PD1) and its isomer protectin DX (PDX). An overview on the dihydroxy-docosatrienes described to date. *Biochimie*. 2014;99:1-7.

384. Hong S, Gronert K, Devchand PR, Moussignac RL, Serhan CN. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J Biol Chem*. 2003;278(17):14677-14687.

385. Mukherjee PK, Marcheselli VL, Serhan CN, Bazan NG. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc Natl Acad Sci U S A*. 2004;101(22):8491-8496.

386. Brash AR. A review of possible roles of the platelet 12-lipoxygenase. *Circulation*. 1985;72(4):702-707.

387. Aharony D, Smith JB, Silver MJ. Regulation of arachidonate-induced platelet aggregation by the lipoxygenase product, 12-hydroperoxyeicosatetraenoic acid. *Biochim Biophys Acta*. 1982;718(2):193-200.

388. Konopatskaya O, Gilio K, Harper MT, et al. PKCalpha regulates platelet granule secretion and thrombus formation in mice. *J Clin Invest*. 2009;119(2):399-407.

389. Chari R, Getz T, Nagy B, Jr., et al. Protein kinase C[delta] differentially regulates platelet functional responses. *Arterioscler Thromb Vasc Biol*. 2009;29(5):699-705.

390. Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost*. 2010;8(3):454-462.

391. Falker K, Haglund L, Gunnarsson P, Nylander M, Lindahl TL, Grenegard M. Protease-activated receptor 1 (PAR1) signalling-desensitization is counteracted via PAR4 signalling in human platelets. *Biochem J*. 2011.

392. Kim SY, Kim S, Kim JM, et al. PKC inhibitors RO 31-8220 and Go 6983 enhance epinephrine-induced platelet aggregation in catecholamine hypo-responsive platelets by enhancing Akt phosphorylation. *BMB Rep*. 2011;44(2):140-145.

393. Unsworth AJ, Smith H, Gissen P, Watson SP, Pears CJ. Submaximal inhibition of protein kinase C restores ADP-induced dense granule secretion in platelets in the presence of Ca2+. *J Biol Chem*. 2011.

394. Strehl A, Munnix IC, Kuijpers MJ, et al. Dual role of platelet protein kinase C in thrombus formation: stimulation of pro-aggregatory and suppression of procoagulant activity in platelets. *J Biol Chem*. 2007;282(10):7046-7055.

395. Kenyon V, Rai G, Jadhav A, et al. Discovery of potent and selective inhibitors of human platelet-type 12- lipoxygenase. *J Med Chem*. 2011;54(15):5485-5497.

396. Lee SH, Williams MV, DuBois RN, Blair IA. Targeted lipidomics using electron capture atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun Mass Spectrom*. 2003;17(19):2168-2176.

397. Reed KA, Tucker DE, Aloulou A, et al. Functional Characterization of Mutations in Inherited Human cPLA(2) Deficiency. *Biochemistry*. 2011;50(10):1731-1738.

398. Holinstat M, Boutaud O, Apopa PL, et al. Protease-activated receptor signaling in platelets activates cytosolic phospholipase A2alpha differently for cyclooxygenase-1 and 12-lipoxygenase catalysis. *Arterioscler Thromb Vasc Biol*. 2011;31(2):435-442.

399. Mahanonda N. Anti-thrombotic and anticoagulant treatment in interventional cardiology. *J Med Assoc Thai*. 1998;81(1):42-46.

400. Varon D, Spectre G. Antiplatelet agents. *Hematology Am Soc Hematol Educ Program*. 2009:267-272.

401. Diener HC, Bogousslavsky J, Brass LM, et al. Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. *Lancet*. 2004;364(9431):331-337.

402. Durand-Zaleski I, Bertrand M. The value of clopidogrel versus aspirin in reducing atherothrombotic events: the CAPRIE study. *Pharmacoeconomics*. 2004;22 Suppl 4:19-27.

403. Gurbel PA, Tantry US, Shuldiner AR, Kereiakes DJ. Genotyping: one piece of the puzzle to personalize antiplatelet therapy. *J Am Coll Cardiol*. 2010;56(2):112-116.

404. Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med*. 2005;353(22):2373-2383.

405. Pidgeon GP, Lysaght J, Krishnamoorthy S, et al. Lipoxygenase metabolism: roles in tumor progression and survival. *Cancer Metastasis Rev*. 2007;26(3-4):503-524.

406. Ding XZ, Tong WG, Adrian TE. 12-lipoxygenase metabolite 12(S)-HETE stimulates human pancreatic cancer cell proliferation via protein tyrosine phosphorylation and ERK activation. *Int J Cancer*. 2001;94(5):630-636.

407. Szekeres CK, Tang K, Trikha M, Honn KV. Eicosanoid activation of extracellular signal-regulated kinase1/2 in human epidermoid carcinoma cells. *J Biol Chem*. 2000;275(49):38831-38841.

408. Chen FL, Wang XZ, Li JY, Yu JP, Huang CY, Chen ZX. 12-lipoxygenase induces apoptosis of human gastric cancer AGS cells via the ERK1/2 signal pathway. *Dig Dis Sci*. 2008;53(1):181-187.

409. Konopatskaya O, Poole AW. Protein kinase Calpha: disease regulator and therapeutic target. *Trends Pharmacol Sci*. 2010;31(1):8-14.

410. McPhail LC, Clayton CC, Snyderman R. A potential second messenger role for unsaturated fatty acids: activation of Ca2+-dependent protein kinase. *Science*. 1984;224(4649):622-625.

411. Shearman MS, Naor Z, Sekiguchi K, Kishimoto A, Nishizuka Y. Selective activation of the gamma-subspecies of protein kinase C from bovine cerebellum by arachidonic acid and its lipoxygenase metabolites. *FEBS Lett*. 1989;243(2):177-182.

412. Liu B, Khan WA, Hannun YA, et al. 12(S)-hydroxyeicosatetraenoic acid and 13(S)-hydroxyoctadecadienoic acid regulation of protein kinase C-alpha in melanoma cells: role of receptor-mediated hydrolysis of inositol phospholipids. *Proc Natl Acad Sci U S A*. 1995;92(20):9323-9327.

413. Seth RK, Haque MS, Zelenka PS. Regulation of c-fos induction in lens epithelial cells by 12(S)HETE-dependent activation of PKC. *Invest Ophthalmol Vis Sci*. 2001;42(13):3239-3246.

414. Raso E, Tovari J, Toth K, et al. Ectopic alphaIIbbeta3 integrin signaling involves 12-lipoxygenase- and PKC-mediated serine phosphorylation events in melanoma cells. *Thromb Haemost*. 2001;85(6):1037-1042.

415. Samiei M, Sanghera JS, Pelech SL. Activation of myelin basic protein and S6 peptide kinases in phorbol ester- and PAF-treated sheep platelets. *Biochim Biophys Acta*. 1993;1176(3):287-298.

416. Kramer RM, Roberts EF, Strifler BA, Johnstone EM. Thrombin induces activation of p38 MAP kinase in human platelets. *J Biol Chem*. 1995;270(46):27395-27398.

417. Bugaud F, Nadal-Wollbold F, Levy-Toledano S, Rosa JP, Bryckaert M. Regulation of c-jun-NH2 terminal kinase and extracellular-signal regulated kinase in human platelets. *Blood*. 1999;94(11):3800-3805.

418. Borsch-Haubold AG, Kramer RM, Watson SP. Inhibition of mitogen-activated protein kinase kinase does not impair primary activation of human platelets. *Biochem J*. 1996;318 (Pt 1):207-212.

419. Nadal-Wollbold F, Pawlowski M, Levy-Toledano S, Berrou E, Rosa JP, Bryckaert M. Platelet ERK2 activation by thrombin is dependent on calcium and conventional protein kinases C but not Raf-1 or B-Raf. *FEBS Lett*. 2002;531(3):475-482.

420. Khan WA, Blobe G, Halpern A, et al. Selective regulation of protein kinase C isoenzymes by oleic acid in human platelets. *J Biol Chem*. 1993;268(7):5063-5068.

421. Grabarek J, Ware JA. Protein kinase C activation without membrane contact in platelets stimulated by bryostatin. *J Biol Chem*. 1993;268(8):5543-5549.

422. Verstraete M, Zoldhelyi P. Novel antithrombotic drugs in development. *Drugs*. 1995;49(6):856-884.

423. Furie B, Furie BC. Mechanisms of thrombus formation. *N Engl J Med*. 2008;359(9):938-949.

424. Elwood PC, Renaud S, Sharp DS, Beswick AD, O'Brien JR, Yarnell JW. Ischemic heart disease and platelet aggregation. The Caerphilly Collaborative Heart Disease Study. *Circulation*. 1991;83(1):38-44.

425. Lincoff AM. Important triad in cardiovascular medicine: diabetes, coronary intervention, and platelet glycoprotein IIb/IIIa receptor blockade. *Circulation*. 2003;107(11):1556-1559.

426. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 1999;340(2):115-126.

427. Marcus AJ. Aspirin as prophylaxis against colorectal cancer. *N Engl J Med*. 1995;333(10):656-658.

428. Hamberg M, Samuelsson B. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc Natl Acad Sci U S A*. 1974;71(9):3400-3404.

429. Marcus AJ. The role of lipids in platelet function: with particular reference to the arachidonic acid pathway. *J Lipid Res*. 1978;19(7):793-826.

430. Ozeki Y, Ito H, Nagamura Y, Unemi F, Igawa T. 12(S)-HETE plays a role as a mediator of expression of platelet CD62 (P-selectin). *Platelets*. 1998;9(5):297-302.

431. Quintana LF, Guzman B, Collado S, Claria J, Poch E. A coding polymorphism in the 12-lipoxygenase gene is associated to essential hypertension and urinary 12(S)-HETE. *Kidney Int*. 2006;69(3):526-530.

432. Zink MH, Oltman CL, Lu T, et al. 12-lipoxygenase in porcine coronary microcirculation: implications for coronary vasoregulation. *Am J Physiol Heart Circ Physiol*. 2001;280(2):H693-704.

433. Siegel MI, McConnell RT, Porter NA, Cuatrecasas P. Arachidonate metabolism via lipoxygenase and 12L-hydroperoxy-5,8,10,14-icosatetraenoic acid peroxidase sensitive to anti-inflammatory drugs. *Proc Natl Acad Sci U S A*. 1980;77(1):308-312.

434. Ruzicka T. The role of the epidermal 12-hydroxyeicosatetraenoic acid receptor in the skin. *Eicosanoids*. 1992;5 Suppl:S63-65.

435. Herbertsson H, Kuhme T, Evertsson U, Wigren J, Hammarstrom S. Identification of subunits of the 650 kDa 12(S)-HETE binding complex in carcinoma cells. *J Lipid Res*. 1998;39(2):237-244.

436. Morgan AH, Dioszeghy V, Maskrey BH, et al. Phosphatidylethanolamine-esterified eicosanoids in the mouse: tissue localization and inflammation-dependent formation in Th-2 disease. *J Biol Chem*. 2009;284(32):21185-21191.

437. Brune B, Ullrich V. 12-hydroperoxyeicosatetraenoic acid inhibits main platelet functions by activation of soluble guanylate cyclase. *Mol Pharmacol*. 1991;39(5):671-678.

438. Eynard AR, Galli G, Tremoli E, Maderna P, Magni F, Paoletti R. Aspirin inhibits platelet 12-hydroxy-eicosatetraenoic acid formation. *J Lab Clin Med*. 1986;107(1):73-78.

439. Pidgeon GP, Tang K, Cai YL, Piasentin E, Honn KV. Overexpression of platelet-type 12-lipoxygenase promotes tumor cell survival by enhancing alpha(v)beta(3) and alpha(v)beta(5) integrin expression. *Cancer Res*. 2003;63(14):4258-4267.

440. Kaur G, Jalagadugula G, Mao G, Rao AK. RUNX1/core binding factor A2 regulates platelet 12-lipoxygenase gene (ALOX12): studies in human RUNX1 haplodeficiency. *Blood*. 2010;115(15):3128-3135.

441. Yeung J, Holinstat M. 12-lipoxygenase: a potential target for novel anti-platelet therapeutics. *Cardiovasc Hematol Agents Med Chem*. 2011;9(3):154-164.

442. Kenyon V, Chorny I, Carvajal WJ, Holman TR, Jacobson MP. Novel human lipoxygenase inhibitors discovered using virtual screening with homology models. *J Med Chem*. 2006;49(4):1356-1363.

443. Holinstat M, Voss B, Bilodeau ML, Hamm HE. Protease-activated receptors differentially regulate human platelet activation through a phosphatidic acid-dependent pathway. *Mol Pharmacol*. 2007;71(3):686-694.

444. Holinstat M, Voss B, Bilodeau ML, McLaughlin JN, Cleator J, Hamm HE. PAR4, but not PAR1, signals human platelet aggregation via Ca2+ mobilization and synergistic P2Y12 receptor activation. *J Biol Chem*. 2006;281(36):26665-26674.

445. Neeves KB, Maloney SF, Fong KP, et al. Microfluidic focal thrombosis model for measuring murine platelet deposition and stability: PAR4 signaling enhances shear-resistance of platelet aggregates. *J Thromb Haemost*. 2008;6(12):2193-2201.

446. Konopatskaya O, Matthews SA, Harper MT, et al. Protein kinase C mediates platelet secretion and thrombus formation through protein kinase D2. *Blood*. 2011;118(2):416-424.

447. Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. *Platelets*. 2001;12(5):261-273.

448. Unsworth AJ, Smith H, Gissen P, Watson SP, Pears CJ. Submaximal inhibition of protein kinase C restores ADP-induced dense granule secretion in platelets in the presence of Ca2+. *J Biol Chem*. 2011;286(24):21073-21082.

449. Margalit A, Granot Y. Endogenous hepoxilin A3, produced under short duration of high shear-stress, inhibits thrombin-induced aggregation in human platelets. *Biochim Biophys Acta*. 1994;1190(1):173-176.

450. Margalit A, Sofer Y, Grossman S, Reynaud D, Pace-Asciak CR, Livne AA. Hepoxilin A3 is the endogenous lipid mediator opposing hypotonic swelling of intact human platelets. *Proc Natl Acad Sci U S A*. 1993;90(7):2589-2592.

451. Italiano JE, Jr., Battinelli EM. Selective sorting of alpha-granule proteins. *J Thromb Haemost*. 2009;7 Suppl 1:173-176.

452. Singh NK, Kundumani-Sridharan V, Rao GN. 12/15-Lipoxygenase gene knockout severely impairs ischemia-induced angiogenesis due to lack of Rac1 farnesylation. *Blood*. 2011;118(20):5701-5712.

453. Dailey LA, Imming P. 12-Lipoxygenase: classification, possible therapeutic benefits from inhibition, and inhibitors. *Curr Med Chem*. 1999;6(5):389-398.

454. Cannon CP. Effectiveness of clopidogrel versus aspirin in preventing acute myocardial infarction in patients with symptomatic atherothrombosis (CAPRIE trial). *Am J Cardiol*. 2002;90(7):760-762.

455. Glading A, Koziol JA, Krueger J, Ginsberg MH. PEA-15 inhibits tumor cell invasion by binding to extracellular signal-regulated kinase 1/2. *Cancer Res*. 2007;67(4):1536-1544.

456. Jamieson DG, Parekh A, Ezekowitz MD. Review of antiplatelet therapy in secondary prevention of cerebrovascular events: a need for direct comparisons between antiplatelet agents. *J Cardiovasc Pharmacol Ther*. 2005;10(3):153-161.

457. Zucker MB, Nachmias VT. Platelet activation. *Arteriosclerosis*. 1985;5(1):2-18.

458. Cheng CM, Hawiger J. Affinity isolation and characterization of immunoglobulin G Fc fragment-binding glycoprotein from human blood platelets. *J Biol Chem*. 1979;254(7):2165-2167.

459. Looney RJ, Anderson CL, Ryan DH, Rosenfeld SI. Structural polymorphism of the human platelet Fc gamma receptor. *J Immunol*. 1988;141(8):2680-2683.

460. Kelton JG, Sheridan D, Santos A, et al. Heparin-induced thrombocytopenia: laboratory studies. *Blood*. 1988;72(3):925-930.

461. Hauch TW, Rosse WF. Platelet-bound complement (C3) in immune thrombocytopenia. *Blood*. 1977;50(6):1129-1136.

462. Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor gamma-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem*. 1997;272(38):23528-23531.

463. Suzuki-Inoue K, Fuller GL, Garcia A, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood*. 2006;107(2):542-549.

464. Bergmeier W, Stefanini L. Platelet ITAM signaling. *Curr Opin Hematol*. 2013;20(5):445-450.

465. Kasirer-Friede A, Kahn ML, Shattil SJ. Platelet integrins and immunoreceptors. *Immunol Rev*. 2007;218:247-264.

466. Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood*. 1987;69(6):1712-1720.

467. Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest*. 1989;84(5):1440-1445.

468. Arai M, Yamamoto N, Moroi M, Akamatsu N, Fukutake K, Tanoue K. Platelets with 10% of the normal amount of glycoprotein VI have an impaired response to collagen that results in a mild bleeding tendency. *Br J Haematol*. 1995;89(1):124-130.

469. Suzuki-Inoue K, Inoue O, Ozaki Y. Novel platelet activation receptor CLEC-2: from discovery to prospects. *J Thromb Haemost*. 2011;9 Suppl 1:44-55.

470. Takai T. Roles of Fc receptors in autoimmunity. *Nat Rev Immunol*. 2002;2(8):580-592.

471. Meyer T, Robles-Carrillo L, Robson T, et al. Bevacizumab immune complexes activate platelets and induce thrombosis in FCGR2A transgenic mice. *J Thromb Haemost*. 2009;7(1):171-181.

472. Cox D, Kerrigan SW, Watson SP. Platelets and the innate immune system: mechanisms of bacterial-induced platelet activation. *J Thromb Haemost*. 2011;9(6):1097-1107.

473. Reilly MP, Sinha U, Andre P, et al. PRT-060318, a novel Syk inhibitor, prevents heparin-induced thrombocytopenia and thrombosis in a transgenic mouse model. *Blood*. 2011;117(7):2241-2246.

474. Yeung J, Apopa P, Vesci J, et al. Protein kinase C regulation of 12-lipoxygenase-mediated human platelet activation. *Molecular pharmacology*. 2012;81(3):420-430.

475. Coffey MJ, Jarvis GE, Gibbins JM, et al. Platelet 12-lipoxygenase activation via glycoprotein VI: involvement of multiple signaling pathways in agonist control of H(P)ETE synthesis. *Circ Res*. 2004;94(12):1598-1605.

476. Morgan LT, Thomas CP, Kuhn H, O'Donnell VB. Thrombin-activated human platelets acutely generate oxidized docosahexaenoic-acid-containing phospholipids via 12-lipoxygenase. *Biochem J*. 2010;431(1):141-148.

477. Katoh A, Ikeda H, Murohara T, Haramaki N, Ito H, Imaizumi T. Platelet-derived 12-hydroxyeicosatetraenoic acid plays an important role in mediating canine coronary thrombosis by regulating platelet glycoprotein IIb/IIIa activation. *Circulation*. 1998;98(25):2891-2898.

478. Luci DK, Jameson JB, 2nd, Yasgar A, et al. Synthesis and structure-activity relationship studies of 4-((2-hydroxy-3-methoxybenzyl)amino)benzenesulfonamide derivatives as potent and selective inhibitors of 12-lipoxygenase. *J Med Chem*. 2014;57(2):495-506.

479. van Triest M, de Rooij J, Bos JL. Measurement of GTP-bound Ras-like GTPases by activation-specific probes. *Methods Enzymol*. 2001;333:343-348.

480. Bledzka K, Smyth SS, Plow EF. Integrin alphaIIbbeta3: from discovery to efficacious therapeutic target. *Circ Res*. 2013;112(8):1189-1200.

481. Phillips DR, Agin PP. Platelet membrane defects in Glanzmann's thrombasthenia. Evidence for decreased amounts of two major glycoproteins. *J Clin Invest*. 1977;60(3):535-545.

482. Lee HS, Lim CJ, Puzon-McLaughlin W, Shattil SJ, Ginsberg MH. RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences. *J Biol Chem*. 2009;284(8):5119-5127.

483. Watanabe N, Bodin L, Pandey M, et al. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin alphaIIbbeta3. *J Cell Biol*. 2008;181(7):1211-1222.

484. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC, 2nd. Rap1b is required for normal platelet function and hemostasis in mice. *J Clin Invest*. 2005;115(3):680-687.

485. Bertoni A, Tadokoro S, Eto K, et al. Relationships between Rap1b, affinity modulation of integrin alpha IIbbeta 3, and the actin cytoskeleton. *J Biol Chem*. 2002;277(28):25715-25721.

486. Brass LF, Manning DR, Shattil SJ. GTP-binding proteins and platelet activation. *Prog Hemost Thromb*. 1991;10:127-174.

487. Holmsen H, Weiss HJ. Secretable storage pools in platelets. *Annu Rev Med*. 1979;30:119-134.

488. Fukami MH, Salganicoff L. Human platelet storage organelles. A review. *Thromb Haemost*. 1977;38(4):963-970.

489. Israels SJ, McNicol A, Robertson C, Gerrard JM. Platelet storage pool deficiency: diagnosis in patients with prolonged bleeding times and normal platelet aggregation. *Br J Haematol*. 1990;75(1):118-121.

490. McNicol A, Israels SJ. Platelet dense granules: structure, function and implications for haemostasis. *Thromb Res*. 1999;95(1):1-18.

491. Asselin J, Gibbins JM, Achison M, et al. A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1. *Blood*. 1997;89(4):1235-1242.

492. Blake RA, Schieven GL, Watson SP. Collagen stimulates tyrosine phosphorylation of phospholipase C-gamma 2 but not phospholipase C-gamma 1 in human platelets. *FEBS Lett*. 1994;353(2):212-216.

493. Gibbins J, Asselin J, Farndale R, Barnes M, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. *J Biol Chem*. 1996;271(30):18095-18099.

494. Yanaga F, Poole A, Asselin J, et al. Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor. *Biochem J*. 1995;311 (Pt 2):471-478.

495. Yacoub D, Theoret JF, Villeneuve L, et al. Essential role of protein kinase C delta in platelet signaling, alpha IIb beta 3 activation, and thromboxane A2 release. *J Biol Chem*. 2006;281(40):30024-30035.

496. Jankun J, Aleem AM, Malgorzewicz S, et al. Synthetic curcuminoids modulate the arachidonic acid metabolism of human platelet 12-lipoxygenase and reduce sprout formation of human endothelial cells. *Mol Cancer Ther*. 2006;5(5):1371-1382.

497. Kandouz M, Nie D, Pidgeon GP, Krishnamoorthy S, Maddipati KR, Honn KV. Platelet-type 12-lipoxygenase activates NF-kappaB in prostate cancer cells. *Prostaglandins Other Lipid Mediat*. 2003;71(3-4):189-204.

498. Nie D, Krishnamoorthy S, Jin R, et al. Mechanisms regulating tumor angiogenesis by 12-lipoxygenase in prostate cancer cells. *J Biol Chem*. 2006;281(27):18601-18609.

499. Greinacher A, Althaus K, Krauel K, Selleng S. Heparin-induced thrombocytopenia. *Hamostaseologie*. 2010;30(1):17-18, 20-18.

500. Solomon EI, Zhou J, Neese F, Pavel EG. New insights from spectroscopy into the structure/function relationships of lipoxygenases. *Chem Biol*. 1997;4(11):795-808.

501. Serhan CN. Lipoxin biosynthesis and its impact in inflammatory and vascular events. *Biochim Biophys Acta*. 1994;1212(1):1-25.

502. Nakano H, Inoue T, Kawasaki N, et al. Synthesis and biological activities of novel antiallergic agents with 5-lipoxygenase inhibiting action. *Bioorg Med Chem*. 2000;8(2):373-380.

503. Ghosh J, Myers CE. Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc Natl Acad Sci U S A*. 1998;95(22):13182-13187.

504. Steele VE, Holmes CA, Hawk ET, et al. Lipoxygenase inhibitors as potential cancer chemopreventives. *Cancer Epidemiol Biomarkers Prev*. 1999;8(5):467-483.

505. Hussain H, Shornick LP, Shannon VR, et al. Epidermis contains platelet-type 12-lipoxygenase that is overexpressed in germinal layer keratinocytes in psoriasis. *Am J Physiol*. 1994;266(1 Pt 1):C243-253.

506. Olas B, Wachowicz B, Stochmal A, Oleszek W. Inhibition of blood platelet adhesion and secretion by different phenolics from Yucca schidigera Roezl. bark. *Nutrition*. 2005;21(2):199-206.

507. Yeung J, Apopa PL, Vesci J, et al. Protein Kinase C Regulation of 12-lipoxygenase-mediated Human Platelet Activation. *Mol Pharmacol*. 2011.

508. Ma K, Nunemaker CS, Wu R, Chakrabarti SK, Taylor-Fishwick DA, Nadler JL. 12-Lipoxygenase Products Reduce Insulin Secretion and {beta}-Cell Viability in Human Islets. *J Clin Endocrinol Metab*. 2010;95(2):887-893.

509. Tohjima T, Honda N, Mochizuki K, et al. Decreased activity of arachidonate 12-lipoxygenase in platelets of Japanese patients with non-insulin-dependent diabetes mellitus. *Metabolism*. 1998;47(3):257-263.

510. Connolly JM, Rose DP. Enhanced angiogenesis and growth of 12-lipoxygenase gene-transfected MCF-7 human breast cancer cells in athymic nude mice. *Cancer Lett*. 1998;132(1-2):107-112.

511. Natarajan R, Nadler J. Role of lipoxygenases in breast cancer. *Front Biosci*. 1998;3:E81-88.

512. Harats D, Shaish A, George J, et al. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2000;20(9):2100-2105.

513. Kamitani H, Geller M, Eling T. Expression of 15-lipoxygenase by human colorectal carcinoma Caco-2 cells during apoptosis and cell differentiation. *J Biol Chem*. 1998;273(34):21569-21577.

514. Chen X-S, Brash AR, Funk CD. Purification and characterization of recombinant histidine-tagged human platelet 12-lipoxygenase expressed in a baculovirus/insect cell system. *Eur J Biochem*. 1993;214:845-852.

515. Boeglin WE, Kim RB, Brash AR. A 12R-lipoxygenase in human skin: mechanistic evidence, molecular cloning, and expression. *Proc Natl Acad Sci U S A*. 1998;95(12):6744-6749.

516. Burke JE, Dennis EA. Phospholipase A2 biochemistry. *Cardiovasc Drugs Ther*. 2009;23(1):49-59.

517. Barre DE, Holub BJ. The effect of borage oil consumption on the composition of individual phospholipids in human platelets. *Lipids*. 1992;27(5):315-320.

518. Johnson MM, Swan DD, Surette ME, et al. Dietary supplementation with gamma-linolenic acid alters fatty acid content and eicosanoid production in healthy humans. *J Nutr*. 1997;127(8):1435-1444.

519. Kris-Etherton P, Daniels SR, Eckel RH, et al. Summary of the scientific conference on dietary fatty acids and cardiovascular health: conference summary from the nutrition committee of the American Heart Association. *Circulation*. 2001;103(7):1034-1039.

520. Simopoulos AP. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomed Pharmacother*. 2006;60(9):502-507.

521. Kapoor R, Huang YS. Gamma linolenic acid: an antiinflammatory omega-6 fatty acid. *Curr Pharm Biotechnol*. 2006;7(6):531-534.

522. Segraves EN, Holman TR. Kinetic investigations of the rate-limiting step in human 12- and 15-lipoxygenase. *Biochemistry*. 2003;42(18):5236-5243.

523. Wecksler AT, Jacquot C, van der Donk WA, Holman TR. Mechanistic Investigations of Human Reticulocyte 15- and Platelet 12-Lipoxygenases with Arachidonic Acid. *Biochemistry*. 2009.

524. Amagata T, Whitman S, Johnson TA, et al. Exploring sponge-derived terpenoids for their potency and selectivity against 12-human, 15-human, and 15-soybean lipoxygenases. *J Nat Prod*. 2003;66(2):230-235.

525. Chen XS, Funk CD. Structure-function properties of human platelet 12-lipoxygenase: chimeric enzyme and in vitro mutagenesis studies. *FASEB J*. 1993;7(8):694-701.

526. Murphy RC, Barkley RM, Zemski Berry K, et al. Electrospray ionization and tandem mass spectrometry of eicosanoids. *Anal Biochem*. 2005;346(1):1-42.

527. Graff G, Anderson LA, Jaques LW. Preparation and purification of soybean lipoxygenase-derived unsaturated hydroperoxy and hydroxy fatty acids and determination of molar absorptivities of hydroxy fatty acids. *Anal Biochem*. 1990;188(1):38-47.

528. Hoye TR, Jeffrey CS, Shao F. Mosher ester analysis for the determination of absolute configuration of stereogenic (chiral) carbinol carbons. *Nat Protoc*. 2007;2(10):2451-2458.

529. Holinstat M, Preininger AM, Milne SB, Hudson WJ, Brown HA, Hamm HE. Irreversible platelet activation requires protease-activated receptor 1-mediated signaling to phosphatidylinositol phosphates. *Mol Pharmacol*. 2009;76(2):301-313.

530. Gong H, Shen B, Flevaris P, et al. G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling. *Science*. 2010;327(5963):340-343.

531. Ivanov I, Heydeck D, Hofheinz K, et al. Molecular enzymology of lipoxygenases. *Arch Biochem Biophys*. 2010;503(2):161-174.

532. Knapp MJ, Seebeck FP, Klinman JP. Steric control of oxygenation regiochemistry in soybean lipoxygenase-1. *J Am Chem Soc*. 2001;123(12):2931-2932.

533. Gomolka B, Siegert E, Blossey K, Schunck WH, Rothe M, Weylandt KH. Analysis of omega-3 and omega-6 fatty acid-derived lipid metabolite formation in human and mouse blood samples. *Prostaglandins Other Lipid Mediat*. 2011;94(3-4):81-87.

534. Harper CR, Edwards MJ, DeFilippis AP, Jacobson TA. Flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans. *J Nutr*. 2006;136(1):83-87.

535. Holy EW, Forestier M, Richter EK, et al. Dietary alpha-linolenic acid inhibits arterial thrombus formation, tissue factor expression, and platelet activation. *Arterioscler Thromb Vasc Biol*. 2011;31(8):1772-1780.

536. Siess W, Siegel FL, Lapetina EG. Dihomogammalinolenic acid, but not eicosapentaenoic acid, activates washed human platelets. *Biochim Biophys Acta*. 1984;801(2):265-276.

537. Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X. A molecular switch that controls cell spreading and retraction. *J Cell Biol*. 2007;179(3):553-565.

538. von Schacky C, Weber PC. Metabolism and effects on platelet function of the purified eicosapentaenoic and docosahexaenoic acids in humans. *J Clin Invest*. 1985;76(6):2446-2450.

539. Bergstroem S, Danielsson H, Klenberg D, Samuelsson B. The Enzymatic Conversion of Essential Fatty Acids into Prostaglandins. *J Biol Chem*. 1964;239:PC4006-4008.

540. Falardeau P, Hamberg M, Samuelsson B. Metabolism of 8,11,14-eicosatrienoic acid in human platelets. *Biochim Biophys Acta*. 1976;441(2):193-200.

541. Gan QF, Browner MF, Sloane DL, Sigal E. Defining the arachidonic acid binding site of human 15-lipoxygenase. Molecular modeling and mutagenesis. *J Biol Chem*. 1996;271(41):25412-25418.

542. Rickert KW, Klinman JP. Nature of Hydrogen Transfer in Soybean Lipoxygenase-1: Separation of Primary and Secondary Isotope Effects. *Biochemistry*. 1999;38:12218-12228.

543. Guo Y, Zhang W, Giroux C, et al. Identification of the orphan G protein coupled receptor GPR31 as a receptor for 12(S)hydroxyeicosatetraenoic acid. *Journal of Biological Chemistry*. 2011;286(Papers in Press).

544. Zafiriou MP, Deva R, Ciccoli R, Siafaka-Kapadai A, Nigam S. Biological role of hepoxilins: upregulation of phospholipid hydroperoxide glutathione peroxidase as a cellular response to oxidative stress? *Prostaglandins Leukot Essent Fatty Acids*. 2007;77(3-4):209-215.

545. Kernoff PB, Willis AL, Stone KJ, Davies JA, McNicol GP. Antithrombotic potential of dihomo-gamma-linolenic acid in man. *Br Med J*. 1977;2(6100):1441-1444.

546. Fan YY, Chapkin RS. Importance of dietary gamma-linolenic acid in human health and nutrition. *J Nutr*. 1998;128(9):1411-1414.

547. Surette ME, Koumenis IL, Edens MB, et al. Inhibition of leukotriene biosynthesis by a novel dietary fatty acid formulation in patients with atopic asthma: a randomized, placebo-controlled, parallel-group, prospective trial. *Clin Ther*. 2003;25(3):972-979.

548. Levin G, Duffin KL, Obukowicz MG, et al. Differential metabolism of dihomo-gamma-linolenic acid and arachidonic acid by cyclo-oxygenase-1 and cyclo-oxygenase-2: implications for cellular synthesis of prostaglandin E1 and prostaglandin E2. *Biochem J*. 2002;365(Pt 2):489-496.

549. Takai S, Jin D, Kawashima H, et al. Anti-atherosclerotic effects of dihomo-gamma-linolenic acid in ApoE-deficient mice. *J Atheroscler Thromb*. 2009;16(4):480-489.

550. Willis AL, Smith DL. Therapeutic impact of eicosanoids in atherosclerotic disease. *Eicosanoids*. 1989;2(2):69-99.

551. Aragona P, Bucolo C, Spinella R, Giuffrida S, Ferreri G. Systemic omega-6 essential fatty acid treatment and pge1 tear content in Sjogren's syndrome patients. *Invest Ophthalmol Vis Sci*. 2005;46(12):4474-4479.

552. Laidlaw M, Holub BJ. Effects of supplementation with fish oil-derived n-3 fatty acids and gamma-linolenic acid on circulating plasma lipids and fatty acid profiles in women. *Am J Clin Nutr*. 2003;77(1):37-42.

553. Hurtado de Catalfo GE, de Gomez Dumm IN. Spontaneously hypertensive rats: eicosa-8,11,14-trienoic acid metabolism and arachidonic acid biosynthesis. *Biochem Mol Biol Int*. 1996;40(4):759-768.

554. Manku MS, Oka M, Horrobin DF. Differential regulation of the formation of prostaglandins and related substances from arachidonic acid and from dihomogammalinolenic acid. II. Effects of vitamin C. *Prostaglandins Med*. 1979;3(2):129-137.

555. Chen ZM, Jiang LX, Chen YP, et al. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. *Lancet*. 2005;366(9497):1607-1621.

556. Palacio S, Hart RG, Pearce LA, Benavente OR. Effect of addition of clopidogrel to aspirin on mortality: systematic review of randomized trials. *Stroke*. 2012;43(8):2157-2162.

557. Diener HC, Bogousslavsky J, Brass LM, et al. Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. *Lancet*. 2004;364(9431):331-337.

558. Farrow JW, Willis AL. Proceedings: Thrombolytic and anti-thrombotic properties of dihomo-gamma-linolenate in vitro. *Br J Pharmacol*. 1975;55(2):316P-317P.

559. Willis AL, Comai K, Kuhn DC, Paulsrud J. Dihomo-gamma-linolenate suppresses platelet aggregation when administered in vitro or in vivo. *Prostaglandins*. 1974;8(6):509-519.

560. Lands WE, Samuelsson B. Phospholipid precursors of prostaglandins. *Biochim Biophys Acta*. 1968;164(2):426-429.

561. Borsch-Haubold AG, Kramer RM, Watson SP. Cytosolic phospholipase A2 is phosphorylated in collagen- and thrombin-stimulated human platelets independent of protein kinase C and mitogen-activated protein kinase. *J Biol Chem*. 1995;270(43):25885-25892.

562. Srivastava KC. Metabolism of arachidonic acid by platelets: utilization of arachidonic acid by human platelets in presence of linoleic and dihomo-gamma-linolenic acids. *Z Ernahrungswiss*. 1978;17(4):248-261.

563. Bunting S, Gryglewski R, Moncada S, Vane JR. Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac ateries and inhibits platelet aggregation. *Prostaglandins*. 1976;12(6):897-913.

564. Bunting S, Moncada S, Needleman P, Vane JR. Proceedings: Formation of prostaglandin endoperoxides and rabbit aorta contracting substance (RCS) by coupling two enzyme systems. *Br J Pharmacol*. 1976;56(3):344P-345P.

565. Moncada S, Gryglewski R, Bunting S, Vane JR. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*. 1976;263(5579):663-665.

566. Reheman A, Gross P, Yang H, et al. Vitronectin stabilizes thrombi and vessel occlusion but plays a dual role in platelet aggregation. *J Thromb Haemost*. 2005;3(5):875-883.

567. Reheman A, Yang H, Zhu G, et al. Plasma fibronectin depletion enhances platelet aggregation and thrombus formation in mice lacking fibrinogen and von Willebrand factor. *Blood*. 2009;113(8):1809-1817.

568. Wang Y, Reheman A, Spring CM, et al. Plasma fibronectin supports hemostasis and regulates thrombosis. *J Clin Invest*. 2014;124(10):4281-4293.

569. Simons K, Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol*. 2000;1(1):31-39.

570. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood*. 2004;104(6):1606-1615.

571. Falati S, Gross P, Merrill-Skoloff G, Furie BC, Furie B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med*. 2002;8(10):1175-1181.

572. de Oliveira Otto MC, Wu JH, Baylin A, et al. Circulating and dietary omega-3 and omega-6 polyunsaturated fatty acids and incidence of CVD in the Multi-Ethnic Study of Atherosclerosis. *J Am Heart Assoc*. 2013;2(6):e000506.

573. Dyerberg J, Bang HO, Stoffersen E, Moncada S, Vane JR. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? *Lancet*. 1978;2(8081):117-119.

574. Simon LM, Edelstein LC, Nagalla S, et al. Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. *Blood*. 2014;123(16):e37-45.

575. Welsh JD, Muthard RW, Stalker TJ, Taliaferro JP, Diamond SL, Brass LF. A systems approach to hemostasis: 4. How hemostatic thrombi limit the loss of plasma-borne molecules from the microvasculature. *Blood*. 2016;127(12):1598-1605.

576. Tateson JE, Moncada S, Vane JR. Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins*. 1977;13(3):389-397.

577. Gorman RR, Bunting S, Miller OV. Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins*. 1977;13(3):377-388.

578. Noe L, Peeters K, Izzi B, Van Geet C, Freson K. Regulators of platelet cAMP levels: clinical and therapeutic implications. *Curr Med Chem*. 2010;17(26):2897-2905.

579. Miller OV, Gorman RR. Modulation of platelet cyclic nucleotide content by PGE1 and the prostaglandin endoperoxide PGG2. *J Cyclic Nucleotide Res*. 1976;2(2):79-87.

580. Haslam RJ. Roles of cyclic nucleotides in platelet function. *Ciba Found Symp*. 1975;35:121-151.

581. Haslam RJ, Davidson MM, Davies T, Lynham JA, McClenaghan MD. Regulation of blood platelet function by cyclic nucleotides. *Adv Cyclic Nucleotide Res*. 1978;9:533-552.

582. Haslam RJ, Davidson MM, Fox JE, Lynham JA. Cyclic nucleotides in platelet function. *Thromb Haemost*. 1978;40(2):232-240.

583. Armstrong RA, Jones RL, MacDermot J, Wilson NH. Prostaglandin endoperoxide analogues which are both thromboxane receptor antagonists and prostacyclin mimetics. *Br J Pharmacol*. 1986;87(3):543-551.

584. Riva CM, Morganroth ML, Ljungman AG, et al. Iloprost inhibits neutrophil-induced lung injury and neutrophil adherence to endothelial monolayers. *Am J Respir Cell Mol Biol*. 1990;3(4):301-309.

585. Turcato S, Clapp LH. Effects of the adenylyl cyclase inhibitor SQ22536 on iloprost-induced vasorelaxation and cyclic AMP elevation in isolated guinea-pig aorta. *Br J Pharmacol*. 1999;126(4):845-847.

586. Butt E, Abel K, Krieger M, et al. cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. *J Biol Chem*. 1994;269(20):14509-14517.

587. Gilman AG. Guanine nucleotide-binding regulatory proteins and dual control of adenylate cyclase. *J Clin Invest*. 1984;73(1):1-4.

588. Smigel M, Katada T, Northup JK, Bokoch GM, Ui M, Gilman AG. Mechanisms of guanine nucleotide-mediated regulation of adenylate cyclase activity. *Adv Cyclic Nucleotide Protein Phosphorylation Res*. 1984;17:1-18.

589. Zhang L, Brass LF, Manning DR. The Gq and G12 families of heterotrimeric G proteins report functional selectivity. *Mol Pharmacol*. 2009;75(1):235-241.

590. Samuelsson B. Role of basic science in the development of new medicines: examples from the eicosanoid field. *J Biol Chem*. 2012;287(13):10070-10080.

591. Capodanno D, Gargiulo G, Buccheri S, Giacoppo D, Capranzano P, Tamburino C. Meta-Analyses of Dual Antiplatelet Therapy Following Drug-Eluting Stent Implantation: Do Bleeding and Stent Thrombosis Weigh Similar on Mortality? *J Am Coll Cardiol*. 2015;66(14):1639-1640.

592. Ahrens I, Peter K. Humanizing mouse thrombi. *Nat Biotechnol*. 2008;26(1):62-63.

593. Lee H, Sturgeon SA, Mountford JK, Jackson SP, Hamilton JR. Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice. *Br J Pharmacol*. 2012;166(7):2188-2197.

594. Tourdot BE, Adili R, Isingizwe ZR, et al. 12-HETrE inhibits platelet reactivity and thrombosis in part through the prostacyclin receptor. *Blood Advances*. 2017;1(15):1124-1131.

595. Phipps RP, Blumberg N. Statin islands and PPAR ligands in platelets. *Arterioscler Thromb Vasc Biol*. 2009;29(5):620-621.

596. Nguyen CH, Stadler S, Brenner S, et al. Cancer cell-derived 12(S)-HETE signals via 12-HETE receptor, RHO, ROCK and MLC2 to induce lymph endothelial barrier breaching. *Br J Cancer*. 2016;115(3):364-370.

597. Bolick DT, Orr AW, Whetzel A, et al. 12/15-lipoxygenase regulates intercellular adhesion molecule-1 expression and monocyte adhesion to endothelium through activation of RhoA and nuclear factor-kappaB. *Arterioscler Thromb Vasc Biol*. 2005;25(11):2301-2307.

598. Gardiner EE, Arthur JF, Kahn ML, Berndt MC, Andrews RK. Regulation of platelet membrane levels of glycoprotein VI by a platelet-derived metalloproteinase. *Blood*. 2004;104(12):3611-3617.

599. Gardiner EE, Karunakaran D, Arthur JF, et al. Dual ITAM-mediated proteolytic pathways for irreversible inactivation of platelet receptors: de-ITAM-izing FcgammaRIIa. *Blood*. 2008;111(1):165-174.

600. Stephens G, Yan Y, Jandrot-Perrus M, Villeval JL, Clemetson KJ, Phillips DR. Platelet activation induces metalloproteinase-dependent GP VI cleavage to down-regulate platelet reactivity to collagen. *Blood*. 2005;105(1):186-191.

601. Honn KV, Tang DG, Gao X, et al. 12-lipoxygenases and 12(S)-HETE: role in cancer metastasis. *Cancer Metastasis Rev*. 1994;13(3-4):365-396.

602. Dilly AK, Tang K, Guo Y, et al. Convergence of eicosanoid and integrin biology: Role of Src in 12-LOX activation. *Exp Cell Res*. 2017;351(1):1-10.

603. Senis YA, Mazharian A, Mori J. Src family kinases: at the forefront of platelet activation. *Blood*. 2014;124(13):2013-2024.

604. Luci D, Jameson JB, II, Yasgar A, et al. Discovery of ML355, a Potent and Selective Inhibitor of Human 12-Lipoxygenase. Probe Reports from the NIH Molecular Libraries Program. Bethesda (MD); 2010.

605. Adili R, Tourdot BE, Mast K, et al. First Selective 12-LOX Inhibitor, ML355, Impairs Thrombus Formation and Vessel Occlusion In Vivo With Minimal Effects on Hemostasis. *Arterioscler Thromb Vasc Biol*. 2017;37(10):1828-1839.

606. Cawello W, Schweer H, Muller R, Bonn R, Seyberth HW. Metabolism and pharmacokinetics of prostaglandin E1 administered by intravenous infusion in human subjects. *Eur J Clin Pharmacol*. 1994;46(3):275-277.

607. Dorris SL, Peebles RS, Jr. PGI2 as a regulator of inflammatory diseases. *Mediators Inflamm*. 2012;2012:926968.

608. Kovacs IB, Mayou SC, Kirby JD. Infusion of a stable prostacyclin analogue, iloprost, to patients with peripheral vascular disease: lack of antiplatelet effect but risk of thromboembolism. *Am J Med*. 1991;90(1):41-46.

609. Turner M, Schweighoffer E, Colucci F, Di Santo JP, Tybulewicz VL. Tyrosine kinase SYK: essential functions for immunoreceptor signalling. *Immunol Today*. 2000;21(3):148-154.

610. Bohmer R, Neuhaus B, Buhren S, et al. Regulation of developmental lymphangiogenesis by Syk(+) leukocytes. *Dev Cell*. 2010;18(3):437-449.

611. Coopman PJ, Do MT, Barth M, et al. The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature*. 2000;406(6797):742-747.