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METHODS

Capturing Proteins that Bind Polyunsaturated Fatty Acids: Demonstration Using Arachidonic Acid and Eicosanoids

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Abstract Polyunsaturated fatty acids (PUFA) and their biological derivatives, including the eicosanoids, have numerous roles in physiology and pathology. Although some eicosanoids are known to act through receptors, the molecular actions of many PUFA remain obscure. As the three-dimensional structure of eicosanoids allows them to specifically bind and activate their receptors, we hypothesized that the same structure would allow other proteins to associate with PUFA and eicosanoids. Here, we demonstrate that biotinylation of arachidonic acid and its oxygenated derivatives 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene (LT) B_4 can be used to pull down associated proteins. Separation of proteins by two-dimensional gel electrophoresis indicated that a large number of proteins bound each lipid and that proteins could distinguish between two enantiomers of 5-HETE. Individual proteins, identified by matrix assisted laser desorption/ionization-time of flight mass spectrometry, included proteins that are known to bind lipids, including albumin and phosphatidylethanolamine-binding protein, as well as several novel proteins. These include cytoskeletal proteins, such as actin, moesin, stathmin and coactosin-like protein, and G protein signaling proteins, such as Rho GDP dissociation inhibitor 1 and nucleoside diphosphate kinase B. This method, then, represents a relatively simple and straightforward way to screen for proteins that directly associate with, and are potentially modulated by, PUFA and their derivatives.

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Abbreviations

ARA	Arachidonic acid
ACH	Arachidic acid
HETE	Hydroxyeicosatetraenoic acid
HpETE	Hydroperoxyeicosatetraenoic acid
LT	Leukotriene
MALDI-TOF	Matrix assisted laser desorption/ionization-time of flight
PUFA	Polyunsaturated fatty acid
RBL	Rat basophilic leukemia

Introduction

Polyunsaturated fatty acids (PUFA) play important roles in health and disease. For example, increasing the consumption of ω-3 PUFA reduces the risk of coronary heart disease [1–3] and decreases ischemic heart disease [4, 5]. On the other hand, the ω-6 PUFA arachidonic acid (ARA) can form ω-6 eicosanoids that exacerbate arrhythmogenic events [6, 7] and decreasing excessive ω-6 eicosanoid action can reduce the risk of ischemic heart disease [8–10]. Although these PUFA have profound effects on physiology, the specific actions of PUFA at the molecular and cellular levels are diverse and poorly understood.

Chemically, PUFA include long chain hydrocarbon molecules with numerous carbon–carbon double bonds. The double bonds represent sites of chemical reactivity

with potential for enzymatic modification. For example, ARA is a twenty carbon PUFA with four double bonds (20:4). It can be modified by the enzyme 5-lipoxygenase, which inserts molecular oxygen at the fifth carbon to produce 5(*S*)-hydroperoxyeicosatetraenoic acid (5(*S*)-HpETE; reviewed in [11]). This PUFA can be modified by peroxidases to produce 5(*S*)-hydroxyeicosatetraenoic acid (5(*S*)-HETE), a biologically active monohydroxylated PUFA. Alternatively, 5(*S*)-HpETE can be further metabolized along the 5-lipoxygenase pathway to produce the dihydroxylated PUFA leukotriene (LT) B_4 or the glutathione-containing LTC_4. Whereas ARA induces apoptosis in neurons [12] and in leukemic cells [13, 14] or activates a variety of cell types [15, 16], LTB_4 and 5(*S*)-HETE are best known as chemoattractants and activators of leukocytes, and LTC_4 is recognized to promote contraction of smooth muscle and secretion of mucus from goblet cells (reviewed in [17]). Thus, enzymes recognize specific PUFA and alter them to produce a variety of novel constructs that have unique functions. The multitude of products that can be produced from ARA are called eicosanoids, whereas the smaller group produced solely by the 5-lipoxygenase pathway are the LT.

Structurally, the carbon–carbon double bonds in PUFA provide a degree of rigidity that is not found in saturated FA. As a result, each of the PUFA and their derivatives has a unique three-dimensional structure. This structure allows these lipids to interact with specific receptors. For example, LTB_4 binds to and activates two G-protein coupled receptors, the high affinity BLT_1 [18] and the lower affinity BLT_2 [19]. Similarly, LTC_4 activates two other receptors, $CysLT_1$ and $CysLT_2$. Consistent with the high specificity of lipid for protein, LTB_4 does not activate $CysLT_1$ or $CysLT_2$ at physiological concentrations, and LTC_4 does not activate BLT_1 or BLT_2. Relevant to this study, the interaction of these eicosanoids with their receptors results in conformational changes of the receptors with their consequential activation. This suggests that, if PUFA associate with non-receptor proteins, they may alter protein shape, resulting in modification of protein function.

We hypothesized that the distinctive structure of PUFA and eicosanoids should allow them to interact with specific soluble proteins other than traditional receptors. For example, it is known that ARA can bind directly to protein phosphatase 5 [20], binds to [21] and activates [22] S100A8/A9, and directly activates a GTP-binding protein in neutrophils [23]. Here, we describe a method for objectively capturing proteins that associate with PUFA and eicosanoids. We use proteins from the rat basophilic leukemia (RBL-1) cell line because it is mast cell like, in that it releases ARA, makes LT and responds to LT.

Materials and Methods

Cells and Cell Lysates

RBL cells (RBL-1, American Type Culture Collection, Manassas, VA) were grown under 5% CO_2 in Minimal Essential Medium alpha (Invitrogen) with 10% fetal bovine serum, 182 units/mL penicillin G sodium, 182 μg/mL streptomycin sulfate, 455 μg/mL amphotericin B. 2×10^7 cells were suspended in lysis buffer (137 mM NaCl, 10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 1 mM EGTA, protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), sonicated, centrifuged at $14{,}000\times g$ (5 min, 4 °C) and supernatant collected as total soluble proteins from RBL cells.

Isolation of PUFA-Binding Proteins

PUFA (ARA, ACH, 5(*S*)-HETE, 5(*R*)-HETE, or LTB_4) were biotinylated using the EZ-Link Biotin PEO-Amine kit (Pierce, Rockford IL), according to manufacturer's directions. Briefly, PUFA were diluted to 10 mg/mL in 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer, pH 4.5, mixed with biotin (21 mg/mL) and 1-ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride crosslinker (20 mg/mL), with mixing on rotator for 2 h at RT. Total soluble proteins from RBL cells were mixed with FA-biotin solution, without purification of the FA-biotin preparation, at an approx 1:1 molar ratio, on rotator for 1 h at RT. Samples of the cell protein/FA-biotin preparation were mixed with avidin immobilized on beaded agarose (Pierce) for 1 h at RT and centrifuged 1 min at $3{,}000\times g$. The resulting pellet was washed three times with TBS and FA-binding proteins recovered by elution with 400 mM NaCl in PBS. In some experiments, the supernatant, partially depleted of FA-binding proteins, was mixed with additional immobilized avidin and centrifuged to remove remaining FA-binding proteins, producing a protein fraction that was highly depleted of FA-binding proteins.

Gel Electrophoresis and Protein Identification

Protein samples were dialyzed overnight in PBS with stirring at 4 °C, mixed with SDS-PAGE sample buffer, boiled, separated by SDS-PAGE using a 12% Tris-HCl gel and stained with Coomassie. For two-dimensional gel electrophoresis, samples were mixed with isoelectric focusing buffer and separated using ReadyStrip IPG strips, pH 3–10, in the Protean IEF Cell (Bio-Rad Laboratories, Hercules CA). After focusing, strips were equilibrated

using iodoacetamide, separated by SDS-PAGE using a 12% Tris-HCl gel and stained with Coomassie. Isolated proteins were subjected to matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) ms and tandem ms, with fragments sequenced by the Michigan Proteome Consortium at the University of Michigan. Initial analysis involved searching the NCBInr database; subsequent analysis included BLAST searching the SwissProt database for proteins matching all peptide sequences from tandem mass spectrometry. Calculated kDa and p*I* values for proteins were obtained through SwissProt.

Densitometric Analysis

Regions of images from SDS-PAGE results were analyzed using ImageJ 1.33, with quantitation of grayscale values of pixels measured by ImageJ and plotted using GraphPad Prism 3.00.

Results

Isolation of Fatty Acid-Associated Proteins

Soluble proteins were used in this study, excluding membrane-associated proteins, which have extensive hydrophobic regions that would interact non-specifically with various FA (although many soluble proteins will also have accessible hydrophobic regions). As a first evaluation, proteins from these total soluble protein preparations were separated into those proteins that could be pulled down using different FA and those that were resistant to capture. Here, the polyunsaturated ARA (20 carbon, 4 double bonds) was compared with the saturated arachidic acid (ACH, 20:0). An initial evaluation of the proteins captured by fatty acids indicated that a surprisingly large number of proteins associated with both ARA and ACH (Fig. 1a). Prominent bands could be readily identified in the fatty acid-associated samples, which were largely absent from the fatty acid-depleted samples. Densitometric analysis of a region of the ARA-depleted sample (lane 2) and the ARA-associated samples (lane 3) indicated two major peaks that were unique to the latter sample (Fig. 1b). In addition, while many bands were associated with both ARA (lane 3) and ACH (lane 5), some bands appeared to be more abundant in one than the other (Fig. 1c), suggesting some degree of specificity. In control experiments, essentially no protein bound to beads alone or to beads after incubation with biotin alone without fatty acid, as determined by SDS-PAGE analysis of fractions eluted from washed beads processed in parallel (not shown).

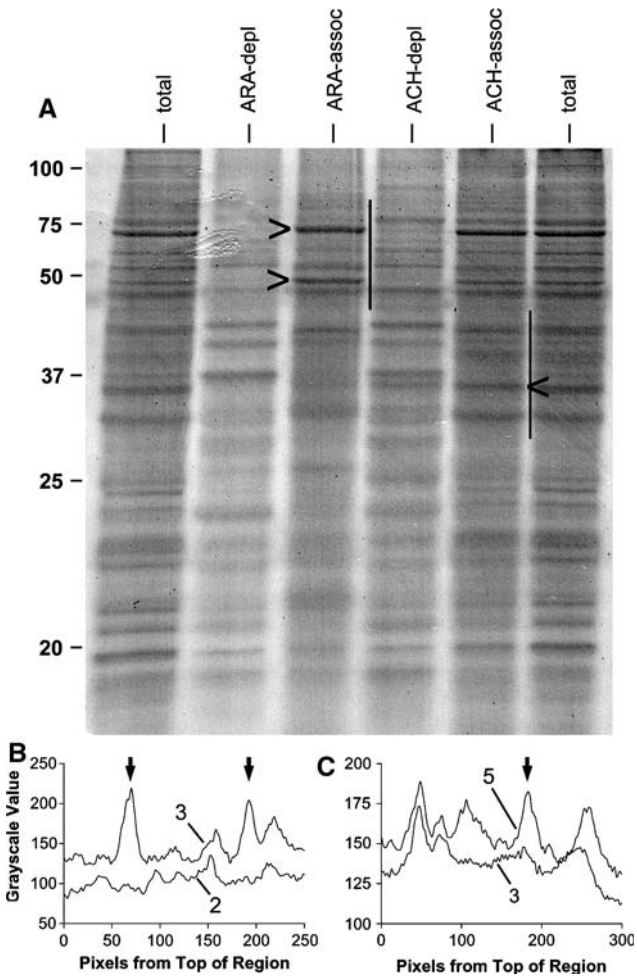

Fig. 1 Separation of ARA-associated proteins from proteins that do not associate with ARA from total soluble proteins of RBL1 cells. **a** SDS-PAGE analysis. Total soluble proteins of RBL1 cells ("total") were incubated with either ARA-biotin or ACH-biotin and the proteins that associated ("assoc") with the fatty acids were captured using avidin agarose. The remaining proteins, depleted of fatty acid-associated proteins, are also presented ("depl"). >, more abundant in ARA-assoc than ARA-depleted; <, more abundant in ACH-assoc than ARA-assoc. **b** Densitometric analysis of regions of ARA-depleted (*2*) and ARA-associated (*3*) lanes; region denoted by bar adjacent to *lane 3*. **c** Densitometric analysis of regions of ARA-associated (*3*) and ACH-associated (*5*) lanes; region corresponds to bar at *lane 5*

Identification of Some ARA-Associating Proteins

The large number of proteins that were pulled down by FA in this protocol indicated that additional separation steps would be required to identify individual FA-associating proteins. Parallel separation of total proteins and ARA-associated proteins by 2-dimensional gel electrophoresis again supported the conclusion that this protocol was capturing a subset of total proteins that bound ARA (Fig. 2).

Some of the most abundant (heavily staining) proteins that associated with ARA were identified by MALDI-TOF

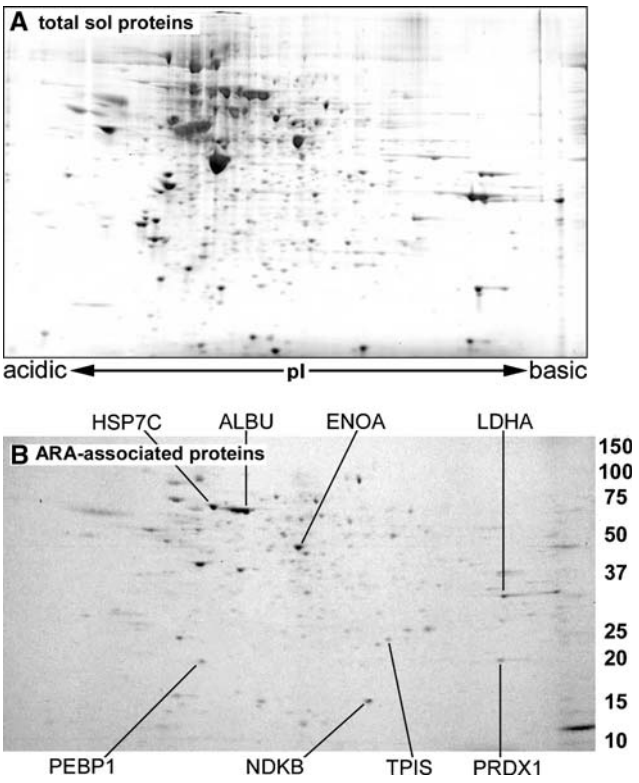

Fig. 2 Two-dimensional separation of total soluble proteins compared with ARA-associating proteins from RBL1 cells. **a** Total soluble proteins. **b** ARA-associating proteins. Coomassie-stained spots were analyzed by MALDI-TOF ms/ms and sequenced peptides used to identify proteins. Abbreviations refer to proteins detailed in Table 1. Migration of molecular weight markers indicated at right of (**b**)

mass spectrometric analysis followed by database searches. In each case, individual peptide sequences were further queried by BLAST search for unique identities. For example, one protein provided the peptides FEELNADLFR, TTPSYVAFTDTER, IINEPTAAAIAYGLDK and TFTNAVVTVPAYFNDSQR; the first three peptides are shared by various heat shock proteins, including heat shock-related 70 kDa protein 2 (HSP72), but the fourth peptide is unique to heat shock cognate 71 kDa protein (HSP7C). The position of this protein on the two-dimensional gel, and some others that were positively identified, is indicated in Fig. 2b. Additional proteins that were identified as ARA-associating proteins are provided in Table 1, grouped according to function, with predicted kDa and p*I* values. Supporting their identification were correlations between the kDa and calculated p*I* values of the proteins and the position of the spots on the gels. Also, when peptide sequences were species specific, the identified isoform was from rat (or from mouse, if a rat protein sequence was not in SwissProt), consistent with these proteins being produced in a rat cell line.

Identification of Proteins Associating with 5-HETE and LTB_4

As noted above, 5(*S*)-HETE can be a significant product of 5-LO metabolism of ARA. Several studies have examined the importance of the stereochemistry on function by comparing the effects of 5(*S*)- versus 5(*R*)-HETE. In short, some effects can be induced by both [24], whereas others are stereospecific [25, 26]. We compared 5(*S*)- vs. 5(*R*)-HETE in their capacity to bind proteins. Remarkably, both enantiomers retained many proteins (Fig. 3). Also, the similarity between the two sets of proteins was very high. Importantly, the major differences between the proteins were seen in horizontal groups of proteins (boxed in Fig. 3), which commonly represent proteins with different degrees of post-translational modification. Indeed, identification of selected pairs of spots using MALDI-TOF mass spectrometry verified that they were identical, recognized as Rho GDP-dissociation inhibitor 1 (GDIR), eukaryotic translation initiation factor 5A-2 (IF5A2), coactosin-like protein (COTL1) and SH3 domain-binding glutamic acid-rich-like protein 3 (SH3L3). Typically, 5(*S*)-HETE selectively captured the more acidic version and 5(*R*)-HETE bound the more basic protein. Additional proteins identified from the 5(*S*)-HETE gel are listed in Table 2.

Finally, two-dimensional gel separation of proteins that associated with LTB_4 revealed at least 20 proteins (not shown). A partial list of the most abundant proteins is given in Table 2. These are grouped according to known roles.

Discussion

This study presents a relatively simple and straightforward approach for capturing proteins that associate with PUFA and their derivatives. An asset of the approach is that it allows novel interactions to be discovered objectively. Through this approach, we have identified groups of proteins involved in protein synthesis, cytoskeletal function, Rho functioning and glycolysis that can associate with ARA. Some of these same proteins and roles are also shared with 5-HETE, suggesting that it can act like a second messenger akin to ARA. Perhaps most surprisingly, several proteins were found to associate with LTB_4, a lipid mediator whose actions are thought to be solely receptor-mediated. This suggests that at least some of the effects of LTB_4, as well as 5-HETE and ARA, may be through direct lipid-protein interactions.

Lipids, due to their hydrophobic nature, are first thought to associate with membranes. However, PUFA may have higher affinities for certain proteins than for membranes. For example, PUFA will partition into liposomal membranes in an aqueous solution, but will leave those

Table 1 Identification of some of the proteins that associate with ARA

Enzyme	Abbrev.	Accession	kDa	p*I*	Role(s)
Elongation factor 1-gamma	EF1G	Q68FR6	50	6.31	Protein synthesis; probably anchors the complex to other cellular components
Elongation factor 2	EF2	P05197	95.3	6.41	Protein synthesis; promotes the GTP-dependent translocation of the nascent protein chain
Eukaryotic translation initiation factor 5A-2	IF5A2	Q9GZV4	16.8	5.38	Protein synthesis; promotes the formation of the first peptide bond
Actin, cytoplasmic 1	ACTB	P60711	41.7	5.29	Motility
Moesin	MOES	O35763	67.7	6.16	Probably involved in connections of major cytoskeletal structures to the plasma membrane
Stathmin	STMN1	P13668	17.3	5.76	Prevents assembly and promotes disassembly of microtubules; Binds to two alpha/beta-tubulin heterodimers
Rho GDP-dissociation inhibitor 1	GDIR	Q99PT1	23.4	5.12	Inhibits dissociation of GDP from and the subsequent binding of GTP to Rho proteins
Nucleoside diphosphate kinase B	NDKB	P19804	17.3	6.91	Synthesis of nucleoside triphosphates other than ATP; negatively regulates Rho activity
Triosephosphate isomerase	TPIS	P48500	26.8	6.89	D-glyceraldehyde 3-phosphate = glycerone phosphate
Alpha-enolase	ENOA	P04764	47.1	6.16	Glycolysis, growth control, hypoxia tolerance, allergic responses, fibrinolysis
L-lactate dehydrogenase A chain	LDHA	P04642	36.5	8.45	Anaerobic glycolysis; (*S*)-lactate + NAD+ = pyruvate + NADH
Peroxiredoxin-1	PRDX1	Q63716	22.1	8.27	Redox regulation; eliminating peroxides; regulating H2O2
Heat shock cognate 71 kDa protein	HSP7C	P63018	70.9	5.37	Chaperone
Phosphatidylethanolamine-binding protein 1	PEBP1	P31044	20.8	5.47	Binds ATP, opioids and PE; Serine protease inhibitor
Serum albumin [Precursor]	ALBU	P02770	68.7	6.09	Regulates colloidal osmotic pressure of blood: binds water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs

Proteins were identified by BLAST search of SwissProt using at least two peptide sequences obtained by MALDI-TOF ms/ms analysis of two-dimensional gel samples. Abbreviation and SwissProt accession numbers, as well as predicted kDa and p*I* obtained using sequence analysis tools at SwissProt, are included

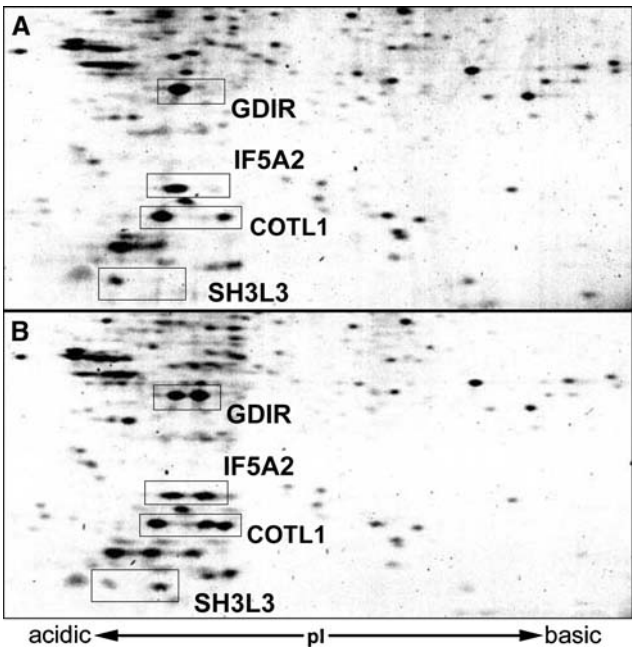

Fig. 3 Selected region of 2-D PAGE analysis of proteins that associate with 5(*S*)-HETE versus 5(*R*)-HETE. **a** 5(*S*)-HETE-associated proteins. **b** 5(*R*)-HETE-associated proteins. Region corresponds to approx. 10–37 kDa and p*I* 4.5–8. Abbreviations refer to proteins detailed in Table 2

membranes to associate with added albumin [27]. Furthermore, ARA will leave albumin to associate with classical fatty acid-binding proteins (FABP), again due to differences in affinity. This indicates that soluble proteins, like albumin and FABP, can have higher affinities for PUFA than do liposomal membranes.

An important question may be whether previous studies support any of the PUFA-protein interactions here. That is, are any of these interactions biologically important, or do they just reflect the interaction of a lipid with a hydrophobic portion of a protein? First, the unique profile of proteins observed for each of the PUFA/eicosanoids argues against all of the interactions being non-specific interactions. Second, the capture of albumin, while not surprising, serves as a positive control. Albumin is recognized to serve the physiologically important function of regulating the colloidal osmotic pressure of blood through its binding of fatty acids, cations, hormones, bilirubin and drugs. Perhaps most intriguing is the case of the Rho GDP-dissociation inhibitor 1 (GDIR). It is well established that PUFA, including ARA, activate the production of reactive oxygen species in leukocytes [28, 29]. The GTP-bound (activated) form of Rac is required for activation of NADPH oxidase [30]. In resting leukocytes, GDIR inhibits the activation of Rac by directly binding to the GDP-bound (inactive) form of Rac, preventing guanine–nucleotide exchange and activation. In a comparison of the ability of different lipids to alter GDIR binding, ARA and other PUFA, but not saturated fatty acids, caused almost complete dissociation of GDIR from Rac, leading to activation of Rac [31]. The identification of the same protein binding to 5(*S*)-HETE suggests that this derivative of ARA may also modulate Rac activation through GDIR. Finally, similar approaches using biotinylation of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 have been used to show that this PGD_2 metabolite interacts with cytoskeletal [32, 33] and mitochondrial [34] proteins. These results underscore the versatility of this approach and strengthen the assertion that ARA metabolites may directly interact with proteins.

The role of PUFA to alter protein–protein interactions, demonstrated for GDIR [31], may also apply to other results from this study. Heat shock cognate 71 kDa protein (HSP7C) was found to bind ARA. ARA has been shown to cause the dissociation of two HSP proteins, approx. 70 and 90 kDa, from protein phosphatase 5, leading to inactivation and degradation of the phosphatase [35], suggesting, at least that the interaction of ARA with HSP7C may serve to dissociate it from target proteins. In contrast with this, ARA has been shown to promote a physical interaction between phospholipase C-γ and a multimeric activating protein, AHNAK [36]. Similarly, ARA promotes translocation of p47-phox [37] and assembly of the NADPH oxidase complex [38]. Thus, PUFA may alter protein functions by facilitating the assembly or disassembly of protein complexes.

Many of the oxidized lipids that are produced from PUFA are relatively unstable and reactive. For example, the 5-LO product LTA_4 has been shown to form stable adducts with nucleotides through covalent binding [39, 40]. Also, lipid hydroperoxides may be decomposed by antioxidants to produce DNA-reactive electrophiles [41]. Relevant to this study, biotinylated derivatives of 15-HETE have been shown to form complexes with proteins that are stable enough to withstand SDS-PAGE and electrophoretic transfer [42]. This suggests that some lipid-protein associations may be very stable.

Another interesting finding relates to L-lactate dehydrogenase A chain (LDHA), which is known to mediate the conversion of lactate to pyruvate, with concomitant generation of NADH from NAD^+. Either transient hypoxia [43] or ischemia [44] produces a rapid release of arachidonic acid associated with a marked increase in lactate and decreases in pyruvate and ATP. It seems possible that arachidonic acid might directly associate with LDHA and inhibit lactate dehydrogenase activity.

Proteins also show specificity for specific PUFA. For example, the different FABP clearly favor certain PUFA over others [45]. Similarly, 5-lipoxygenase, which initiates the synthesis of LTs from ARA, prefers ARA over other PUFA [46] and was detected in ARA-associated proteins

Table 2 Identification of some of the proteins that associate with 5(*S*)-HETE and LTB_4

Enzyme	Abbrev.	Accession	kDa	p*I*	Role(s)
5s-HETE binding prots					
Elongation factor 2	EF2	P05197	95.3	6.41	Protein synthesis; promotes the GTP-dependent translocation of the nascent protein chain
Eukaryotic translation initiation factor 5A-2	IF5A2	Q9GZV4	16.8	5.38	Protein synthesis; promotes the formation of the first peptide bond
Coactosin-like protein	COTL1	Q9CQI6	15.9	5.28	Binds to F-actin in a calcium-independent manner; has no direct effect on actin depolymerization
Tropomyosin alpha-4 chain	TPM4	P09495	28.5	4.66	Binds to actin filaments in muscle and non-muscle cells; in non-muscle cells, implicated in stabilizing cytoskeleton actin filaments
Stathmin	STMN1	P13668	17.3	5.76	Destabilizes microtubules; binds to two alpha/beta-tubulin heterodimers
Rho GDP-dissociation inhibitor 1	GDIR	Q99PT1	23.4	5.12	Inhibits dissociation of GDP from and the subsequent binding of GTP to Rho proteins
SH3 domain-binding glutamic acid-rich-like protein 3	SH3L3	Q91VW3	10.5	5.02	Could act as a modulator of glutaredoxin biological activity
LTB4 binding prots					
Actin, cytoplasmic 2	ACTG	P63259	41.8	5.31	Motility
Profilin-1	PROF1	P62963	15	8.46	Complexes with actin
Nucleoside diphosphate kinase B	NDKB	P19804	17.3	6.91	Synthesis of nucleoside triphosphates other than ATP; negatively regulates Rho activity
Fructose-bisphosphate aldolase A	ALDOA	P05064	39.4	8.3	Glycolysis: D-fructose 1,6-bisphosphate = glycerone phosphate + D-glyceraldehyde 3-phosphate
Peroxiredoxin-1	PRDX1	Q63716	22.1	8.27	Redox regulation; eliminating peroxides; regulating H2O2
Serum albumin (Precursor)	ALBU	P02770	68.7	6.09	Regulates colloidal osmotic pressure of blood: binds water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs

Proteins were identified by BLAST search of SwissProt using at least two peptide sequences obtained by MALDI-TOF ms/ms analysis of two-dimensional gel samples. Abbreviation and SwissProt accession numbers, as well as predicted kDa and p*I* obtained using sequence analysis tools at SwissProt, are included

but not in the ACH-associated proteins (not shown). Also, certain proteins were more abundant in the ACH-associated fraction than in the ARA-associated fractions (Fig. 1), suggesting specificity. This specificity must, at least in part, be due to the shape and structure of the PUFA. This specificity of protein for PUFA may be so exquisite that it may distinguish between stereochemistry of a hydroxyl group, as seen in the difference between the proteins that bind 5(*S*)- and 5(*R*)-HETE (Fig. 3).

The specificity of protein for PUFA points to a drawback of the approach used in this study. Since the biotin moiety is at the carboxyl terminus, proteins that specifically target that site will be missed. For example, FA associate with classical FABP, like the human epidermal-FABP, with the carboxyl group of the FA directly interacting with side chains of key amino acids within the FABP [47]. As a result, this approach did not capture these well-known FABP, and perhaps many other proteins.

Another drawback of this approach is that it will not reveal which proteins interact directly with lipids, as opposed to those that associate indirectly by binding to a true PUFA-associating protein. For example, it is possible that the different cytoskeletal proteins (e.g., actin, moesin, stathmin, coactosin-like protein, profilin) may have been pulled down together, with only one of these proteins truly associating with ARA.

In summary, we present a relatively simple stepwise approach for the discovery of proteins that associate with PUFA and their derivatives. This approach can be used with any lipid with a free carboxyl terminus. Given the large number of proteins that were observed on the two-dimensional gels presented here, it appears that PUFA interact with many proteins. The diversity of PUFA and PUFA derivatives suggests that numerous interactions can be discovered with this approach.

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