GPCR-dependent NF-κB Signaling in Endothelial Dysfunction: A Critical Role for the CARMA3/Bcl10/MALT1 Signaling Complex

by

Phillip Charles Delekta

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Doctoral Committee:

Assistant Professor Peter C. Lucas, Chair
Professor Christin Carter-Su
Professor Mark L. Day
Professor Ormond A. MacDougald
Professor Gabriel Nunez

To my amazing wife, Valerie Ann Delekta, you are always there for me.

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“My most brilliant achievement was my ability to

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Winston Churchill

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Abstract

Atherosclerosis is a complex inflammatory disease. Atherosclerotic lesions are characterized by the accumulation of lipid particles and immune cells in the subendothelial space, resulting in the narrowing of the arterial lumen restricting blood flow. Endothelial cell activation, upon exposure to oxidized lipids and pro-inflammatory stimuli, plays an important role in the pathogenesis of atherosclerosis. This damage leads to endothelial dysfunction in the vessel wall, resulting in the expression of chemotactic factors and adhesion molecules, attracting monocytes to migrate into the subendothelial space. Here, they differentiate into macrophages and phagocytose the lipids, becoming lipid-laden foam cells. A significant factor in the pathogenesis of atherosclerosis is the chronic activation of G protein-coupled receptors (GPCR) such as Protease activated receptor -1 (PAR-1). GPCRs are the largest family of cell surface receptors and are expressed in all vascular cell types. In response to agonists, these receptors signal to induce pro-inflammatory genes important to atherogenesis via the NF-κB cell signaling pathway. The NF-κB pathway is a driving force in the formation and progression of atherosclerotic plaques. Activation of the NF-κB pathway leads to nuclear translocation of the NF-κB transcription factors (p50 and p65) that are kept inactive in the cytoplasm by association with the regulatory protein, IκBα. Activated GPCRs stimulate the IκB kinase which phosphorylates IκBα, leading to IκBα ubiquitination and

proteasomal degradation. The transcription factors are then released from IκBα, allowing them to accumulate in the nucleus. NF-κB transcription factors activate the expression of genes for key steps in plaque initiation such as the recruitment of monocytes, stimulation of lymphocytes, and the proliferation and migration of underlying vascular smooth muscle cells. Utilizing the PAR-1, we establish a critical link in GPCR-dependent NF-κB signaling in endothelial dysfunction. This involves a three protein complex consisting of CARMA3, a scaffolding protein with several protein interaction domains, Bcl10, a linker protein, and MALT1, an effector protein that appears to be involved in ubiquitin-mediated activation of the IKK complex. The CARMA3/Bcl10/MALT1 complex is a pivotal point in PAR-1 induced endothelial dysfunction and the disruption of this complex blocks PAR-1 induced NF-κB signaling and preserves normal endothelial cell function.

Chapter I

Thrombin-dependent NF-κB Signaling in Atherosclerosis

Introduction

Atherosclerosis is an obstruction of the artery's lumen by the formation of plaques composed of lipids, extracellular matrix proteins, immune cells and vascular smooth muscle cells which cause the expansion of the vessel wall (1,2). In 2004, atherosclerosis and its related diseases, stroke and myocardial infarction, collectively referred to as cardiovascular disease, accounted for 31.5% of all female deaths and 26.8% of all male deaths, making it the most common cause of disease-related deaths in the world. Cardiovascular diseases showed the largest gender bias of any disease group studied with women being nearly 5% more likely to die from cardiovascular disease and the highest geographic presence was in Europe (3).

The development of plaques occur in the arteries (4). These plaques decrease blood flow to the organs, with the heart and brain being particularly affected. The most severe health risk is due to the instability of these plaques: rupture can result in a blood clot causing stroke or myocardial infarction (1). Plaque formation is a complex process involving many molecular influences and the interactions of multiple cell types with the endothelial cell layer of the vessel being a key player; for the purposes of this review, we will focus on the contributions of endothelial cells and monocytes (5). Atherosclerosis is now viewed as chronic inflammation of the vasculature with the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a classic

moderator of inflammation, as a potentially major driving force in the disease (Figure 1-1) (6). Understanding the role of NF-κB will help to better understand the molecular mechanisms of atherosclerosis.

Protease Activated Receptor-1

Protease Activated Receptor-1 (PAR-1) is a member of the G-protein-coupled receptor (GPCR) superfamily, which is the largest cell surface receptor family, consisting of over 900 known members (7-9). GPCRs are structurally defined by an extracellular N-terminal tail, seven trans-membrane domains linked together by 6 alternating intracellular and extracellular loops and a C-terminal tail (10,11). Members of this receptor family respond to a diverse array of ligands such as peptides, amines, glycoproteins and enzymes (10). These receptors relay extracellular signals by activating multiple intracellular signaling pathways including extracellular signal-regulated kinases (ERK), Akt, and NF-κB through heterotrimeric G protein-dependent and -independent mechanisms and through cross activation of growth factor receptors (9,10).

Both PAR-1 and its agonist thrombin are major participants in endothelial cell biology and atherogenesis, affecting cell signaling, gene expression, endothelial permeability, angiogenesis and vascular tone (7,8). Thrombin is a protease that binds to a hirudin-like domain in the extracellular N-terminus of PAR-1 and cleaves a specific sequence exposing the PAR-1 cryptic ligand, SFLLRN-NH2. The newly exposed amino acid sequence acts as a tethered ligand binding to the extracellular loop two and permanently activating the receptor. This binding causes a conformational change to the receptor, revealing the cytoplasmic surfaces to bind to the heterotrimeric G-proteins (8,10,12,13). A man-made peptide ligand called SFLLRN, also known as TRAP-6, can be used to induce the same response from PAR-1 as thrombin (8,12).

Unlike most GPCR ligands which are hormones, PAR-1's agonist is a serine protease, thrombin (10,14). Besides thrombin's role in the clotting cascade, it is known to activate pro-inflammatory responses, especially in endothelial cells which contribute to atherogenesis (Figure 1-1) (15). Prothrombin is the inactive precursor of thrombin and is produced by the liver or at the site of injury and circulates in the blood. Prothrombin is converted to thrombin via cleavage by a complex consisting of tissue factor Xa and factor Va, which are normally not present in the vessel, and calcium (16). These tissue factors become released into the vessel lumen when the endothelium integrity is compromised and can also be expressed by stimulated endothelial cells and leukocytes within an atherosclerotic plaque (8,12). Several *in vivo* studies using mice have pointed to a role for thrombin in atherogenesis. A mouse model for atherosclerosis (*ApoE*$^{-/-}$) was treated with the thrombin inhibitor melagatran, which had a protective effect by reducing atherosclerotic plaque size and progression and increasing plaque stability. Using the same mouse model, the gene for *HCII*, a protein inhibitor of thrombin, was knocked out and this resulted in significantly increased plaque size (16). This *in vivo* data indicates a potentially significant role for thrombin in atherogenesis in the organism.

Secondary messengers of PAR-1

Stimulated PAR-1 in the endothelium causes activation of a complex series of secondary messengers, leading to multiple responses in the cell. Unstimulated GPCR, through its intracellular loops, binds to Gβγ, a heterotrimeric G-protein (guanine nucleotide binding protein), which binds to the inactive form of Gα (Gα-GDP). Once a ligand binds to a GPCR, the GPCR performs as a guanine nucleotide exchange factor (GEF), causing the Gα to exchange the GDP for GTP which releases the Gβγ. Both the Gα-GTP and Gβγ are then active until the Gα's guanosine triphosphatase activity hydrolyzes GTP to GDP, allowing it to bind to Gβγ again to block further signaling (17).

The activated PAR-1 can couple to several Gα subtypes ($G\alpha_q$, $G\alpha_{12/13}$, $G\alpha_i$) and the Gβγ subunits that act as effectors of multiple cell signaling pathways (8,10,12,13).

For the purpose of this thesis, we will focus on the role of the $G\alpha_q$ and Gβγ subunits, which are essential for PAR-1 induction of the NF-κB signaling pathway and expression of cell adhesion molecules (CAM) in endothelial cells (discussed in detail below) (18,19). Only a blockade of $G\alpha_q$, by overexpression of regulator of G protein signaling-3T or overexpression of dominant negative mutants of $G\alpha_{q,}$ inhibits PAR-1 dependent NF-κB (18). Among the multiple constitutively active G-protein mutants transfected into endothelial cells, only $G\alpha_q$ and Gβγ were able to induce NF-κB signaling (Figure 1-2) (18).

Active GTP-$G\alpha_q$ and Gβγ dimers then stimulate phospholipase C-β isozymes (PLC-β), a membrane-bound enzyme. PLC-β hydrolyzes phosphatidylinositol bisphosphate (PIP_2), a membrane phospholipid, to produce inositol trisphosphate (IP_3), a soluble lipid secondary messenger that spreads throughout the cell and diacylglycerol (DAG) which remains membrane-bound (8,17,20,21). DAG acts as a secondary messenger along with Ca^{2+} for stimulation of conventional PKCs (α, β, γ) and as the sole secondary messenger for novel PKCs (δ, ε, θ, ή), both activating and localizing the PKC to the plasma membrane (19,22). PKC-δ is a primary PKC that regulates PAR-1 dependent NF-κB transcription in endothelial cells while other PKCs may contribute to augmentation of this pathway (Figure 1-2) (19,23-25).

PAR-1-induced production of IP_3 via PLC-β increases intracellular Ca^{2+} levels (19,26). The increasing IP_3 levels activate the IP_3-receptors on the endoplasmic reticulum, causing the opening of channels that release Ca^{2+} stores into the cytosol. This initial short-lived Ca^{2+} spike is augmented by the longer lasting Ca^{2+} influx response in which extracellular Ca^{2+} enters the cell through the transient receptor potential channel

1, a cation channel, whose expression is induced in thrombin-dependent NF-κB activity (21,26,27).

Thrombin, by activation of PAR-1, induces release of the Gβγ from the $G\alpha_q$ protein, allowing Gβγ to dimerize and recruit phosphatidylinositol 3-kinases (PI3K) to the cell membrane to be activated. The active PI3K then goes on to phosphorylate PIP_2, thereby producing PIP_3, which serves to recruit PH-domain containing proteins like Akt and PDK1 (17). PI3K has been placed as an upstream effector of NF-κB in endothelial cells after PAR-1 stimulation while the role of Akt has remained debatable (18,23).

Canonical NF-κB signaling pathway

Endothelium reacts to injury, infection or other insults by activating an inflammatory transcriptional profile releasing growth factors and cytokines and expressing cell adhesion molecules (CAMs) to aid in the attraction, binding and infiltration of leukocytes (28,29). Thrombin produced at the site of injury induces the expression of cytokines such as interleukin 8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), then CAMs allow leukocytes to adhere to the site of inflammation (15,30). Most of these pro-inflammatory mediators are dependent on the NF-κB signaling pathway for their expression and can also induce NF-κB signaling (Figure 1-1) (29,30). Cytokines produced by the endothelium act in autocrine and paracrine manners to perpetuate the initial inflammatory response, partly by a feed-forward mechanism activating the NF-κB pathway and furthering atherogenesis (29).

The canonical NF-κB pathway is traditionally considered to be responsible for many of the effects of pro-inflammatory stimulation in the cell. Canonical NF-κB signaling can be activated by several different receptor families such as the tumor necrosis factor or IL-1 receptor families which, along with GPCR, contribute to atherogenesis primarily through activation of the NF-κB pathway (29, 32) (Figure 1-2). This means that receptor-

proximal events vary by receptor type but all canonical NF-κB signaling eventually terminates in the nuclear localization of the NF-κB transcription factors. The point at which these diverse receptor signals converge into the common canonical NF-κB pathway is at the I kappa B kinase (IKK) complex (31).

The I kappa B kinase complex

The IKK complex consists of three proteins. IKKγ is the non-catalytic regulator protein bound to two serine/threonine kinase sub-units, IKKα and IKKβ (Figure 1-2) (31,32). IKKγ is one of the two proteins of the complex required for canonical signaling. *Ikkγ*$^{-/-}$ mice are embryonic lethal at E12.5-E13. The mouse embryonic fibroblasts (MEFs) derived from these embryos demonstrate total loss of canonical NF-κB signaling when treated with known NF-κB inducers (33). For example, the IL-1 receptor causes activation of an E3 ligase, TRAF6, in response to pro-inflammatory stimulation of the cell and is a key component of turning these signals into an activated NF-κB pathway. TRAF6 causes K63-linked polyubiquitination of IKKγ in a non-degradative fashion that is necessary for IKK complex kinase activity and it is thought to assist in the recruitment of the IKK kinase. Other receptors capable of inducing NF-κB signaling may use a different E3 ligase, such as TRAF2 in the case of TNFR, to perform the same function (Figure 1-2) (31,34).

The effector of canonical signaling is the IKKβ subunit. This kinase is responsible for the phosphorylation of Inhibitor of κB (IκBα) (Ser$^{32/36}$) of the IκBα complex (Figure 1-2) (32,35). Overexpression of IKKβ alone can drive NF-κB signaling in transfected cells and *Ikkβ*$^{-/-}$ mice show an inhibition of canonical IκBα signaling in the fetus (35). IKKβ needs to be phosphorylated (Ser$^{177/181}$) to become fully activated to then phosphorylate IκBα (31). The search for an IKK kinase able to phosphorylate IKKβ remains unresolved, but of the many kinases proposed, TAK1 now appears to be one of the more likely

candidates (31,32). IKKα appears to mainly play a role in non-canonical signaling and is not redundant with IKKβ in the cell (review of IKKα see Israel, A., 2010) (31).

IκBα complex and transcription factors of the canonical NF-κB pathway

NF-κB transcription factors stand at the ready in the cytoplasm of cells, retained there by their regulator protein, IκB (Figure 1-2). IκB binds to the various NF-κB transcriptional dimers, inhibiting their ability to bind to target genes by concealing their nuclear localization sequence (34). The release of the transcription factors is regulated by activation of IKK complex which phosphorylates IκBα (36). This phosphorylation results in the recruitment of SCD-βTrCP and UBC4/5, E3 and E2 ligases, respectively, which causes K48-linked polyubiquitination at lysines 21 and 22 of IκBα. This leads to expedient targeting of IκBα to the proteasome for degradation, which releases the transcription factors (34,37).

NF-κB transcription factors denote a family of proteins involved in both canonical and non-canonical NF-κB signaling. These proteins include p50, p52, p65 (RelA), c-Rel and RelB and they share a high degree of homology in their N-termini, called the Rel Homology Domain (RHD). The RHD directs the protein's DNA binding, nuclear localization signal, and dimerization properties (38). While these transcription factors can form multiple combinations of homo- and heterodimers in terms of canonical NF-κB signaling, p65/p65 or p65/p50 dimers are most common (39). Once the transcription factors are released, they enter the nucleus and bind to the NF-κB enhancer sequence of GGGRNNYYCC, (N= nonspecific base, R=purine, and Y=pyrimidine) where they help to form the transcription complex (Figure 1-2) (32,38). NF-κB transcription factors regulate the expression of over 100 genes, many of which are pro-inflammatory and pro-atherogenic, such as cell adhesion molecules and cytokines (38).

Immunohistochemistry performed on human atherosclerotic plaques demonstrated increased p65 nuclear localization in most of the cell types in the plaque, including the endothelial cells, in comparison to almost no NF-κB activity in normal tissue. These results were confirmed by electrophoretic mobility shift assay (EMSA) for NF-κB activity from nuclear extracts prepared from these plaques (40). Additional *in vivo* evidence for the role of endothelial-specific NF-κB signaling to atherogenesis comes from two mouse models. An endothelial-specific *Ikkγ*$^{-/-}$ mouse was created to disrupt any NF-κB signaling in this cell type [41]. These *Ikkγ*$^{-/-}$ mice were crossed with a mouse model of atherosclerosis (*ApoE*$^{-/-}$) and fed a high cholesterol diet. After ten weeks on this diet, these mice showed a 30% reduction in plaque size, retardation of progression to advanced plaques and a 40% reduction of T-cells in the plaque when compared to *Ikkγ*$^{+/+}$*/ApoE*$^{-/-}$ mice under the same treatment. To further demonstrate the role of NF-κB in the endothelium, an additional transgenic mouse model was created expressing a dominant negative IκBα in the endothelium [41]. These mice were once again crossed to the *ApoE*$^{-/-}$ mice and placed on a high cholesterol diet for ten weeks. The *IκBα dominant negative/ApoE*$^{-/-}$ mice showed a 60% reduction in plaque size and a significant reduction in plaque progression when compared to *ApoE*$^{-/-}$ with functional NF-κB signaling in their endothelium. The endothelium of the *IκBα dominant negative/ApoE*$^{-/-}$ mice were also almost completely free of most cytokine, chemokine and CAM (VCAM-1, ICAM-1) expression (41). Taken together, it appears that the NF-κB pathway, particularly in the endothelium, is a major contributor to atherogenesis (Figure 1-1).

The endothelium: a vital component of vascular health

A healthy vascular system functions as the efficient network by which the body moves nutrients and oxygen into and biological byproducts out of all the body's tissues (42). The endothelium is a flat and unbroken, single-cell-thick, selectively permeable

lining of the vessel separating the underlying vascular smooth muscle cells from the blood in the vessel lumen (Figure 1-3) (5,43). Endothelial cell layers represent the meeting of multiple cell types: the circulating blood cells, endothelium and vascular smooth muscle cells. This also places endothelial cells at the center of a diverse array of signals to its receptors and in a position prone to overstimulation and dysfunction (Figure 1-1, 1-3) (44,45). Once thought to be a passive barrier, endothelial cells are now known to be at the center of homeostasis in the circulatory system, regulating vascular tone, angiogenesis, perfusion, thrombogenesis and inflammatory response (5,45,46). Atherogenesis is a slowly developing inflammatory disease with the endothelium at its epicenter, and can be separated into several broadly defined stages: endothelial dysfunction, fatty streak or plaque initiation, intermediate plaque and, lastly, vulnerable/advanced plaque.

Inflammation and endothelial dysfunction

Endothelial dysfunction and atherosclerosis share many common risk factors such as hypercholesterolemia, smoking, hypertension and diabetes. Treatment for endothelial dysfunction, like that of atherosclerosis, is to reduce hypertension and lower lipid levels, along with diet and exercise (5,45). Endothelial dysfunction is now considered the earliest biological condition leading to atherosclerosis and is broadly defined as the activation of the endothelium to the harm of the organism (5,47). This activation, often attributed to high levels of oxidized low-density lipids (LDL), causes a prolonged inflammatory response that significantly alters endothelial function via the expression of selectins, CAMs, cytokines, and by altering vascular tone (Figure 1-3) (48).

LDL can rapidly invade through the functional endothelium and become confined in the extracellular matrix protein rich space below the endothelium, known as the intima,

through association with proteins such as proteoglycans (49). Once deposited, the aggregating lipids are oxidized by free radicals or enzymatically modified, releasing bioactive phospholipids. These phospholipids can act as ligands for several receptors, helping to initiate the inflammatory response (1,50,51). Macrophages that are residing in the intima engulf the lipids as part of the innate immune response, but cannot metabolize the lipids and become foam cells (44). This model of LDL-induced inflammation of the endothelial cell layer has been proven in both *in vitro* and *in vivo* models of atherosclerosis (Figure 1-1, 1-3) (51).

Polymorphonuclear neutrophils and leukocytes are attracted to areas of inflammation by NF-κB-regulated chemoattractants such as MCP-1 and IL-8 produced by the endothelial cells to contribute to atherogensis. Once there, the leukocytes need to be honed and retained by the activated endothelial cells to infiltrate through the endothelium to the source of inflammation (29,52). This is done through a set of NF-κB responsive inflammatory genes known as cell adhesion molecules that facilitate the binding between leukocytes and the endothelium. The ability of the dysfunctional endothelium to attract leukocytes is a key step early on and throughout atherogenesis (Figure 1-3).

Leukocyte adhesion a key result of endothelial dysfunction

Rolling action is due to a weak tethering of leukocytes to the endothelium by selectin glycoproteins on both the leukocytes and endothelial cells (Figure 1-3). Leukocytes constitutively express L-selectin (30). The endothelium controls rolling through the expression of the L-selectin binding partners, MadCAM-1, Gly-CAM-1 and CD-34 and the expression of its own P- and E-selectins in response to inflammatory mediators such as thrombin (16,29,30). Thrombin can induce P-selectins which are responsible for the early response to inflammation, since they are stored in Weilbel-

Palade bodies in the endothelial cell and can quickly be moved to the cell surface. At the same time, thrombin-dependent NF-κB signaling induces E-selectin transcription and translation for the longer lasting late rolling response (16,30). This rolling action by leukocytes allows the cell to become further activated by the cytokine-rich environment and to firmly adhere to the endothelium (29).

Firm adhesion: the role of ICAM-1 and VCAM-1

For firm adhesion, the leukocyte integrins CD11a/CD18 and CD11b/CD18 β_2 bind to members of the immunoglobulin superfamily of adhesion molecules (ICAM-1, VCAM-1) on the endothelium (Figure 1-3) (29). Under non-inflammatory conditions, ICAM-1 expression is low on the endothelium surface (30). Pro-inflammatory stimuli such as thrombin activation of PAR-1 cause a dramatic increase in ICAM-1 expression on the endothelium. This increase in expression level is achieved by an early type I phase and slower type II phase induction (53,54). Type I occurs within thirty minutes of stimulation of endothelial cells with thrombin and is insensitive to cycloheximide, a protein synthesis inhibitor, indicating this early ICAM-1 is kept in an inactive state until thrombin induction (53,55).

The later type II induction is cycloheximide-sensitive and results in an increase in *ICAM-1* mRNA levels within two hours of thrombin treatment. ICAM-1 protein levels stay elevated for twenty-four hours after treatment (54). Type II induction of the *ICAM-1* gene is dependent on NF-κB signaling in endothelial cells (18,30,56). Disruption of the NF-κB pathway downstream of PAR-1 through the use of a chemical inhibitor or the use of RNA interference (RNAi) to knock down key proteins in the NF-κB pathway blocks thrombin-dependent ICAM-1 expression (54,56).

The *ICAM-1* promoter contains four κB enhancer elements at base pair -533 and -223 and intronic κB enhancer sites in intron 1 at +70bp and +611bp from the

transcriptional start site (54,57). Interestingly, it was discovered that thrombin-dependent NF-κB activation of *ICAM-1* only required the use of two out of the four possible NF-κB binding sites, -233bp and +70 sites. A series of 5' deletions of the *ICAM-1* promoter in a reporter construct identified the -233bp κB enhancer site as essential to its transcriptional activation. In two separate series of experiments, chromatin immunoprecipitation (ChIP) demonstrated the necessity of the +70 site in thrombin-induced endothelial cell cultures. Both the -233 and +70bp binding sites are needed to achieve maximum induction of the ICAM-1 expression in endothelial cells (54,57).

Using EMSA, thrombin induced protein binding to oligonucleotides based on *ICAM-1*'s NF-κB binding sites. Supershift EMSA were performed to identify the combination of NF-κB transcription factors that were binding to the *ICAM-1* promoter. For both the -233 and +70bp binding sites, the only antibody to produce a supershift was the anti-p65 antibody (54,57). Using siRNA to knock down p65 in the cells, both thrombin's and TNFα's abilities to cause an increase in ICAM-1 protein levels in endothelial cells were severely inhibited (57).

Thrombin-dependent ICAM-1 expression in endothelial cell culture induces the adhesion of polymorphonuclear leukocytes, recreating key steps in atherogenesis. This response of the endothelium was blocked by pretreating the endothelial cells with an ICAM-1 antibody before incubation with the polymorphonuclear leukocytes (54). The NF-κB pathway is vital for leukocyte/endothelium adhesion and a blockade of NF-κB caused a 75% reduction in the number of polymorphonuclear leukocytes that adhered to the endothelial cells (54,57). The binding of polymorphonuclear leukocytes to ICAM-1 causes ICAM-1 to stimulate several cell signaling events leading to gene expression and cytoskeletal rearrangements of the endothelial cells that aid in transendothelial migration of the leukocytes into intimal space (22).

VCAM-1 binds to mononuclear leukocytes, particularly monocytes, for firm adhesion. When expressed on the inflamed endothelium, this aids in the transendothelial migration of the mononuclear leukocytes into intimal space for plaque development (Figure 1-3) (30). Thrombin activation of PAR-1 in endothelial cells mediates an increase in *VCAM-1* transcription and translation after four hours of treatment (55,58). When treated with thrombin or TRAP-6, the endothelial cells caused an ~3.5 fold increase in *VCAM-1* promoter activity with the use of a reporter construct, and thrombin-induced expression of VCAM-1 in endothelial cells is NF-κB dependent (58).

The *VCAM-1* promoter contains two κB enhancer sites at -75 and -65bp from the transcriptional start site. When either of these sites in the *VCAM-1* promoter reporter was mutated, promoter activity was lost (58). Thrombin induction of *VCAM-1* transcription was successfully blocked by the overexpression of a dominant negative IκBα or knockdown of the p65 transcription factor in endothelial cells (55). Thrombin only induced the binding of p65 to the *VCAM-1* promoter-based oligonucleotide as measured by supershift EMSA (55,58).

When endothelial cell cultures were stimulated with thrombin, monocytes subsequently added to the culture were able to adhere. This action could be blocked by pretreatment with a VCAM-1 antibody but not by an ICAM-1 or E-selectin antibody (55). The use of a dominant negative IκBα or knockdown of p65 caused a 73-83% reduction in the number of monocytes that were able to bind (55). VCAM-1 is an important mediator of plaque formation. Mice were genetically engineered to not express the 4th immunoglobulin domain of VCAM-1, which is important to the binding of integrins on the monocytes to initiate adhesion. These mice were crossed with the atherosclerosis mouse model, *ApoE*$^{-/-}$. The decreased expression of VCAM-1 in these mice resulted in almost no monocyte adhesion to the endothelium and an 84% reduction in plaque formation (59).

Fatty streak

Fatty streak is a waxy yellow deposit in the subendothelial space that is the pathological outcome of endothelial dysfunction. The fatty streak is formed by the accumulation of lipid-laden macrophage, foam cells, recruited during endothelial dysfunction into the intimal space (Figure 1-3). While these fatty streaks are recognized as an early step in plaque formation, they have the potential to involute and the negative health impact can be averted (49). Foam cells have an active role in plaque progression by further feeding the inflammatory response in endothelial cells. This leads to the recruitment of even more monocytes by increasing the expression of integrin, adhesion molecules and cytokines, and it also drives the maturation of monocytes into macrophages (50,51).

Intermediate lesion

As the plaque progresses, macrophages proliferate and release multiple pro-inflammatory factors known to mediate NF-κB cell signaling pathways in the surrounding cells. The expression of cytokines and CAMs attract T-lymphocytes to the plaque where they become T-helper cells. The T-helper cells produce additional cytokines and chemokines, attracting even more leukocytes to the maturing plaque (Figure 1-3) (49,50).

Importantly, vascular smooth muscle cells (VSMCs) then start to invade the intima in response to increasing concentration of growth factors released from the plaque (50,52). VSMCs contribute to plaque size by invasion, proliferation and production of extracellular matrix proteins. These extracellular matrix proteins form the protective fibrous cap that separates inflammation within the intima and the endothelial cell layer (1,49,50). While the plaque is growing, the vessel undergoes compensatory

remodeling to try to maintain its lumen size, causing an overall increase in the outside diameter of the vessel (49,51).

Advanced / vulnerable lesion

As the plaque grows, it becomes increasingly less stable and more prone to rupture due to a multitude of factors (Figure 1-3). The microvascular outgrowth from the vessel wall, which is thought to be promoted by thrombin, contributes to the instability of the plaque. These microvessels ease the leukocyte and nutrient entry and cause internal hemorrhaging due to the weakness of the vessel. This hemorrhaging then causes local increases in thrombin levels, leading to clots and activating the PAR-1 receptor (16,49). Foam cells die from their inability to metabolize the oxidized LDL, causing a necrotic core within the plaque. The increase of cellular debris and crystalline cholesterol continues to bring more leukocytes to weaken the plaque (Figure 1-3) (1,51). The plaque is especially unstable where the plaque meets the normal vessel. This frail area is known to release pro-thrombogenic material such as tissue factors and stimulate thrombus formation in the lumen. This thrombosis can lead to clots and blocked arteries and can cause heart attack (1).

Rupture also involves the breakdown of the extracellular matrix that makes up the protective fibrous cap. Thrombin and other pro-inflammatory factors stimulate endothelial cell, T-lymphocyte and macrophage expression of extracellular proteases, such as matrix metalloproteinase family members, which break down the fibrous cap (1,15,50). T-lymphocytes uniquely contribute to plaque instability via the production of IFN-γ which then down regulates VSMC extracellular matrix production (49). Ultimately, plaque growth and healing after rupture narrow the vessel lumen to the point at which the compensatory remodeling cannot maintain adequate blood flow to the tissue (1,51).

Connecting the pieces: CARMA3/Bcl10/MALT1 (CBM) complex in GPCR-dependent NF-κB signaling

CARD-containing MAGUK-1 (CARMA1) is a member of a superfamily of scaffolding proteins that use multiple protein interaction domains that gather receptors and cytosolic signaling proteins at the cell membrane (Figure 1-4) (60,61). MAGUK members contain three defining interaction domains: the PSD-95/Dlg/ZO-1 homologous (PDZ) domain, the Src-homology (SH3) domain, and the guanylate kinase (GUK)-like domain. CARMA1 is expressed solely in lymphocytes and it diverges from other MAGUK proteins by having coiled-coil and caspase recruitment domains (CARD) (Figure 1-5) (62).

In the adaptive immune system, foreign antigens are recognized by lymphocytes via the antigen receptors (63). Activated receptors initiate cell signaling through several signaling pathways, but stimulation of NF-κB is critical (64). CARMA1 is an indispensable component of the antigen-dependent NF-κB signaling pathway in lymphocytes (Figure 1-4). This has been demonstrated through the use of multiple molecular tools including a dominant negative CARMA1, CARMA1 siRNA knockdown and CARMA1-defective T-cell cultures created by chemical mutagenesis, each of which resulted in the loss of antigen-dependent NF-κB activity (65-67). Multiple groups have generated *Carma1*$^{-/-}$ mouse strains, which displayed lymphocyte deficiencies associated with the loss of antigen-dependent NF-κB signaling (68-71).

The stimulated T-cell receptor recruits 3-phosphoinositide-dependent kinase 1 (PDK1) via production of PIP_3 at the cell membrane by stimulated PI3K. PDK1 then serves to scaffold for both activated PKCθ and CARMA1 (Figure 1-4). The ability of PDK1 to interact with CARMA1 characterizes a newly discovered role for this protein (72). PDK1 is known to phosphorylate PKC in its activating loop, allowing it to respond to Ca^{2+} and/or DAG (73-75). PDK1 binding to both PKC and CARMA1 allows PKCθ to

phosphorylate CARMA1 between the coiled-coil and PDZ domains, resulting in the exposure of the CARD domain (76,77). Phosphorylated CARMA1 acts as a molecular bridge by recruiting Bcl10, which acts as an adaptor protein to recruit MALT1. MALT1 recruits the proteins needed to activate the IKK complex (63). Finally, the IKK complex is recruited and can thereby be activated by the fully assembled CBM complex to then activate the NF-κB signaling pathway (Figure 1-4, 1-5) (78-80). There are notable parallels between the antigen- and GPCR-dependent NF-κB signaling pathways. The scrutiny of this lymphocyte pathway has provided significant understandings into the molecular mechanisms by which thrombin might activate the NF-κB signaling pathway in endothelial cells (Figure 1-6).

CARMA3

Until recently, GPCR-dependent canonical NF-κB signaling was not fully understood. However, GPCR-dependent canonical NF-κB signaling was shown to share a number of similarities with antigen receptor-dependent CARMA1/Bcl10/MALT1 complex-induced canonical NF-κB signaling. The most notable of these similarities is that PKC and IKK complex activation are needed for both receptor types (Figure 1-6). This led to speculation that CARMA3 might be involved in a GPCR-dependent NF-κB signaling pathway in a manner similar to CARMA1 in antigen receptor-dependent NF-κB. The innovative work to discover that the CARMA3/Bcl10/MALT1 complex was needed for GPCR-induced NF-κB signaling was initially done with angiotensin II type I receptor (AGTR1) and lysophosphatidic acid (LPA) receptor (81,82).

CARMA3 is a member of the CARMA subfamily of MAGUK proteins (71). CARMA1 and CARMA3 proteins are similar with the CARD and the coiled-coil domains having 58% and 50% sequence homology, respectively, while the PDZ, SH3 and GUK domains share ~20-30% sequence homology (Figure 1-5) (83). The similarities between

CARMA3 and CARMA1 are illustrated by the fact that CARMA3 can rescue antigen-induced NF-κB in CARMA1-deficient T-cells (76). CARMA3 shows an expression pattern that is much broader than CARMA1 and is expressed in vascular tissue (62,83,84). CARMA3, like CARMA1, functions as a scaffold protein to recruit multiple other proteins needed for signaling to the receptor and does not itself have any known transcriptional or enzymatic properties (84).

Overexpression of CARMA3 alone induces canonical NF-κB activity, which is blocked by co-expression of a dominant negative IκBα (62,83). Deletion of the CARD domain of CARMA3 fully blocked the induction of canonical NF-κB signaling, while deletion of PDZ and SH3 only partially blocked the induction. Using a mammalian two-hybrid screen, it was determined that the CARD domain of CARMA3 specifically interacted with the CARD domain of a known NF-κB effector protein, Bcl10 (see below) (83). This interaction was confirmed by the ability of CARMA3 to co-immunoprecipitate with Bcl10, which could be blocked by the overexpression of either CARD domain (Figure 1-5) (83). Overexpression of CARMA3 in *Bcl10*$^{-/-}$ MEFs blocked CARMA3's ability to induce NF-κB, further showing that CARMA3 and Bcl10 work in the same pathway to activate canonical NF-κB signaling (62).

PMA/ionomycin treatment of cells is known to activate NF-κB in a PKC-dependent manner. When cells over-expressing a dominant negative form of CARMA3 were treated with PMA/ionomycin, NF-κB induction was blocked. While this did not identify the specific PKC involved, it does place PKC upstream of CARMA3 (62). This is consistent with data that downstream of the antigen receptor, CARMA1 binds to and is phosphorylated by a PKC which results in conformational changes, allowing it to bind to Bcl10 and induce canonical NF-κB signaling (84). Further, a point mutation to Ala at Ser^{520} in CARMA3, an area homologous to a PKC phosphorylation site in CARMA1, blocked CARMA3-dependent NF-κB activation (76).

MEFs derived from *Carma3*$^{-/-}$ mice were treated with LPA to gauge whether canonical NF-κB signaling by this GPCR was affected by the loss of Carma3. Results showed that LPA receptor-induced NF-κB transcription factor nuclear localization was inhibited in the absence of Carma3 while TNFα-dependent NF-κB signaling remained unaffected. Carma3 was also necessary for the LPA-induced phosphorylation of IκBα, but interestingly not for the phosphorylation of IKKα/β, thus indicating a possible role for a parallel pathway (81).

In a series of independent experiments performed at the same time, another research group explored whether a different GPCR, AGTR1, needed CARMA3 for NF-κB activation. Using exogenously expressed AGTR1 in cell culture, Ang II-dependent NF-κB induction was inhibited by shRNA knockdown or expression of a dominant negative mutant of CARMA3 (82). Overexpression of a dominant negative CARMA3 was able to block K63-linked polyubiquitination of IKKγ (82). Several PKCs (α, θ, and ζ) have now been implicated in GPCR CARMA3-dependent canonical NF-κB activation, although any direct binding or phosphorylation to CARMA3 remains to be investigated (Figure 1-6) (85,86).

Traditionally, β-arrestin 1 and 2 are known for their role in down regulating activated GPCR through endocytosis of the receptor, leading to recycling or degradation. Activated GPCRs are phosphorylated by various G-protein receptor kinases (GRKs), which then recruit arrestins to the receptors. This helps to uncouple the G-proteins from the receptors and assists in receptor internalization through clathrin-coated pits. More recently, arrestins have become known to be scaffold proteins that facilitate the recruitment and activation of a number of signaling proteins to activated GPCRs (87).

Recently, β-arrestin 2 has been shown to recruit Carma3 to the LPA receptor after stimulation. Knockout MEFs for β-arrestin 1 and β-arrestin 2 showed that a loss of β-arrestin 2, but not β-arrestin 1, inhibited NF-κB activation after LPA treatment. β-

arrestin $2^{-/-}$ MEFs showed blockade of IκBα phosphorylation and transcription factor nuclear localization but no change in IKK phosphorylation, results that are similar to the *Carma3*$^{-/-}$ MEFs (see above). It was then determined that β-arrestin 2 bound to Carma3 through its CARD domain. In co-immunoprecipitation experiments in transfected HEK293T cells, Carma3 could only pull down the LPA receptor when β-arrestin 2 was co-transfected to act as a bridge (88).

Bcl10

B-cell lymphoma/leukemia 10 (Bcl10) is also a scaffold protein that needs to oligomerize to activate NF-κB signaling. Bcl10 acts as an adaptor between the upstream scaffold protein CARMA3 and MALT1. Unlike CARMA1 or CARMA3, Bcl10 is expressed in wide variety of tissues types including both lymphocytes and endothelial cells (82). Bcl10 has an N-terminal CARD domain that can bind to CARMA3; this interaction is necessary for GPCR-dependent NF-κB signaling along with Bcl10's Ser/Thr-rich domain at the C-terminus (Figure 1-5) (84). Deletion of the CARD domain of either CARMA3 or Bcl10 prevents their ability to bind to each other and inhibits CARMA3/Bcl10-dependent NF-κB pathway induction (62,83,89).

Bcl10 was first described as an oncogene in MALT lymphoma where it is overexpressed due to chromosomal translocation, and causes constitutive NF-κB activity (90). This can be reproduced in cell culture by overexpression of Bcl10 which activates the NF-κB pathway and can be augmented with co-expression of CARMA3 or MALT1 (62,91). Bcl10, like CARMA3, is necessary for several GPCRs to induce an NF-κB response (81,82,86). *Bcl10*$^{-/-}$ MEFs, when treated with LPA, lose the ability to induce canonical phosphorylation of IκBα, similarly to the *Carma3*$^{-/-}$ MEFs (81). Likewise, angiotensin II treatment of cell cultures expressing a dominant negative Bcl10 mutant, or

of *Bcl10*$^{-/-}$ primary hepatocytes, resulted in a loss of Ang II-dependent NF-κB activity (82).

MALT1

Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), as the names implies, first came to the attention of researchers as part of an oncogenic fusion protein in MALT lymphoma. The t(11;18)(q21;21) chromosomal translocation results in the formation of the cIAP2/MALT1 fusion oncoprotein which auto-oligomerizes to constitutively stimulate the NF-κB signaling pathway. This fusion oncoprotein leads to a treatment-resistant form of MALT lymphoma (90). MALT1 is expressed in a wide variety of tissues including lymphocyte and endothelial cells (82).

Unlike the other members of the CARMA3/Bcl10/MALT1 (CBM) complex, MALT1 overexpression alone cannot drive canonical NF-κB signaling due to its inability to auto-oligomerize, but its overexpression can augment the NF-κB signaling by Bcl10 (91). This ability to augment increases even more when all three members of the CBM complex are overexpressed together (62). Through a series of co-immunoprecipitation experiments, it was determined that MALT1's Ig-like domains were needed to bind to regions critical to Bcl10-dependent NF-κB activity (AA 107-119) (Figure 1-5). MALT1 failed to augment Bcl10-driven NF-κB activity when this twelve amino acid stretch of Bcl10 needed to bind to MALT1 was deleted. Binding to Bcl10 initiates MALT1 oligomerization, which is sufficient for NF-κB signaling (Figure 1-5) (91).

CBM complex and IKK complex

Precisely how MALT1 influences the IKK complex to activate NF-κB is still an evolving, multi-layered question which has been predominantly explored in the context of antigen receptors. Early hints came from working out the interaction between MALT1

and Bcl10. Despite its ability to bind to Bcl10, a truncated form of MALT1 missing the C-terminus beyond the Ig-like domain, was unable to activate the NF-κB pathway, demonstrating that the CARMA/Bcl10/MALT1 (CBM) complex is dependent on a fully functional MALT1 to activate the NF-κB pathway (91). It now appears that MALT1 serves to recruit proteins for the K63-linked polyubiquitination of IKKγ, a necessary step for activation of the IKK complex (Figure 1-4, 1-6) (92,93).

MALT1 contains two binding sites for TRAF6, an E3 ubiquitin-protein ligase. These sites, when mutated, result in a loss of CBM complex-dependent NF-κB signaling. TRAF6 binding to MALT1 allows TRAF6 to oligomerize and recruit its ubiquitin conjugating enzyme, Ubc13, and its catalytically inactive cofactor, Uev1A, to the CBM complex. TRAF6, once bound to MALT1, then causes K63 polyubiquitination of itself, MALT1and Bcl10 (94,95). Point mutations of the eleven C-terminal lysines to arginine showed a nearly complete loss of ubiquitination of MALT1 and an ~80% fold reduction in CBM-dependent NF-κB activity. This was despite the ability of the CBM complex to still form in response to stimulation and recruit TRAF6 (92). Similarly, *Traf6*$^{-/-}$ MEFs that were treated with LPA were unable to activate the NF-κB pathway as was seen in LPA treated *β-arrestin 2*$^{-/-}$, *CARMA3*$^{-/-}$ and *Bcl10*$^{-/-}$ MEFs (81).

IKKγ contains a ubiquitin binding domain that preferably binds K63 ubiquitination like that produced by active TRAF6 (96). This domain allows IKKγ to be recruited to the CBM complex in response to cellular stimuli through the ubiquitination chains on MALT1 that are necessary for NF-κB activation. A point mutation in the ubiquitin binding domain of IKKγ severely reduces its ability to bind to MALT1 and blocks the ability of the CBM complex to cause the degradation of IκBα (92). Lastly, once IKKγ is recruited to the CBM complex, TRAF6 then ubiquitinates IKKγ, which is needed for activation of the IKK complex to phosphorylate the IκBα complex (Figure 1-4, 1-6) (95). The dependence of GPCRs on the formation of the CBM complex for the activation of the NF-κB pathway

positions CARMA3, Bcl10 and MALT1 as critical mediators of endothelial dysfunction and atherosclerosis.

Conclusion

Cardiovascular disease stands as a pressing health issue to the world. It is a complex and slowly progressing disease that is often associated with a chronic inflammatory response to oxidized LDL by multiple cells in the vessel wall. Given the endothelium's position in the vessel, its functional health has a great effect on the initiation and progression of atherosclerotic plaques. The NF-κB signaling pathway in endothelial cells appears to underlie much of the endothelial dysfunction that occurs in atherosclerosis. PAR-1, a GPCR, is known to activate, through an unclear mechanism, NF-κB in endothelial cells and inhibition of its antagonist thrombin has benefits in *in vivo* studies of atherosclerosis. The CARMA3/BCL10/MALT1 signaling complex is known to activate NF-κB signaling downstream of several GPCRs, making it a possible culprit in thrombin-induced endothelial dysfunction.

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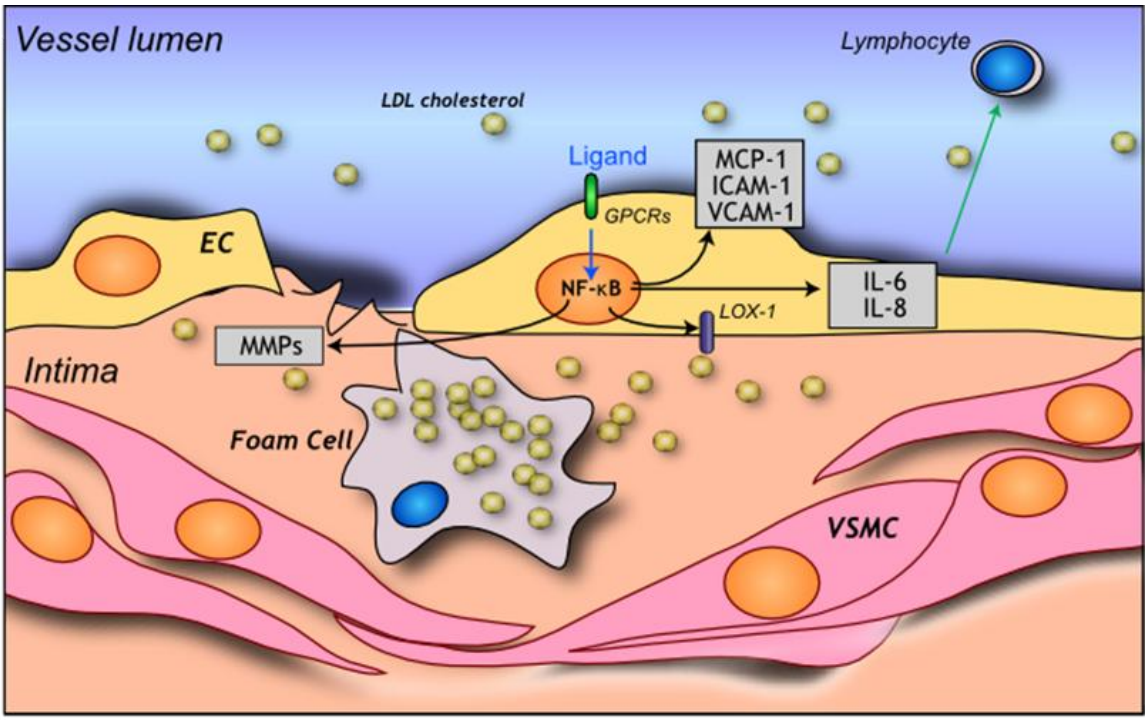

Figure 1-1. The role of endothelial dysfunction in plaque formation is a complex process involving many molecular influences and the interactions of multiple cell types. Atherosclerosis is a chronic inflammation of the vasculature with the transcription factor NF-κB as a major driving force in the disease. Endothelial dysfunction is the earliest biological condition leading to atherosclerosis and is broadly defined as the stimulation of the endothelium to the detriment of the organism. The stimulation of high levels of oxidized low-density lipids causes a sustained inflammatory response that significantly alters endothelial function via the expression of selectins, lectin-type oxidized LDL receptor 1 (LOX-1), CAMs, and cytokines as well as by altering vascular tone. LDL can rapidly invade through the functional endothelium and become restricted in the extracellular matrix protein rich space beneath the endothelium, known as the intima, through association with proteins such as proteoglycans. Once deposited, the aggregating lipids are oxidized by free radicals releasing bioactive phospholipids. Macrophages that are residing in the intima consume the lipids as part of the innate immune response, but cannot metabolize the lipids and become foam cells.

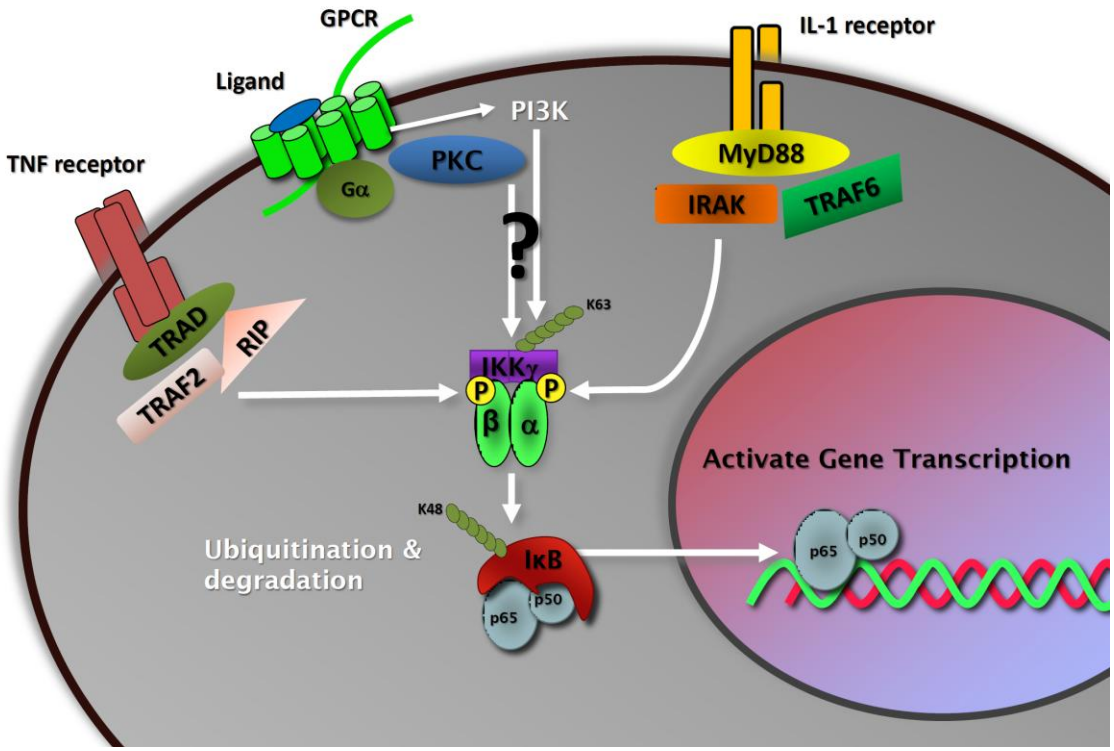

Figure 1-2. The canonical NF-κB pathway is activated by multiple receptor families and is responsible for many of the effects of pro-inflammatory stimulation in the cell. Canonical NF-κB signaling can be activated by several different receptor families such as the tumor necrosis factor receptor family. This means that receptor-proximal events vary by receptor type but all converge at the IKK complex. IKKγ is the non-catalytic regulator protein bound to two kinase sub-units, IKKα and IKKβ. IKKγ is one of the two proteins of the complex required for canonical signaling. The effector of canonical signaling is the IKKβ subunit, and is responsible for the phosphorylation of the IκBα complex. IKKβ needs to be phosphorylated to become fully activated to then phosphorylate IκBα. This phosphorylation results in the recruitment of ligases that are required for polyubiquitination of the IκBα. This leads to expedient targeting of IκBα to the proteasome for degradation, which releases the transcription factors p50 and p65 to the nucleus.

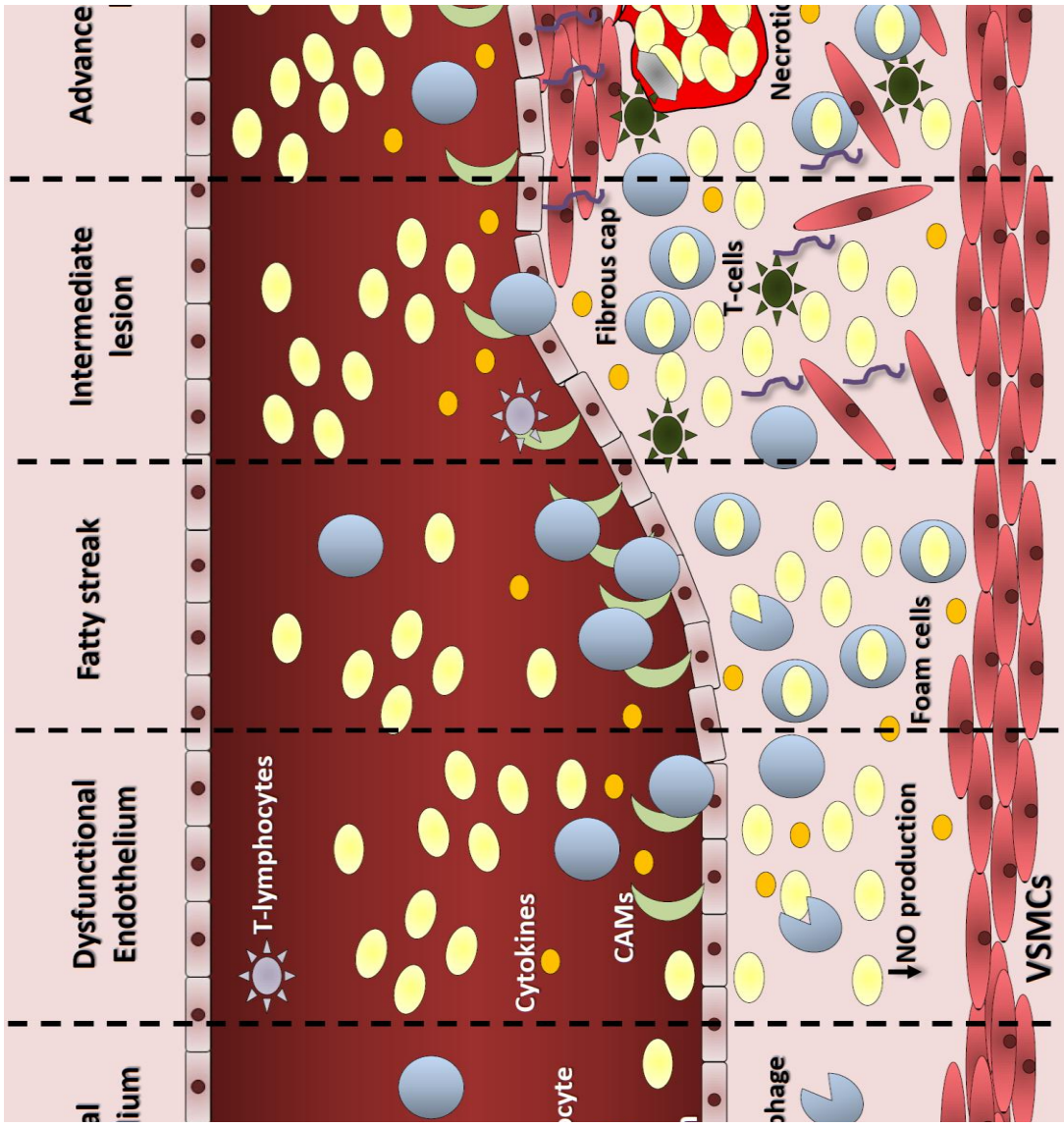

Figure 1-3. The role of the endothelium through the stages of plaque formation. It should be noted that while endothelial dysfunction is the first stage of plaque formation, it continues through all other stages as well. See text for a detailed description of each stage. This figure is modified from Sanz, J. & Z. A. Fayad, 2008.

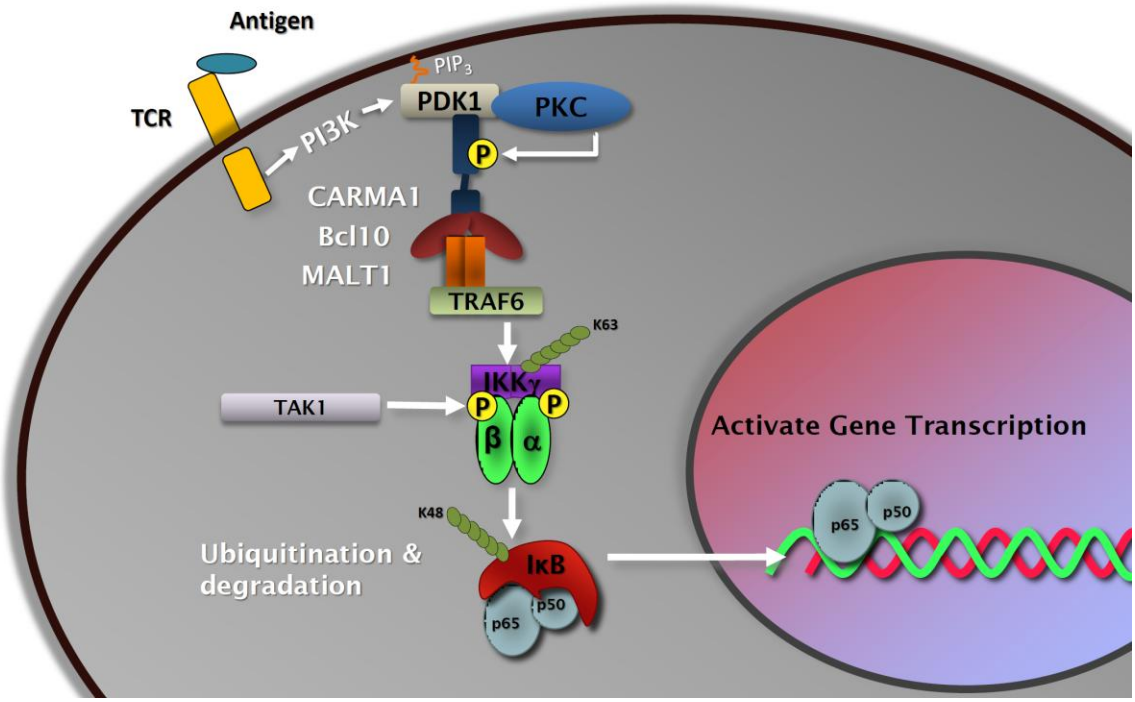

Figure 1-4. T-cell receptor signals through CARMA1/Bcl10/MALT1 complex for the activation of the NF-κB pathway. In the adaptive immune system, foreign antigens are recognized by lymphocytes via the T-cell receptors. CARMA1 is a necessary component of the antigen-dependent NF-κB signaling pathway in lymphocytes. The stimulated T-cell receptor recruits PDK1 via production of PIP_3 at the cell membrane by activation of PI3K. PDK1 then serves to scaffold for both activated PKCθ and CARMA1. PDK1 is known to phosphorylate PKC in its activating loop, allowing it to respond to Ca^{2+} and/or DAG. PDK1 binding to both PKC and CARMA1 allows PKCθ to phosphorylate CARMA1 between the coiled-coil and PDZ domains, resulting in the exposure of the CARD domain. Phosphorylated CARMA1 acts as a molecular bridge by recruiting Bcl10, which acts as an adaptor protein to recruit MALT1. MALT1 recruits other proteins needed to activate the IKK complex to the immunological synapse. The IKK complex is recruited and can thereby be induced by the fully assembled CBM complex to activate the NF-κB signaling pathway. For an review of CARMA1 in CBM complex signaling, please see Rawlings, D.J. and *et al.* 2006.

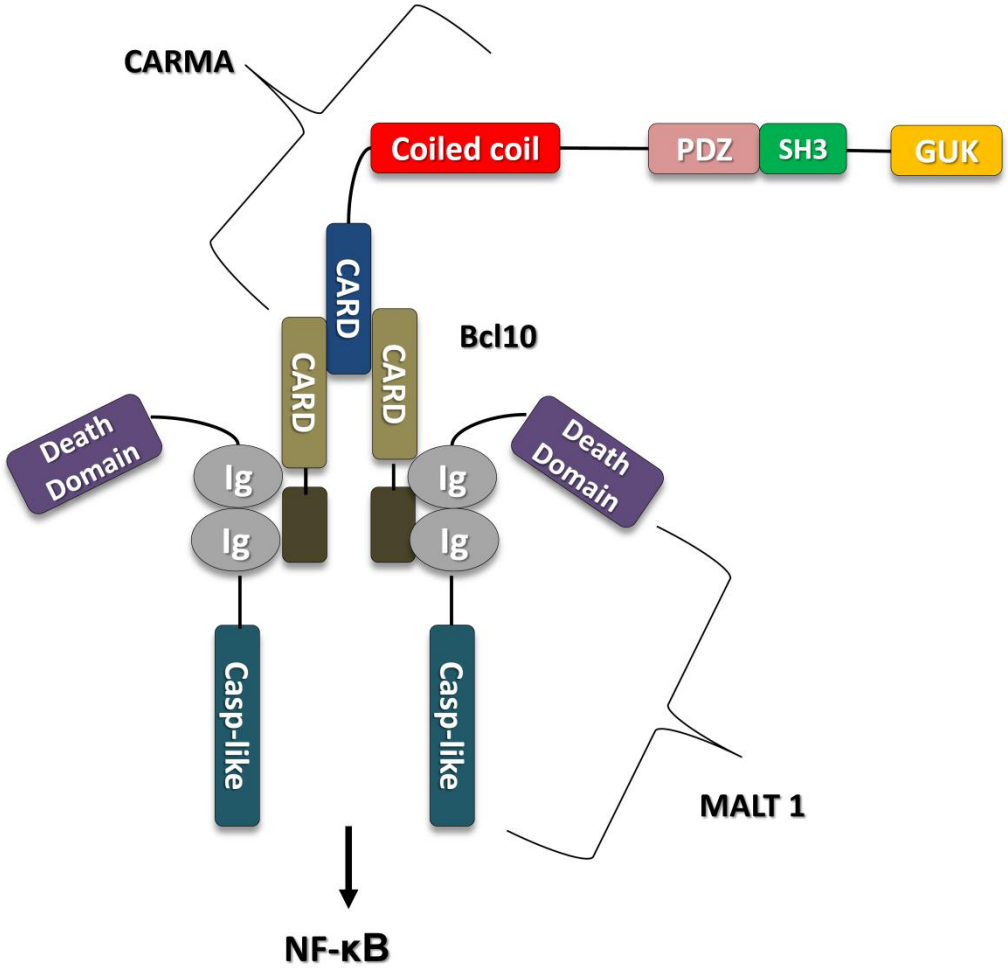

Figure 1-5.The protein-protein interactions of CBM complex-dependent NF-κB activation. CARMA1 and CARMA3 (collectedly referred to as CARMA) is a member of a superfamily of scaffolding proteins that use multiple protein interaction domains that gather receptors and cytosolic signaling proteins at the cell membrane. MAGUK members contain three defining interaction domains: the PSD-95/Dlg/ZO-1 homologous (PDZ) domain, the Src-homology (SH3) domain, and the guanylate kinase (GUK)-like domain. Bcl10 is also a scaffold protein that needs to oligomerize to activate NF-κB signaling. Bcl10 acts as an adaptor between the upstream scaffold protein CARMA and MALT1. Bcl10 has an N-terminus CARD domain that can bind to CARMA; this interaction is necessary for cell receptor-dependent NF-κB signaling along with Bcl10's Ser/Thr-rich domain at the C-terminus. MALT1's Ig-like domains are needed to bind to regions critical to Bcl10-dependent NF-κB activity (AA107-119). MALT1 serves to recruit the proteins necessary for NF-κB signaling. This figure is modified from Lucas, P. *et al*., 2004.

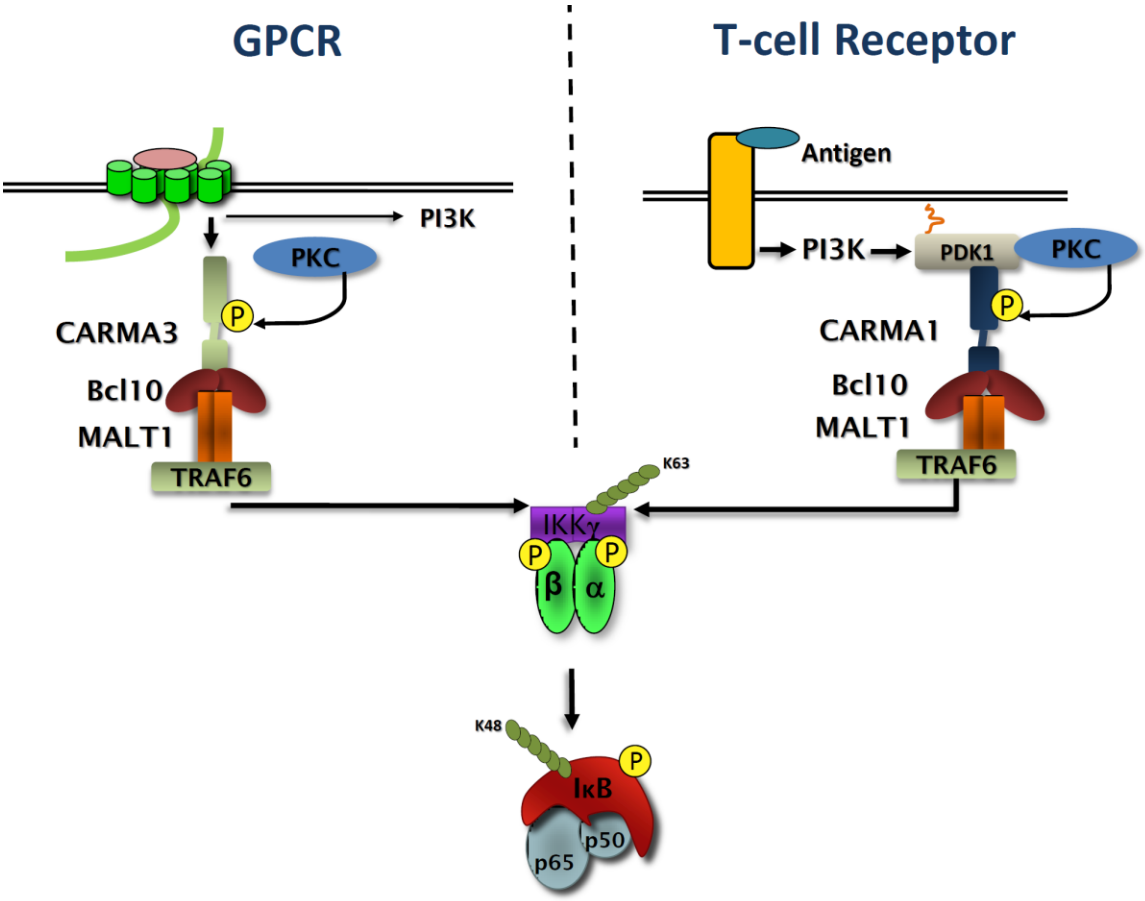

Figure 1-6. A comparison between T-cell receptor/CBM complex activation versus GPCR/CBM complex activation. Until recently, GPCR-dependent canonical NF-κB signaling was not fully understood. However, GPCR-dependent canonical NF-κB signaling was shown to share a number of similarities with antigen receptor-dependent CARMA1/Bcl10/MALT1 complex-induced canonical NF-κB signaling. The most notable of these similarities is that PKC and IKK complex activation are needed for both receptor types. This led to speculation that the CBM complex might be involved in GPCR-dependent NF-κB signaling.

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Chapter II

Thrombin-dependent NF-κB Activation and Monocyte/Endothelial Adhesion are Mediated by the CARMA3/Bcl10/MALT1 Complex

Abstract

Thrombin is a potent modulator of endothelial function, and through stimulation of NF-κB, induces endothelial expression of ICAM-1 and VCAM-1. These cell-surface adhesion molecules recruit inflammatory cells to the vessel wall and thereby participate in the development of atherosclerosis, which is increasingly recognized as an inflammatory condition. The principal receptor for thrombin on endothelial cells is PAR-1, a member of the GPCR superfamily. While it is known that PAR-1 signaling to NF-κB depends on initial PKC activation, the subsequent steps leading to stimulation of the canonical NF-κB machinery have remained unclear. Here, we demonstrate that a complex of proteins containing CARMA3, Bcl10, and MALT1 links PAR-1 activation to stimulation of the IKK complex. IKK in turn phosphorylates IκBα, leading to its degradation and the release of active NF-κB. Further, we find that although this CARMA3/Bcl10/MALT1 complex shares features with a CARMA1-containing complex found in lymphocytes, there are significant differences in how the complexes communicate with their cognate receptors. Specifically, while the CARMA1-containing lymphocyte complex relies on PDK1 for assembly and activation, the CARMA3-containing endothelial complex functions completely independent of PDK1, and instead

relies on β-arrestin 2 for assembly. Finally, we show that thrombin-dependent adhesion of monocytes to endothelial cells requires an intact endothelial CARMA3/Bcl10/MALT1 complex, underscoring the importance of the complex in mediating one of the most significant pro-atherogenic effects of thrombin.

Introduction

Thrombin is a serine protease produced during intravascular coagulation, typically as a consequence of vascular injury (1). While thrombin plays a central role in furthering the coagulation cascade by cleaving fibrinogen to produce fibrin, it also possesses the ability to act like a traditional hormone and elicit responses in a variety of cell types, including circulating platelets and endothelial cells (1). These cellular responses are mediated by a small family of GPCRs that are activated by thrombin through an unusual mechanism. Specifically, thrombin-dependent cleavage removes a target receptor's N-terminal sequence, unmasking a cryptic peptide ligand that is present within the extracellular domain of the receptor itself. This tethered ligand is then able to interact with the ligand-binding pocket of the receptor to stimulate intracellular signaling (1). As a result, the family of receptors that are activated in this manner have been termed protease-activated receptors, or PARs. Currently, four members of the family have been identified, PAR-1 through PAR-4, although not all are directly acted upon by thrombin and instead represent targets of other proteases such as trypsin (2-5).

Because thrombin is short-lived in the circulation, most of its effects are exerted locally, near its site of generation. Consequently, endothelial cells adjacent to a site of tissue injury and coagulation represent a particularly important target of thrombin action. The predominant receptor for thrombin on endothelial cells appears to be PAR-1 (6), and while many specific endothelial responses follow stimulation of PAR-1, most can be characterized as contributing to "endothelial dysfunction" (7,8). This is defined as a

breakdown in the ability of the endothelium to maintain appropriate vascular tone, permeability, metabolism, and production of biologically active substances (9). Central to the phenomenon of endothelial dysfunction is the expression of adhesion molecules for recruitment of inflammatory cells, and the production of pro-inflammatory chemokines and cytokines. All of these effects are key to the pathogenesis of atherosclerosis, in which early vascular injury due to cholesterol deposition leads to thrombin-induced inflammation and propagation of vessel damage.

Activation of the NF-κB transcription factor represents one of the foremost mechanisms responsible for thrombin-induced endothelial dysfunction. As for many activators of NF-κB, this occurs through the so-called canonical pathway, whereby activation depends upon stimulation of the IKK complex (10-19). IKK in turn directs the phosphorylation and subsequent degradation of IκBα, a protein that sequesters NF-κB (particularly in the form of RelA/p65) in the cytoplasm (20,21). Upon IκBα degradation, NF-κB is then free to translocate to the nucleus and stimulate transcription of various pro-inflammatory genes.

While considerable work has been carried out to demonstrate that thrombin induction of the canonical NF-κB pathway in endothelial cells requires prior stimulation of PKCδ (2,18,22-24), the molecular links between PKC and the IKK complex have remained unclear. Recently, we and others demonstrated that a signaling complex composed of a scaffolding protein (CARMA3), a linker protein (Bcl10), and an effector protein (MALT1) mediates IKK complex activation, and subsequent NF-κB stimulation, downstream of a small number of GPCRs (25-33). Much of our understanding of the complex, now referred to as the CBM complex, comes from earlier work in lymphocytes where an analogous complex containing the related CARMA1 protein mediates NF-κB activation downstream of the antigen receptor (34-37). In the case of lymphocytes, antigen stimulation leads to activation of PKC which in turn phosphorylates CARMA1,

causing a conformational change that exposes the CARMA1 caspase recruitment domain (CARD), a region responsible for binding Bcl10 (38,39). MALT1 is then recruited and the intact complex interacts with, and stimulates, the IKK complex.

Since thrombin-dependent NF-κB activation clearly requires PKC activation as an upstream event, we asked whether the CBM complex might function as an integral component of the thrombin-responsive signaling machinery. Here we show that all three proteins, CARMA3, Bcl10, and MALT1 are essential for thrombin to effectively stimulate the canonical NF-κB pathway in endothelial cells. Importantly, we also show that disrupting the CBM complex in endothelial cells has important pathophysiologic consequences, since it effectively blocks the ability of thrombin to induce expression of the adhesion molecules ICAM-1 and VCAM-1, and reduces the thrombin-dependent adhesion of monocytes to these endothelial cells. As such, the thrombin receptor now joins a small number of GPCRs that utilize the CBM complex to signal to NF-κB. Several of these GPCRs share the property of causing endothelial dysfunction and vascular inflammation in the context of atherosclerosis. As a result, the CBM complex can be seen as an increasingly attractive target for pharmaceutical intervention in our attempts to combat multiple common instigators of atherogenesis.

Experimental Procedures

Reagents

Polyclonal antibodies to p-ERK1/2 (Thr^{202}/Tyr^{204}; cat. No. 9101), p-Akt (Ser^{473}; cat. No. 9271 and Thr^{308}; cat. No. 2965), Myc, and MALT1 (cat. No. 2494) were from Cell Signaling Technology (Danvers, MA). Polyclonal antibodies to Bcl10 (sc-5611) and p65 (sc-372-G) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to PDK1 (sc-17765), HDAC1 (sc-81598), and GAPDH (sc-32233) were from

Santa Cruz, Biotechnology. Monoclonal antibody to p-IκBα ($Ser^{32/34}$; cat. No. 9246) was from Cell Signaling Technology. Antibodies to tubulin (cat. No. T5168) and Flag (cat. No. F3165) were from Sigma (St. Louis, MO). Taqman® gene expression primers for quantitative RT-PCR were obtained from Applied Biosystems (Foster City, CA). Gene expression assay IDs for mouse *GAPDH*, *VCAM-1*, *ICAM-1,* and *CARMA3* were Mm99999915, Mm00449197, Mm00516023, and Mm00459941, respectively. Thrombin (cat No. T4393), TRAP-6 (cat. No. T1573), and TNFα (cat. No. T6674) were purchased from Sigma. EGF (cat. No. E-3476) and insulin (cat. No. 12585-014) were from Invitrogen (Carlsbad, CA). IL-1β was from R&D Systems (Minneapolis, MN). The inhibitors AG1478 (cat. No. 658552), LY294002 (cat. No. 440204) and wortmannin (cat. No. 681676) were from EMD Chemicals (Gibbstown, NJ). Gene-specific siRNA oligos were obtained from Dharmacon (Lafayette, CO), utilizing the ON-TARGET*plus* smartpool format. In the case of CARMA3, a second siRNA pool was utilized, obtained from Qiagen (Germantown, MD).

Plasmids

The NF-κB-responsive luciferase reporter plasmid (pNF-κB-luciferase) was purchased from Stratagene (La Jolla, CA). Control renilla plasmid (phRL-TK) was from Promega (Madison, WI). The PDK1-myc and kinase-dead (KD) PDK1-myc plasmids were gifts from C. Sutherland and D. Alessi (University of Dundee). All other expression plasmids encoding tagged versions of Bcl10, CARMA3, or CARMA1 have been described previously (40,41).

Quantitative RT-PCR

Total RNA was prepared from cells using either the RNeasy Mini Kit (Qiagen) or with Trizol (Invitrogen). Equivalent amounts of RNA (500-1000ng) were used for cDNA

synthesis with the Superscript First-Strand Synthesis System (Invitrogen) using random primers. Quantitative PCR was performed using TaqMan® gene expression primers (Applied Biosystems) listed above, on an Applied Biosystems 7500 apparatus. Cycle thresholds (Ct) were determined and normalized with those for reactions performed with GAPDH-specific primers. Relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method as described previously (42).

Cell culture

SVEC4-10 mouse endothelial cells were obtained from ATCC and cultured in DMEM with 10% FBS. The generation of SVEC4-10 cells stably expressing an NF-κB-responsive luciferase reporter plasmid has been described previously (43). Briefly, the reporter plasmid (pNF-κB-luciferase; Stratagene) was transfected along with a pSV2neo helper plasmid from Clontech (Palo Alto, CA). Cells were then cultured in the presence of G418 at a concentration of 500μg/ml. Approximately 3 weeks after transfection, G418-resistant clones were isolated and analyzed for luciferase activity to obtain an optimally responsive line. The resulting line was then maintained in medium containing 300μg/ml G418.

Primary endothelial cells were isolated from adult male Sprague-Dawley rat aorta using a method of sequential collagenase digestion (44). Briefly, rats were anesthetized and aortas were exposed through a midline incision. Aortas were then briefly perfused *in vivo* with PBS before being excised, cleaned of adventitia, and transferred to 10cm plates. Following additional flushing, the lumen of each aorta was filled with a collagenase solution using a syringe (2mg/ml type II collagenase; Worthington). Digestion of the intimal layer was allowed to proceed for 15 minutes at 37°C, after which time digestion was halted by flushing aortas three times with DMEM containing 20% FBS. Released endothelial cells were collected by centrifugation at 170xg and washed

prior to plating in EGM-2 endothelial growth media (Cambrex). To evaluate for contamination of VSMCs in endothelial preps, cells were immunostained using antibody against a-smooth muscle actin (anti-SMA; Sigma). Preps of endothelial cells were only used if <5% of cells stained positively for SMA. Primary endothelial cells were used for no more than 5 passages.

Western analysis

After lysing cells with RIPA buffer containing protease and phosphatase inhibitors, immunoblotting was performed as described (28). When analyzing transient induction of phosphorylated proteins (p-IκBα, p-Akt, p-ERK), cells were starved overnight in serum-free media and then treated for the indicated periods of time with either 0.1U/ml thrombin, 100μM TRAP-6, 0.4ng/ml IL-1β, 0.1μg/ml EGF, 50nM insulin, or 10ng/ml TNFα in serum-free DMEM before harvesting for western analysis.

Immunoprecipitation

HEK293T cells were transfected with expression plasmids encoding tagged versions of Bcl10, CARMA3, CARMA1, or PDK1. Cells were harvested 24 hours later and lysed in 0.2% Nonidet P-40 lysis buffer. Immunoprecipitations were carried out using monoclonal anti-Flag (Sigma) as described (45). The products were then resolved by SDS-PAGE and detected by western blotting with polyclonal anti-myc (Santa Cruz Biotechnology). Samples of total lysate, prior to immunoprecipitation, were analyzed in parallel.

Nuclear fractionation

Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's recommendations

(Thermo Scientific, Rockford, IL). Purity of the nuclear extracts was confirmed by Western blot for HDAC1.

Transfection and luciferase reporter assay

For transient transfection experiments, cells were transfected pNF-κB-luciferase and the phRL-TK control renilla plasmid (Promega) using FuGene6 (Roche). 36 hours later, cells were treated for an additional 5 hours with or without medium containing either 5U/ml thrombin or 0.125ng/ml IL-1β. In some cases, cells were cotransfected with expression vectors encoding wild-type PDK1 or a dominant negative, kinase-dead version of PDK1 (PDK1 KD). Cells were harvested and luciferase activity in lysates was measured with a dual luciferase reporter assay system (Promega) and LMax II^{384} luminometer (Molecular Devices), as described (28).

RNA interference

Individual proteins were targeted with the use of siRNA pools from Dharmacon and Qiagen. In brief, cells were transfected with gene-specific siRNA, or non-targeting siRNA, at a concentration of 10-25nM using Lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's instructions. Cells were allowed to recover for 48-72 hours in order to achieve maximal knock-down.

Monocyte/endothelial adhesion

SVEC4-10 cells were transfected with control or Bcl10-specific siRNAs and allowed to grow to confluence. After an overnight serum starvation, cells were then treated with or without 4U/ml thrombin for 6 hours. WEHI-274.1 mouse monocytes (ATCC) were then added to the culture medium ($6x10^5$ cells/well for a 6-well plate). Monocytes were allowed to attach for 10 minutes, followed by vigorous washing, and the number of

monocytes remaining attached to the endothelial monolayer were counted using an inverted bright-field microscope. For each well, the total number of attached monocytes was tallied in seven, random 20x fields.

Statistics

Data are expressed as mean ± SEM. Differences between groups were compared for significance using paired or unpaired 2-tailed Student's *t* tests, as appropriate, with the assistance of GraphPad InStat software. *P* values of less than 0.05 were considered statistically significant.

Animal models

Animal care was provided in accordance with the Laboratory Animal Welfare Act, and all experimental protocols were approved by the University of Michigan Medical School Committee on the Use and Care of Animals (UCUCA). All mice were housed in a standard temperature controlled facility with a 12-hour light/dark cycle, and were provided with a standard chow diet and water *ad libitum*. Bcl10 deficient mice (*Bcl10*$^{-/-}$) were generated as described and backcrossed onto the C57BL/6J background for more than 10 generations (46). *ApoE* deficient mice (*ApoE*$^{-/-}$) were purchased from The Jackson Laboratory and have also been bred onto the C57BL/6J background. To generate double-knockout mice, the two strains were cross-bred using a standard approach.

Atherosclerosis studies

Male *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice between the ages of 48 and 103 days of age were implanted subcutaneously with osmotic minipumps (Alzet model 2004) designed to deliver Ang II at a continuous rate of 500ng/kg/min. After 28 days, mice were sacrificed

and aortic trees were dissected, opened longitudinally, stained with Oil-Red-O, and pinned on black wax, as previously described (47). For each aorta, the percent of total surface area (ascending, arch, and descending to point of the femoral artery bifurcation) involved by fatty streaks or frank atherosclerosis was quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD) after obtaining images with a Spot Insight color camera system (Diagnostic Instruments). Positive Oil-Red-O staining was used as a guide in the analysis, but care was taken to use standard procedures, as outlined by Daugherty *et al* (48), for identification of all lesions regardless of whether or not they reacted with the stain. Specifically, lesions seen under the dissecting scope as areas of thickening, often due to smooth muscle proliferation or accumulation of non-neutral lipids, were included in the calculation of lesional area.

Results

Thrombin activates the canonical NF-κB pathway in mouse endothelial cells through PAR-1

The immortalized mouse endothelial cell line, SVEC4-10, has proven to be a valuable model system for evaluating pro-inflammatory responses in the vasculature (49). However, little is known about the sensitivity of these cells to thrombin. Here, we found that thrombin causes rapid activation of the canonical NF-κB pathway in SVEC4-10 cells, as evidenced by the appearance of $Ser^{32/36}$ phosphorylated IκBα (p-IκBα) in cell extracts within 10 minutes of treatment (Figure 2-1*A*). Other thrombin-responsive signaling pathways are also activated, including that for ERK (Figure 2-1*A*).

Of the various PAR family members, PAR-1 is the predominant member acting as a thrombin receptor in endothelial cells (6). To confirm that PAR-1 is functional in SVEC4-10 cells, we performed a similar time course with a PAR-1 specific peptide agonist

(TRAP-6; SFLLRN). Results showed that TRAP-6 precisely mimics thrombin in its ability to induce the NF-κB and ERK pathways in SVEC4-10 cells, suggesting that PAR-1 likely mediates the thrombin response in these cells (Figure 2-1*B*).

Most of the signaling cascades activated by PAR-1 are mediated through direct receptor-dependent stimulation of G-proteins and their downstream signaling factors. However, PAR-1 is also known to transactivate the EGF receptor (EGFR), thereby stimulating additional signaling cascades (5,50). To test whether the NF-κB activation observed following thrombin treatment might be secondary to EGFR transactivation, we co-treated cells with AG1478, an EGFR tyrosine kinase antagonist. While AG1478 completely blocked ERK activation by thrombin, suggesting that stimulation of the ERK pathway in SVEC4-10 cells does indeed occur through EGFR transactivation, there was no effect on generation of p-IκBα (Figure 2-1*C*). Taken together, the results shown in Figure 2-1 indicate that SVEC4-10 endothelial cells display a robust canonical NF-κB response to thrombin, an effect that is likely mediated through direct downstream signaling from PAR-1.

The CBM-complex mediates thrombin-dependent NF-κB activation

We next employed an RNA interference approach to determine whether thrombin-mediated induction of p-IκBα occurs through the CBM complex. Due to the lack of a sensitive and specific antibody for CARMA3, we used quantitative RT-PCR to demonstrate that siRNA targeting CARMA3 reduces mRNA levels for the protein by approximately 70% (the *upper right panel* in Figure 2-2*A*). Concomitant with this reduction in CARMA3, we observed a complete blockade in the ability of thrombin to induce p-IκBα (the *upper left panel* in Figure 2-2*A*). Conversely, the ability of TNFα to stimulate p-IκBα production remained intact, indicating specificity for the role of CARMA3 in the thrombin response. Further, the reduction in CARMA3 levels did not impair other

signaling responses induced by thrombin, such as that for ERK activation, again indicating specificity for the role of CARMA3 in a select pathway (the *upper left panel* in Figure 2-2*A*).

As a further confirmation of the essential role for CARMA3 in the thrombin-mediated induction of p-IκBα, we utilized an entirely different siRNA pool, prepared by a different manufacturer (Qiagen vs Dharmacon). This siRNA was nearly identical in its ability to reduce *CARMA3* mRNA levels (the *lower right panel* in Figure 2-2*A*), and was also completely effective in blocking the thrombin induction of p-IκBα (the *lower left panel* in Figure 2-2*A*). Again, the siRNA had no impact on p-IκBα generation in response to an unrelated stimulus (IL-1β), and had no effect on thrombin-mediated ERK activation.

We next tested the role of the other components of the CBM complex, and observed similar results. Knockdown of either Bcl10 or MALT1 completely blocked thrombin induction of p-IκBα, while not affecting the IL-1β response nor the ability of thrombin to activate ERK (Figure 2-2, *B* and *C*). In both cases, western analysis showed near complete reduction in the levels of the Bcl10 and MALT1 proteins, respectively, following siRNA transfection. Similarly, human endothelial cells, Ea.Hy926, were transfected with an siRNA pool targeting Bcl10, resulting in near complete reduction of Bcl10 protein levels. As with Bcl10 knock-down in mouse endothelial cells, SVEC4-10, the reduction in Bcl10 levels completely blocked thrombin-dependent p-IκBα generation (Figure 2-2D).

PDK1 is not needed to bridge PAR-1 to the CBM complex

Others have demonstrated a critical role for PI3K in mediating thrombin-induction of NF-κB responsive genes (2,22-24). Since PI3K activation causes rapid production of plasma membrane-associated phosphatidylinositol 3,4,5-tris-phosphate (PIP_3), and subsequent recruitment of PDK1 through a PIP_3-Pleckstrin Homology (PH) domain

interaction (51), we considered the possibility that the thrombin receptor, PAR-1, might communicate with the CBM complex through PDK1. As such, this would be analogous to the mechanism by which a CARMA1-containing CBM complex is recruited and activated in T lymphocytes, following ligand-activation of the antigen receptor complex, CD3/CD28 (52-54). In the case of T-lymphocytes, PDK1 serves as a scaffold by directly binding both CARMA1 and PKC. This allows PKC to phosphorylate CARMA1, resulting in exposure of the CARMA1 caspase recruitment domain (CARD) which subsequently binds Bcl10/MALT1. Thus, PDK1 is thought to function as a central “nidus” for assembly of the activated CBM complex in the vicinity of the antigen receptor.

To begin exploring the possible involvement of PDK1 in thrombin-dependent recruitment of the CARMA3-containing CBM complex, we used an immunoprecipitation approach to test whether CARMA3, like the lymphocyte-specific CARMA1, interacts with PDK1. Results showed that myc-tagged PDK1 co-immunoprecipitates with flag-tagged CARMA3, suggesting an interaction can occur between the two proteins (Figure 2-3*A*). Importantly, when we compared the ability of CARMA3 and CARMA1 to bind to PDK1, we found that the two CARMA proteins are reasonably similar in their apparent affinity for PDK1 (Figure 2-3*B*).

Since recruitment of PDK1 to the membrane is dependent on PI3K activation and generation of PIP_3, we asked whether chemical inhibition of PI3K would be sufficient to block thrombin-induced generation of p-IκBα, thereby suggesting impaired assembly of the CBM complex. Surprisingly, the well-known PI3K inhibitor, LY294002, had only a minimal effect on thrombin-induced p-IκBα generation in SVEC4-10 cells (Figure 2-4*A*). In contrast, LY294002 was completely effective at blocking thrombin- or insulin-induced phosphorylation of Akt, a known downstream substrate for the PI3K pathway, thereby demonstrating that the inhibitor was fully effective in eliminating PI3K activity in this context (Figure 2-4*A*). Similar results were seen with wortmannin, an inhibitor that blocks

PI3K activity through a mechanism different from that used by LY294002 (Figure 2-5). We also found that LY294002 had a minimal effect on the ability of thrombin to induce nuclear translocation of the p65 subunit of NF-κB, an event that occurs as a consequence of IκBα phosphorylation and degradation (Figure 2-4*B*). Thus, two independent measures of thrombin-induced canonical NF-κB activation are largely unaffected by PI3K inhibition. These results sharply contrast with what has been shown in T-cells stimulated with anti-CD3/CD28; in this case, both LY294002 and wortmannin completely block antigen receptor-dependent signaling to IκBα (53).

As a more direct assessment of the functional role of PDK1 in the thrombin response, we then tested the effect of siRNA-mediated PDK1 knockdown in SVEC4-10 cells. Results showed that although PDK1 levels were almost completely ablated by the siRNA, the thrombin response, as measured by p-IκBα generation, remained intact or was even slightly enhanced (Figure 2-4*C*). Similarly, PDK1 knockdown had no effect on thrombin-induced p65 nuclear translocation, while in contrast, Bcl10 knockdown completely prevented the accumulation of p65 in the nucleus (Figure 2-4*D*). Finally, when transiently expressed in SVEC4-10 cells, a kinase-dead, dominant negative mutant of PDK1 had no effect on thrombin-induced NF-κB activation, further supporting the notion that PDK1 has no significant role in mediating early steps in activation of the IKK complex or in the subsequent process of p65 nuclear translocation (Figure 2-4*E*). These results again contrast with what has been shown for antigen receptor-dependent canonical NF-κB signaling in T-cells, which is entirely dependent upon the presence and activity of PDK1 (52,53).

Recently, Sun and Lin demonstrated that β-arrestin 2 can physically link the LPA receptor with CARMA3 (55). Thus, in the context of GPCR signaling, β-arrestin 2 could represent an alternative to PDK1, with regard to assembly of the CARMA3-containing CBM complex. As a result, we measured thrombin-dependent p-IκBα generation in

MEFs obtained from wild-type mice as compared to MEFs obtained from *β-arrestin 2* knockout mice. While the wild-type MEFs responded with a similar time course and magnitude of p-IκBα induction as was seen with SVEC4-10 cells, the *β-arrestin 2* knockout MEFs were completely unresponsive to thrombin (Figure 2-4*F*). In contrast, both MEF lines showed similar responsiveness to TNFα, indicating that β-arrestin 2 deficiency was not impacting the NF-κB pathway in a global manner.

In conclusion, despite the ability of PDK1 to physically interact with CARMA3 in co-immunoprecipitation experiments, our data suggest that PAR-1 does not require PDK1 for communication with the IKK complex. Instead, we find that PAR-1 depends upon β-arrestin 2 for NF-κB activation. Thus, PAR-1, and perhaps several other GPCRs, may utilize a mechanism that is quite distinct from that utilized by antigen receptors for assembly of the CBM complex.

The CBM complex is critical for thrombin induction of NF-κB-responsive gene transcription in endothelial cells

Having definitively shown a requirement for all components of the CBM complex in mediating an early step in the process of thrombin-induced canonical NF-κB activation, namely IκBα phosphorylation, we next turned to an analysis of the role of the complex in mediating thrombin-dependent regulation of gene transcription. To this end, we evaluated SVEC4-10 cells with stable integration of an NF-κB-luciferase reporter and found that siRNA-mediated knockdown of Bcl10 resulted in profound impairment of thrombin-dependent luciferase expression (Figure 2-6). Induction of luciferase in response to IL-1β, however, was unaffected.

We then asked whether disrupting the CBM complex would affect the ability of thrombin to enhance transcription of endogenous genes, using the *ICAM-1* and *VCAM-1* genes as markers since they are known to be strongly induced by NF-κB (2). Preliminary

studies showed that, as in other endothelial cell systems, thrombin induced these genes in a time-dependent fashion in SVEC4-10 cells, with mRNA levels increasing within 2 hours following thrombin treatment, and peak protein levels following shortly thereafter (*not shown*). We then used siRNA to block Bcl10 expression and found that this dramatically impaired the ability of thrombin to induce expression of both *ICAM-1* and *VCAM-1*, as assessed by quantitative RT-PCR (Figure 2-7, *A* and *B*). Since induction of VCAM-1 is particularly relevant to atherogenesis, as a mechanism for promoting macrophage recruitment to the vessel wall, we also analyzed the impact of Bcl10 knockdown on VCAM-1 protein levels. Both thrombin and IL-1β caused a robust induction of VCAM-1 protein in SVEC4-10 cells within 4-6 hours, but under conditions of Bcl10 knockdown, thrombin induction of VCAM-1 was almost completely blocked while the induction by IL-1β remained unaffected (Figure 2-7*C*). Taken together, these data demonstrate that the CBM complex is not only critical for proximal steps in canonical NF-κB activation, but also for downstream regulation of NF-κB target genes.

The CBM complex controls thrombin-dependent monocyte/endothelial adhesion

Recruitment of monocytes to the vessel wall is initiated largely through the interaction of VCAM-1, on the surface of endothelial cells, with VLA-4, an $\alpha_4\beta_1$ integrin expressed on circulating monocytes (56). This interaction causes either rolling of monocytes on the endothelial surface, or firm attachment when VLA-4 is in an activated conformation (57). Since we demonstrated that the CBM complex mediates thrombin-dependent NF-κB activation and VCAM-1 expression in endothelial cells, we utilized an *in vitro* adhesion assay to determine whether disruption of the endothelial CBM complex would interfere with thrombin-induced monocyte/endothelial attachment. To this end, we grew SVEC4-10 endothelial cells to confluency, treated with thrombin for 6 hours, and then quantified the attachment of WEHI-274.1 mouse monocytes to the monolayer.

Endothelial cells transfected with control siRNA showed a nearly 4-fold increase in monocyte attachment following thrombin treatment, while those transfected with Bcl10-specific siRNA showed only a 1.5-fold increase (Figure 2-8, *A* and *B*). These findings illustrate, for the first time, an essential role for the CBM complex in mediating a critical biologic response to thrombin, one with important implications for endothelial pathophysiology.

Bcl10 deficiency protects *ApoE*$^{-/-}$ mice from GPCR-dependent atherogenesis

We next sought to investigate the role of the CBM complex in mediating *in vivo* pathophysiologic consequences of GPCR-dependent pro-inflammatory signaling in the vasculature. However, systemic administration of thrombin in the *in vivo* setting has proven problematic, due to the potent pro-coagulant effect of thrombin. Previously, we had shown that the receptor for Ang II (AGTR1), which is closely related to PAR-1, utilizes the CBM complex to activate NF-κB in hepatocytes (58). Since Ang II also has a prominent role as a pro-inflammatory factor in vascular biology, promoting endothelial dysfunction in a manner similar to thrombin, we first asked whether Ang II might also stimulate the CBM complex in endothelial cells, and thus represent an alternative to thrombin for *in vivo* testing. To this end, we prepared cultures of primary endothelial cells from fresh rat aorta. We confirmed the role of the CBM complex in primary endothelial cells by showing that siRNA-mediated Bcl10 knockdown was effective at specifically blocking Ang II-dependent IκBα phosphorylation (Figure 2-9*A*). Thus, similar to what we had observed in hepatocytes, the CBM complex appears to mediate Ang II-dependent canonical NF-κB signaling in vascular cells.

We then evaluated the effect of Bcl0 deficiency on Ang II-induced atherogenesis in *ApoE*$^{-/-}$ mice. These mice suffer from premature atherosclerosis due to hyperlipidemia. However, chronic infusion of Ang II, which mimics the relatively common clinical problem

of renin-angiotensin system (RAS) dysfunction, substantially accelerates the process (48). We crossed *ApoE*$^{-/-}$ mice with *Bcl10*$^{-/-}$ mice, and then compared the degree of atherosclerosis in *ApoE*$^{-/-}$ versus *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice infused for 4 weeks with Ang II. We observed a dramatic decrease in the extent of atherosclerosis in *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice as compared to *ApoE*$^{-/-}$ mice (Figure 2-9*B, C*). Thus we show that CBM complex is a mediator of GPCR-dependent atherogenesis *in vivo*.

Discussion

Thrombin is now well-recognized for exerting pro-inflammatory effects on the vasculature, particularly through its actions on endothelial cells. While many of these effects are mediated by activation of the NF-κB transcription factor, and downstream induction of NF-κB -responsive genes, the mechanisms linking thrombin receptors to the canonical NF-κB machinery are incompletely understood. In this study, we show that a signaling complex composed of the CARMA3, Bcl10, and MALT1 proteins (CBM complex) serves as a molecular bridge to link activated thrombin receptors to the IκBα kinase. Further, we demonstrate that the CBM complex is essential for thrombin to activate transcription of key genes that underlie the pathogenesis of atherosclerosis, particularly those that mediate recruitment of inflammatory cells to the vessel wall.

To date, a select group of GPCRs have now been shown to utilize the CBM complex for activation of the canonical NF-κB pathway. In addition to the thrombin receptor (PAR-1), these include the receptors for angiotensin II (Ang II), lysophosphatidic acid (LPA), endothelin-1, platelet activating factor (PAF), IL-8 (CXCL8), and SDF-1(CXCL12) (25-33). PAR-1 is unique among these in that it is not activated by a circulating ligand. Rather, PAR-1 utilizes a tethered ligand that is present within the extracellular domain of the receptor itself, but is masked by an amino-terminal sequence that can be removed via proteolytic cleavage by thrombin (1). Once the amino-terminal

sequence is removed, the tethered ligand is free to interact with the ligand-binding pocket of the receptor and activate downstream signaling. Many other differences exist between these receptors, including their preferences for coupling to specific G protein families including $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (59,60).

Despite their differences, GPCRs in this group do share some common properties. In particular, all are capable of activating PKC isoforms through $G\alpha_q$ (59,60). We speculate that the PKC isoforms activated by these receptors phosphorylate CARMA3, thereby exposing the CARMA3 CARD and allowing for CARD-dependent recruitment of Bcl10 and MALT1. As such, this mechanism for assembly of the CBM complex would be analogous to that which occurs in B- and T-lymphocytes, following stimulation of antigen receptor (34-37).

Clearly, however, many GPCRs are capable of activating PKC, but not all of these GPCRs are effective at stimulating NF-κB and perhaps even fewer will be found to utilize the CBM complex. What are the distinguishing features that identify the group of GPCRs that effectively communicate with the CBM complex? The answer to this question is completely unknown, but may lie in discovering additional signaling intermediates, besides PKC, that bridge selected GPCRs with the CBM complex. To gain further insight, we looked for clues by studying the well-characterized activation of the analogous CARMA1-containing CBM complex in lymphocytes. In T-cells, strong evidence implicates PDK1 as a scaffolding protein that brings together activated PKC and CARMA1 following CD3/CD28 stimulation (52-54). In this case, stimulation of CD3 activates PKCθ, while stimulation of the CD28 co-receptor activates PI3K and induces PDK1 membrane recruitment (Figure 2-10). PDK1 then binds to both PKC and CARMA1, facilitating the ability of PKC to recognize CARMA1 as a substrate and induce its phosphorylation.

Importantly, PAR-1 is known to activate PI3K in addition to PKC (1). Thus, we wondered if this single receptor might be capable of activating two separate signal transduction pathways that converge on CARMA3, a feat that in the lymphocyte requires stimulation of two distinct receptors, CD3 and CD28 (Figure 2-10). We therefore tested whether PI3K and PDK1 might be similarly important for the ability of PAR-1 to communicate with the CARMA3-containing CBM complex. As such, this could represent an additional level of control and potentially provide insight into the mechanisms governing the use of the CBM complex by only selected GPCRs.

However, despite evidence suggesting that a physical interaction can occur between PDK1 and CARMA3, we found that PDK1 knockdown by RNA interference had absolutely no impact on the thrombin-dependent NF-κB response, nor did expression of a PDK1 dominant negative mutant. PI3K inhibitors were also largely ineffective at blocking thrombin-induced p-IκBα generation and p65 nuclear translocation. These results suggest that a significant difference exists between the mechanism utilized by PAR-1 for communication with the CARMA3-containing CBM complex and that utilized by CD3/CD28 for communication with the CARMA1-containing CBM complex.

It is intriguing to speculate that the difference may extend to multiple GPCRs, besides PAR-1. In fact, recent work by Sun and Lin provide an alternative explanation as to how GPCRs may communicate with the CBM complex (55). These researchers demonstrated that MEFs from *β-arrestin 2* knock-out mice were unable to respond to LPA with phosphorylation of IκBα. In addition, β-arrestin 2 was found to not only bind CARMA3, but to also mediate an indirect physical interaction between the LPA receptor and CARMA3. Thus, β-arrestin 2 is an essential mediator of LPA-induced NF-κB activation. We similarly found that thrombin induction of p-IκBα is completely impaired in *β-arrestin 2* knockout MEFs, while wild-type MEFs maintain a robust response. The arrestins are a small family of proteins known for their complex roles in GPCR signaling,

and while some family members function only in the retina, two others, β-arrestin 1 and 2, are expressed ubiquitously and impact GPCR-dependent signaling more broadly (61,62). In some cases, the arrestins work to terminate GPCR signaling activity, but in other cases they function as critical positive mediators of signaling. Due to this complex, nuanced role in regulating GPCR activity, one might imagine that specific arrestins could dictate which GPCRs communicate with the CBM complex, possibly in a cell-type specific manner. Based on our work and that of Sun and Lin, we suggest that in certain non-hematopoietic cells, such as endothelial cells, β-arrestin 2 replaces PDK1 as a scaffold for assembly of CARMA3-containing CBM complexes, in response to ligation of specific GPCRs that include PAR-1 and the LPA receptor (Figure 2-10).

It is important to place our current work into the context of what is already known regarding thrombin induction of both ICAM-1 and VCAM-1. As noted earlier, NF-κB activation is a cornerstone in the regulation of these two genes, but other signal transduction events are also required to achieve optimal induction, some of which are specific to one gene or the other. Most relevant to our work is the fact that thrombin-induced PI3K activation is clearly crucial for the ultimate induction of both genes (2,22-24), even though our studies indicate that PI3K activity is not particularly important for the proximal steps in the canonical NF-κB activation pathway, at least not for those leading to phosphorylation of IκBα and p65 nuclear translocation.

It is therefore likely that the crucial role of PI3K lies in its stimulation of parallel pathways regulating *ICAM-1* and *VCAM-1* transcription. For example, it is clear that *VCAM-1* induction depends not only on NF-κB activation, but also on simultaneous activation of the GATA-2 transcription factor, which is PI3K dependent (Figure 2-10) (2,24,63). Others have shown that Akt activation, following thrombin-induced PI3K stimulation, is important for induction of *ICAM-1* gene expression (23). However, it remains uncertain as to precisely how Akt contributes. While evidence exists that Akt

can directly phosphorylate components of the IκB kinase complex, contributing to its activation, this action of Akt appears to mostly impact IKKα and the non-canonical NF-κB pathway (64-67). Thus, in the setting of thrombin stimulation, Akt-responsive IKKα activation may not be sufficient for robust stimulation of the canonical pathway, leading to IκBα phosphorylation, without the contributions of the CBM complex. Another explanation for the role of Akt stems from the observation that Akt can phosphorylate the p65 subunit of NF-κB, an event that increases its transcriptional capacity (Figure 2-10) (68-70). Finally, PI3K activation can lead to stimulation of NADPH oxidase and generation of reactive oxygen species (ROS) (71). Since NF-κB is a redox-sensitive transcription factor (72), this is yet another mechanism whereby PI3K may play a critical role in the downstream regulation of NF-κB-responsive gene transcription, without playing a dramatic role in CBM complex-dependent IκBα phosphorylation and p65 nuclear translocation.

In summary, we show here that the CBM complex plays a critical role in mediating an important pathophysiologic response to thrombin, namely the induction of adhesion molecules on the surface of endothelial cells exposed to thrombin. This is an important event in a variety of conditions since adhesion molecules mediate the recruitment of inflammatory cells to sites of injury. The decreased rate of atherosclerosis observed in Bcl10-deficient mice and the reduced monocyte/endothelial cell adhesion we observed with Bcl10 knockdown suggest that the CBM complex may be required for both thrombin and Ang II to effectively promote monocyte/macrophage recruitment for atherosclerotic plaque formation. In the case of atherogenesis, injury may be initiated by the chronic deposition of cholesterol in the vessel wall, with subsequent plaque rupture and thrombus formation. Thrombin generated locally in response to this injury stimulates recruitment of a variety of leukocytes through induction of ICAM-1 and VCAM-1, which may further exacerbate the problem. However, similar cycles of injury and thrombosis

may occur at sites of trauma and/or infection, where thrombin induction of adhesion molecules is beneficial and necessary for the wound-healing response. While further work will be required to understand how PAR-1 communicates with the CBM complex, there now exists potential for targeting the complex in treatment of vascular conditions, such as atherosclerosis, where thrombin-induced NF-κB activation plays a pathologic role. This goal is potentially within grasp since an inhibitor of the MALT1 protease, the enzymatically active component of the complex, has now been described (73). Thus, the CBM complex is “drugable”, and therefore represents an attractive target for pharmaceutical development.

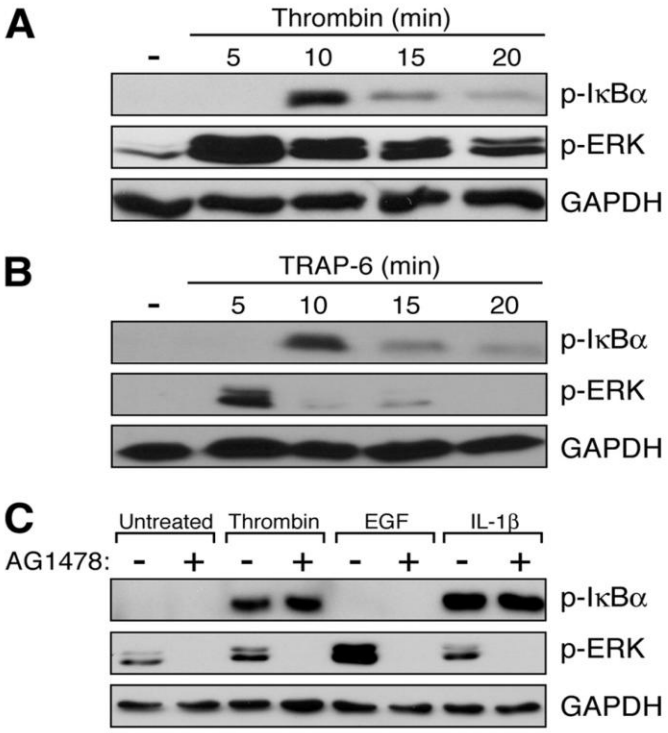

Figure 2-1. Canonical NF-κB activation through PAR-1 in SVEC4-10 endothelial cells. A, SVEC4-10 cells were treated with thrombin for varying periods of time before harvesting and analyzing by western blot. Within 10 minutes, thrombin induces the phosphorylation of IκBα, a marker of canonical NF-κB signaling. ERK activation, as evidenced by the appearance of p-ERK, occurs within 5 minutes. B, SVEC4-10 cells were similarly treated with a peptide that specifically activates PAR-1 (TRAP-6; SFLLRN). The time course for phosphorylation of IκBα and ERK was identical to that observed with thrombin stimulation. C, SVEC4-10 cells were treated with the indicated agonists (thrombin, EGF, or IL-1β) in the presence or absence of 50nM AG1478, an inhibitor of the EGFR tyrosine kinase. AG1478 was effective at completely eliminating both basal and induced levels of p-ERK, indicating that the EGFR is primarily responsible for ERK activation in these cells and that the inhibitor was fully active. In contrast, generation of p-IκBα by thrombin was unaffected, suggesting that thrombin-dependent stimulation of the canonical NF-κB pathway is a direct effect of PAR-1, and not mediated through cross-activation of EGFR.

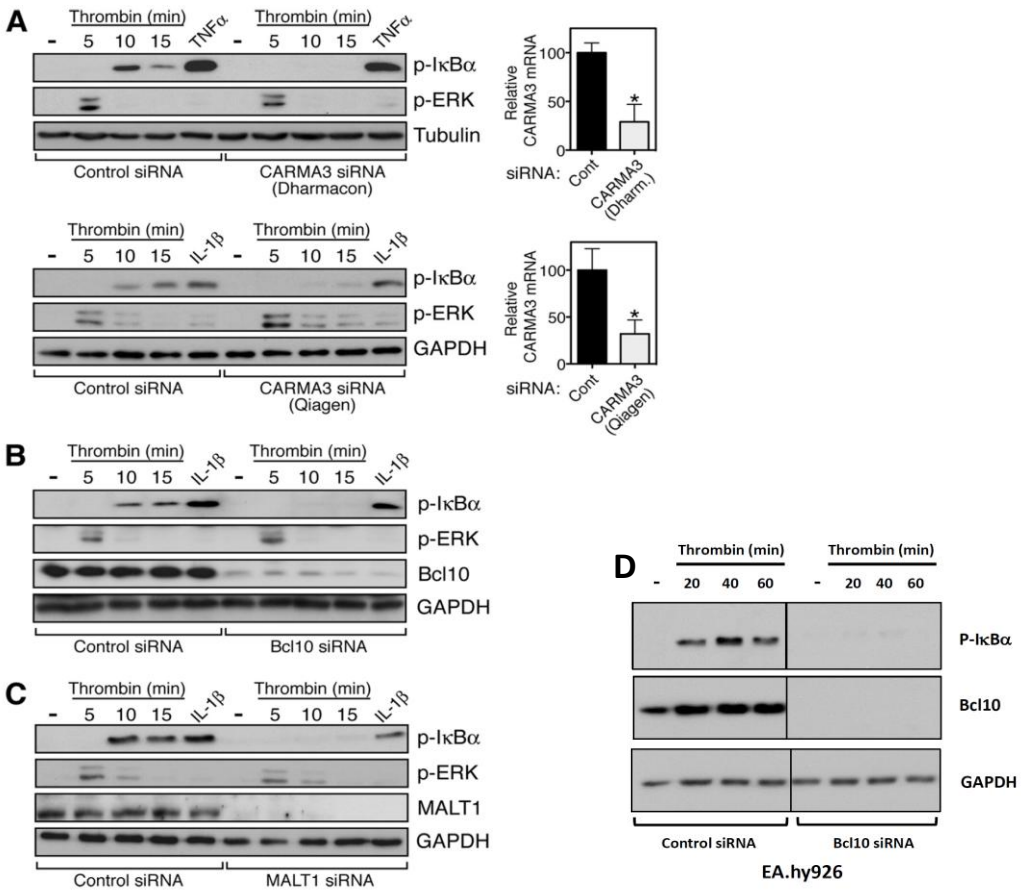

Figure 2-2. The CBM complex mediates thrombin-dependent NF-κB activation in endothelial cells. A, SVEC4-10 cells were transfected with independent siRNA pools targeting CARMA3, designed by Dharmacon (upper panel) or Qiagen (lower panel), at a concentration of 20 and 10nM, respectively. 48 hours following transfection, cells were treated with thrombin for the indicated periods of time, or for 5 minutes with TNFα or IL-1β. Cells were harvested and analyzed by western blotting with the indicated antibodies. CARMA3 knockdown completely abrogated p-IκBα generation following thrombin treatment, but had no effect on the response to either TNFα or IL-1β. In addition, the impact of CARMA3 on thrombin signaling was specific to the canonical NF-κB pathway, since CARMA3 knockdown had no effect on the ability of thrombin to induce p-ERK. CARMA3 mRNA knockdown was assessed by quantitative RT-PCR, shown at right; (*) $p<0.05$. B, SVEC4-10 cells were similarly transfected with an siRNA pool targeting Bcl10 (10nM), resulting in near complete reduction of Bcl10 protein levels as determined by western blotting. As with CARMA3 knock-down, the reduction in Bcl10 levels completely blocked thrombin-dependent p-IκBα generation while not affecting the response to IL-1β, and while not impacting the ability of thrombin to induce p-ERK. C, SVEC4-10 cells were similarly transfected with an siRNA pool targeting MALT1 (10nM), resulting in complete reduction of MALT1 levels as determined by western blotting. As seen in A and B, thrombin-dependent p-IκBα generation was specifically blocked under these conditions. D, Ea.Hy926 (human endothelial cells) were similarly transfected with an siRNA pool targeting Bcl10 (10nM) for 72 hours, resulting in near complete reduction of Bcl10 protein levels as determined by western blotting. As with Bcl10 knock-down in SVEC4-10, the reduction in Bcl10 levels completely blocked thrombin-dependent p-IκBα generation.

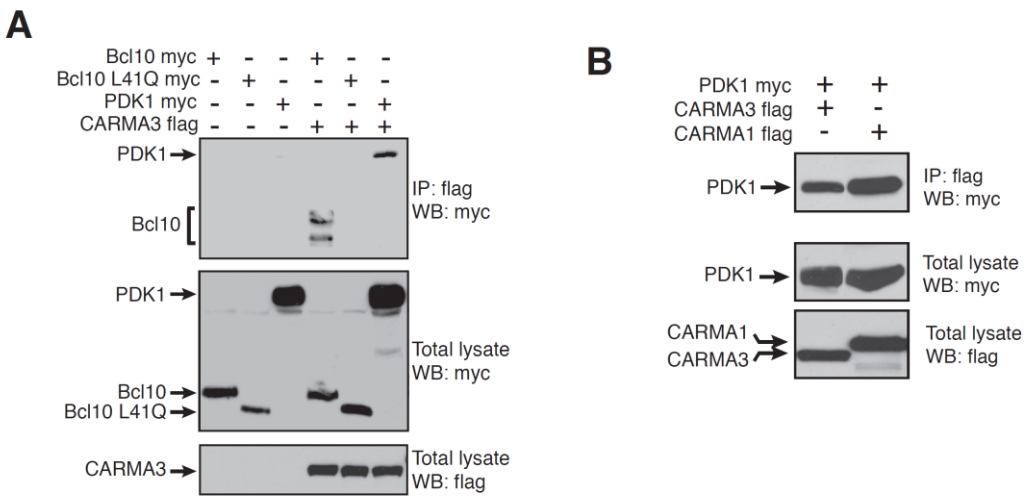

Figure 2-3. Like CARMA1, CARMA3 is capable of interacting with PDK1. A, Flag-tagged CARMA3 was expressed in 293T cells along with potential myc-tagged binding partners, as indicated. Cells were harvested and subjected to immunoprecipitation with anti-flag. The presence of co-immunoprecipitated proteins was analyzed by western blotting (top panel). A small fraction of the total PDK1 effectively co-immunoprecipitated along with CARMA3. In control samples, wild-type Bcl10 was seen to co-immunoprecipitate efficiently, producing a ladder of bands that represent phosphorylated forms of the protein, while as expected, a Bcl10 mutant (L41Q) that has a disrupted CARD interaction domain did not co-immunoprecipitate. B, The relative efficiency of PDK1 co-immunoprecipitation was analyzed for CARMA3, in comparison to CARMA1. While the difference was not dramatic, CARMA3 appeared to be slightly less effective than CARMA1 at interacting. with PDK1.

Figure 2-4. PDK1 is dispensable for thrombin-dependent activation of canonical NF-κB. A, SVEC4-10 cells were pretreated for 1 hour with DMSO vehicle or with 50μM LY294002. Cells were then treated with thrombin for varying periods of time, or with insulin for 5 minutes. Cells were harvested and analyzed by western blotting with the indicated antibodies. The inhibitor had a minimal effect on thrombin induction of p-IκBα, while completely blocking phosphorylation of Akt (p-Akt Ser^{473}) in response to either thrombin or insulin. As expected, thrombin-induced ERK phosphorylation was not affected by LY294002, showing specificity for the effects of the inhibitor at the 50μM dose. B, SVEC4-10 cells were similarly treated with DMSO or 50μM LY294002 prior to thrombin stimulation for 20 minutes. Cells were then lysed and the nuclear fraction was isolated and analyzed for p65 nuclear translocation by western blotting (upper panel). As a loading control, nuclear fractions were also evaluated for the presence of the constitutive nuclear factor, HDAC1. Thrombin-dependent p65 nuclear translocation was largely unaffected by LY294002. As a control, TNFα treatment was also seen to induce nuclear accumulation of p65, an effect that was similarly unaltered by LY294002. In parallel wells, cells were treated with or without thrombin, TNFα, and LY294002, as indicated, prior to preparing whole cell lysates and blotting for p-Akt^{308} (lower panel). As in panel A, this control demonstrates that the LY294002 compound was fully active at blocking PI3K activity within the context of the experiment. C, SVEC4-10 cells were transfected with an siRNA pool targeting PDK1 (25nM; Qiagen), resulting in complete knockdown of PDK1 levels by 48 hours. Cells were then treated with thrombin for the indicated periods of time, or with IL-1β for 5 minutes. PDK1 knockdown had no detrimental effect on the ability of thrombin to stimulate p-IκBα production. D, SVEC4-10 cells were transfected with siRNA pools, as indicated, and treated with thrombin for 20 minutes before harvesting. Nuclear fractions were isolated and probed for p65, while separate cytoplasmic fractions were probed to assess knockdown efficiency for either Bcl10 or PDK1. While Bcl10 knockdown completely abrogated thrombin-induced p65 nuclear translocation, PDK1 knockdown had no effect. E, SVEC4-10 cells were transfected with NF-κB-luciferase and control renilla reporter plasmids, in the presence or absence of co-transfected wild-type (WT) and kinase-dead, dominant negative (KD) PDK1 expression vectors as indicated. Cells were then treated for 5 hours with or without thrombin. Cells were harvested and the resulting NF-κB induction was measured by calculating the luciferase/renilla ratio. The kinase-dead, PDK1 dominant negative mutant was unable to block thrombin-induced NF-κB activation under these conditions. Results are expressed as fold NF-κB induction ± SEM, and are representative of three experiments. Expression of the PDK1 proteins was evaluated by western blotting (insert), using the same extracts as used for the luciferase assay. F, Wild-type and *β-arrestin 2* knockout MEFs were treated with thrombin for the indicated periods of time, or with TNFα for 5 minutes. Cells were harvested and analyzed for IκBα phosphorylation. Results demonstrated that wild-type MEFs respond to both thrombin and TNFα with p-IκBα generation, but *β-arrestin 2* knockout MEFs are completely unresponsive to thrombin, while maintaining their responsiveness to TNFα.

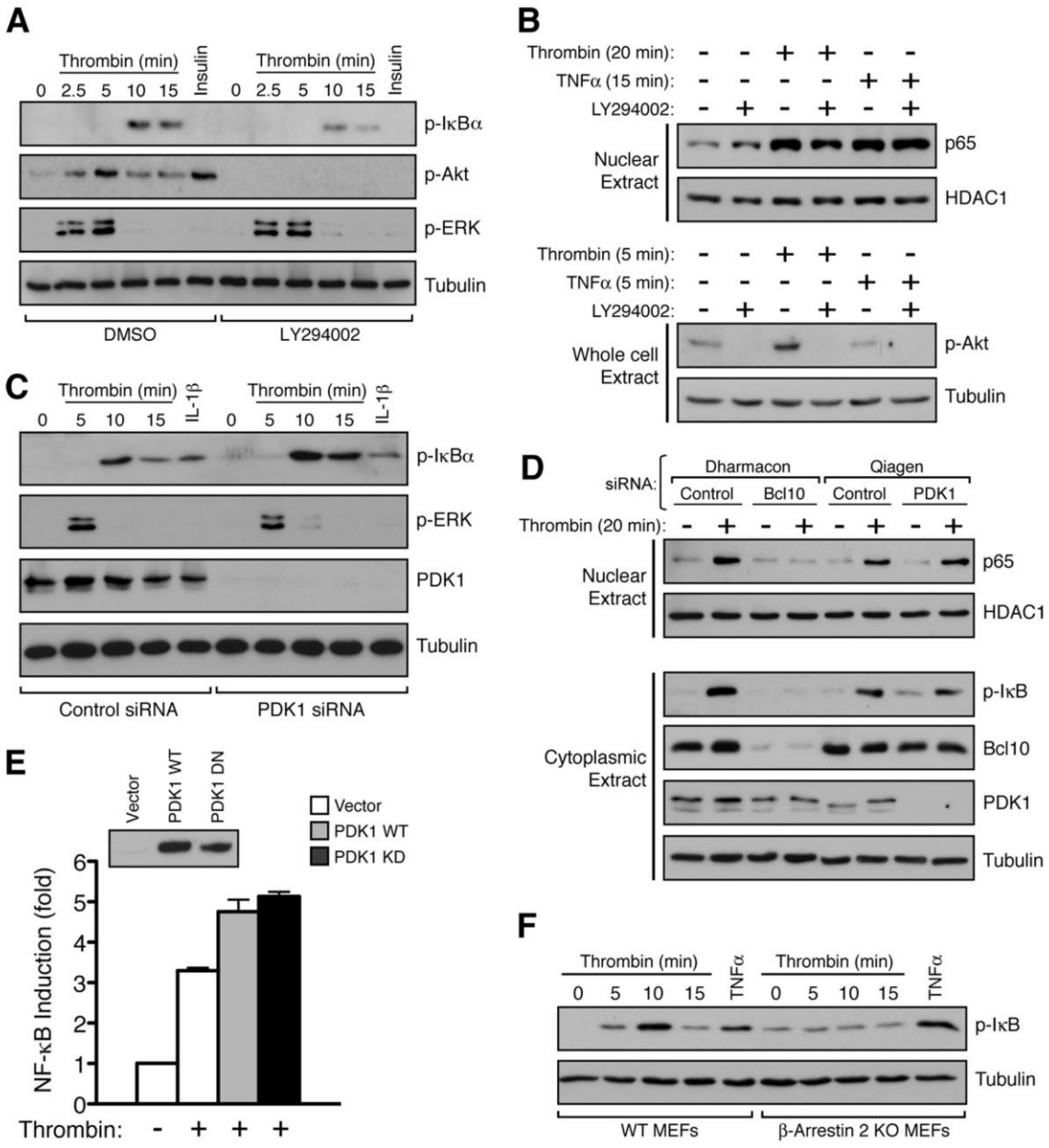

Figure 2-4. PDK1 is dispensable for thrombin-dependent activation of canonical NF-κB.

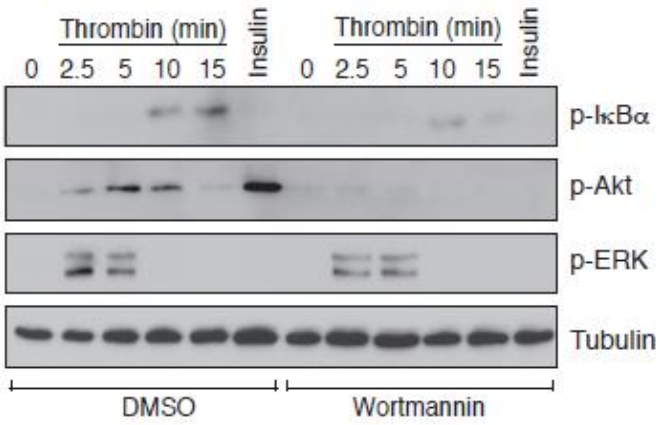

Figure 2-5. The PI3-kinase inhibitor, wortmannin, has little effect on the thrombin-induced p-IκBα response. SVEC4-10 cells were pretreated for 1 hour with DMSO vehicle or with 100nM wortmannin. Cells were then treated with thrombin for varying periods of time, or with insulin for 5 minutes. Cells were harvested and analyzed by western blotting with the indicated antibodies. The inhibitor had only a modest effect on thrombin induction of p-IκBα, while completely blocking phosphorylation of Akt in response to either thrombin or insulin. As expected, thrombin induced ERK phosphorylation was not affected by wortmannin, showing specificity for the effects of the inhibitor at the 100nM dose.

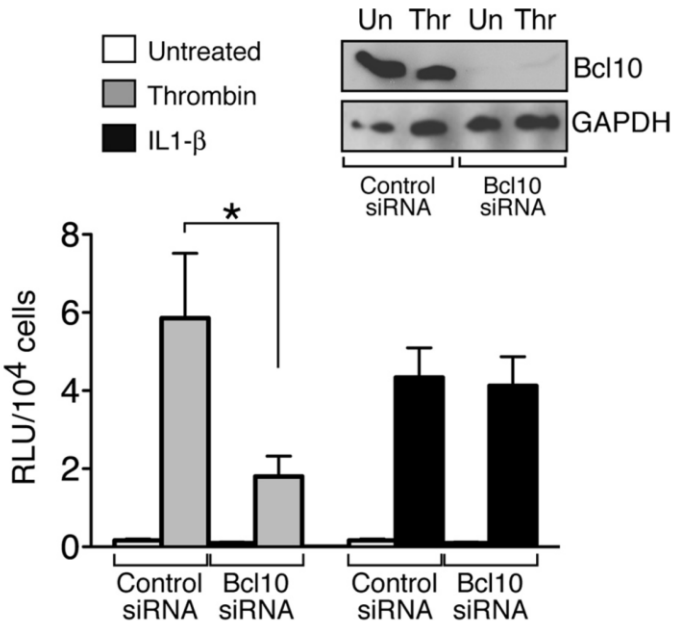

Figure 2-6. Bcl10 knockdown blocks thrombin-responsive NF-κB reporter activity. SVEC4-10 cells stably expressing the NF-κB-luciferase reporter were transfected with either a control siRNA pool or with an siRNA pool targeting Bcl10, as described in Figure 2-2. After 48 hours, cells were treated for an additional 5 hours with either thrombin or IL-1β. Cells were harvested and NF-κB induction was assessed by measuring luciferase levels, corrected for cell number. Lysates were also analyzed for Bcl10 knockdown by western blotting (insert). Results showed that Bcl10 knockdown dramatically reduced NF-κB activation by thrombin, but had no effect on the activation achieved with IL-1β. Results represent the average of 3 independent experiments, ± SEM; (*) $p<0.05$.

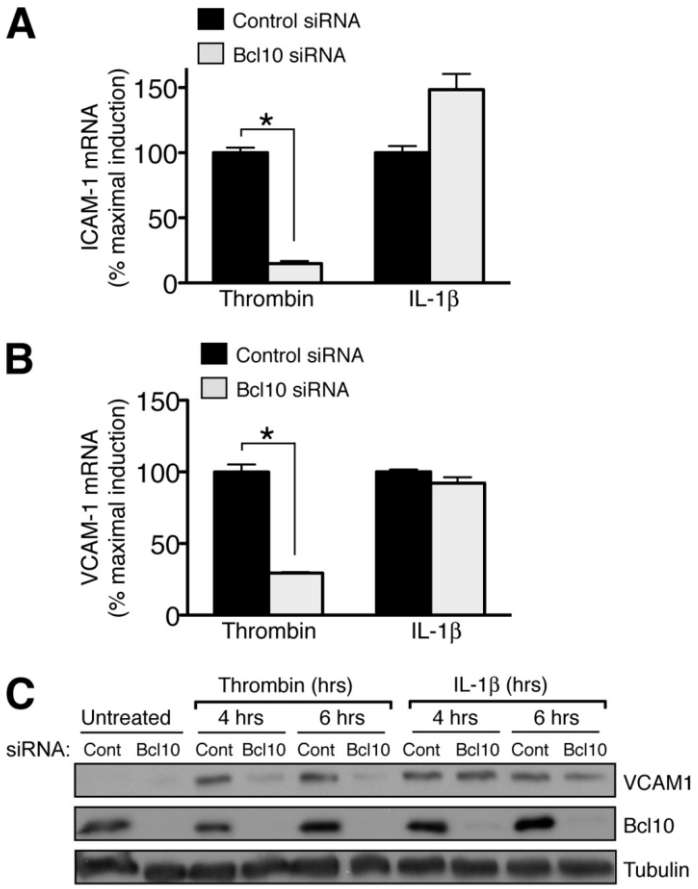

Figure 2-7. Bcl10 knockdown blocks thrombin-responsive induction of endothelial adhesion molecules. A and B, SVEC4-10 cells were transfected with control or Bcl10-specific siRNA as in Figure 2-2. After 48 hours, cells were treated an additional 2 hours with thrombin or IL-1β. Cells were harvested and assayed for *ICAM-1* (A) or *VCAM-1* (B) mRNA expression by quantitative RT-PCR. Results are expressed as percent maximal induction and represent the mean ± SEM for 3 determinations; (*) $p<0.05$. C, Similar to parts A and B above, SVEC4-10 cells were transfected with control or Bcl10 siRNA and allowed to recover for 48 hours. Cells were then treated with thrombin or IL-1β for the indicated periods of time before harvesting and subjecting to western analysis.

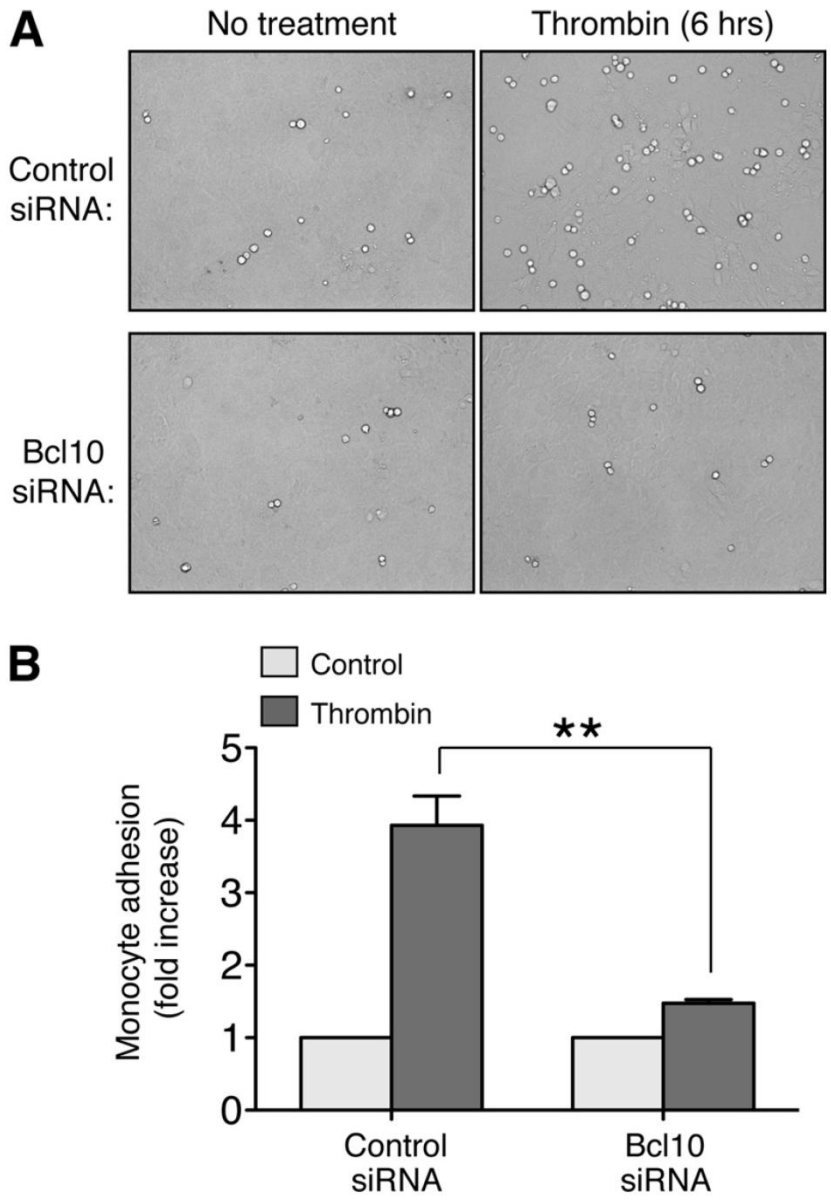

Figure 2-8. Thrombin-induced monocyte/endothelial adhesion is dependent on an intact endothelial CBM complex. A and B, SVEC4-10 cells were transfected with control or Bcl10 siRNA as above, and were allowed to then grow to confluence, producing a monolayer. Cells were then treated with or without thrombin for 6 hours to induce adhesion molecule expression. WEHI-274.1 monocytes were then added to the culture medium and allowed to settle onto the endothelial monolayer. After 10 minutes, unattached monocytes were removed by washing, and monocyte-endothelial attachment was measured by counting adherent monocytes in seven 20x fields per condition. Representative bright-field micrographs of individual 20x fields are shown in (A). Quantification of the fold increase in monocyte adherence is shown in (B). Results represent the average of 9 independent determinations ± SEM; (**) $p<0.01$.

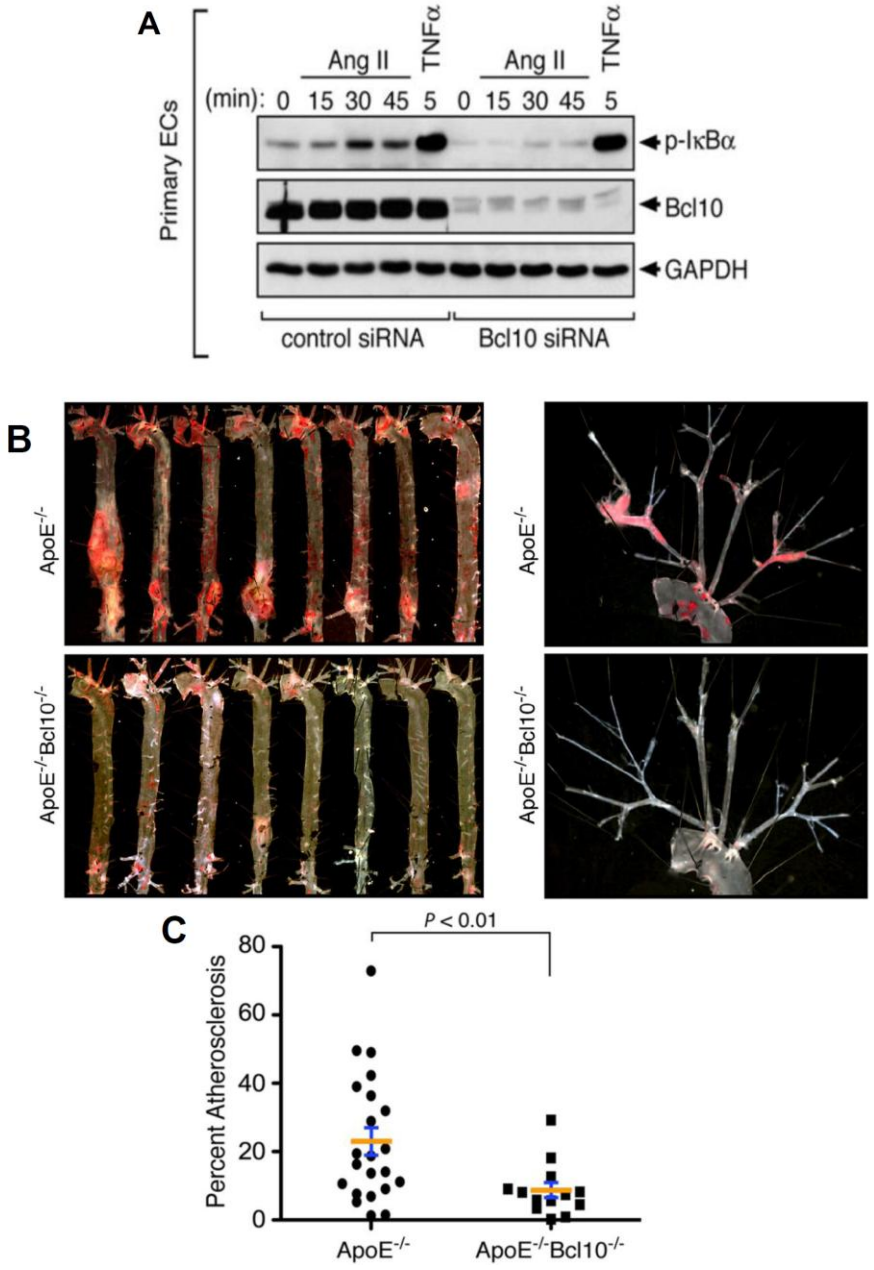

FIGURE 2-9. Bcl10 deficiency protects *ApoE*$^{-/-}$ mice from Ang II-dependent atherogenesis. A, Primary rat aortic endothelial cells were transiently transfected with Bcl10 siRNA as in previous panels, and Ang II/TNFα-dependent p-IκBα generation was assayed as above. B**,** Aortas from the *ApoE*$^{-/-}$ (n=22) and *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ (n=13) groups following 4 weeks of chronic Ang II infusion (500ng/kg/min) and Oil Red O staining. The most significantly affected aortic segments from both groups are shown. The aortic arch and major arterial branches extending from the arch for a representative mouse in each group are also shown. C, Quantification of atherosclerotic lesion area for all mice is shown, presented as the percentage of the surface area of the entire aorta (ascending, arch, and descending to point of the femoral artery bifurcation) stained positively with Oil Red O. The average percentage of area covered by lesion is indicated by an orange bar, and the S.E. is indicated by blue error bars.

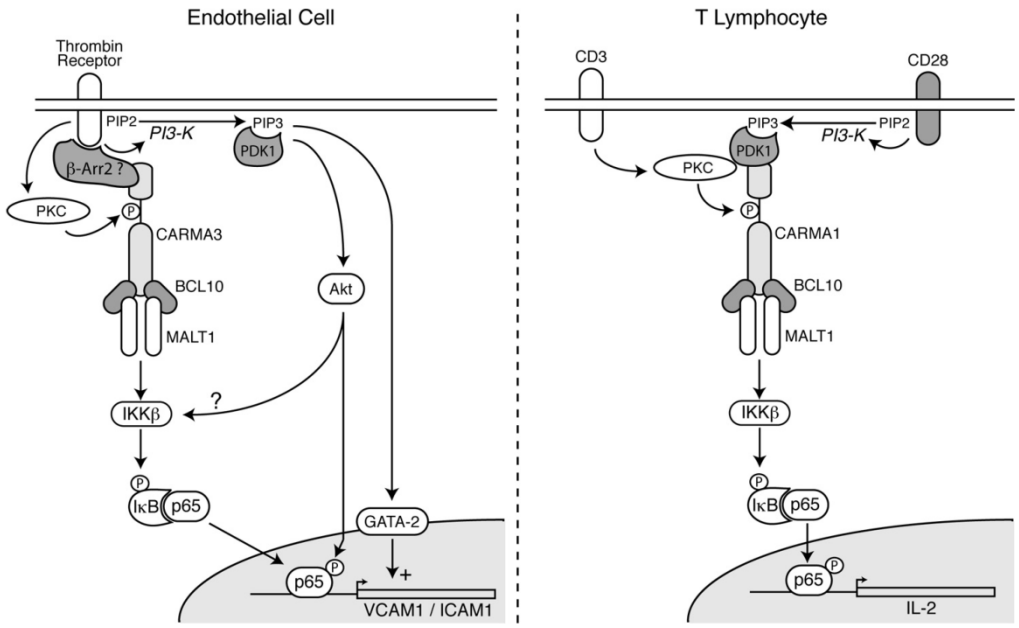

Figure 2-10. Schematic comparing CBM-dependent signaling in endothelial cells and lymphocytes. A schematic diagram illustrating potential differences between the mechanisms for recruitment and activation of the CARMA3-containing complex in endothelial cells (left) and the CARMA1-containing complex in T cells (right). See text for further description. It should be noted that Narayan, P. *et al.* 2006*,* found that while TCR could activate the Akt pathway, it did not phosphorylate IKKβ. Instead, they infer a possible role for Akt kinase activity, modulating activation of CBM complex dependent NF-κB signaling via Akt binding to CARMA1 then phosphorylating Bcl10.This activity was not exclusively necessary for NF-κB signaling. For an excellent review of CARMA1 in CBM complex signaling, please see Rawlings, D.J. and et al. 2006 (35).

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Chapter III

Protease-Activated Receptor 1 Activates Parallel Pathways Which Modulate the Receptor's Impact on NF-κB Signaling

Abstract

Endothelial dysfunction is caused by chronic inflammation and enhanced NF-κB activity that alters the properties of the endothelium. This is recognized as the earliest stage of atherosclerosis. PAR-1 is a member of the G protein-coupled receptor superfamily that activates intracellular signaling pathways through heterotrimeric G protein-dependent and -independent mechanisms that affect gene expression, monocyte adhesion, endothelial permeability, and vascular tone. The canonical role of β-arrestins in GPCR biology is to desensitize activated receptors. This is done through receptor phosphorylation by G protein receptor kinases (GRKs), which recruit β-arrestins to the receptors. Recently, β-arrestins have also become recognized as scaffold proteins that enable the recruitment and activation of signaling pathways downstream of GPCRs, including the NF-κB pathway. After stimulation, β-arrestin 2 recruits CARMA3 to the LPA and thrombin receptors. By facilitating the formation of the CARMA3/Bcl10/MALT1 (CBM) complex, it activates the NF-κB pathway. We demonstrate that multiple parallel pathways, in addition to the β-arrestin 2/CBM complex we previously reported, connect PAR-1 to the activation of the NF-κB pathway. These parallel pathways, RhoA/ROCK and PI3K/Akt, appear to originate from the recruitment of β-arrestin 1 to the receptor and

possibly contribute to the phosphorylation of the IKK complex. In the process of identifying the GRK involved in the β-arrestin recruitment, we identified a novel negative regulator of PAR-1-dependent NF-κB signaling, GRK2. GRK2 negatively regulates PAR-1-dependent NF-κB activation through its ability to desensitize PAR-1 and/or its ability to block PI3K/Akt activity in the NF-κB pathway. All of these pathways represent multiple layers of PAR-1-dependent NF-κB activation that are needed to control a powerful pathway from causing endothelial dysfunction while allowing the plasticity to respond to various stimuli correctly.

Introduction

Endothelial dysfunction is considered the earliest condition leading to atherosclerosis and is broadly defined as the stimulation of the endothelium to the deleterious effect of the organism (1,2). This stimulation, often attributed to high levels of LDL, causes a chronic inflammation with enhanced NF-κB activity that significantly alters endothelial properties, leading to atherosclerosis (3,4). Both PAR-1 and thrombin are major participants in atherogenesis, affecting cell signaling, gene expression, endothelial permeability, and vascular tone (5,6). Thrombin, a protease, binds to the extracellular N-terminus of the GPCR PAR-1. It then cleaves a specific sequence, exposing a cryptic self-ligand that binds to the receptor. This activates intracellular signaling pathways through heterotrimeric G protein activation that are then subsequently desensitized (7,8). Desensitization starts with the phosphorylation of intracellular domains of an activated GPCR by one or more members of the G protein receptor kinase (GRK) family, which recruit β-arrestins to the phosphorylated receptor (9). The canonical role of β-arrestins is to uncouple G proteins by inhibiting the GPCR from constitutively acting as a guanine nucleotide exchange factor (GEF) for heterotrimeric G proteins (10,11). More recently, a “non-canonical” role has been described wherein β-arrestin 1 and β-arrestin 2 act as

scaffold proteins that facilitate the recruitment and activation of a separate set of signaling pathway components downstream of activated GPCRs such as ERK, JNK and p38 MAPK (9,12).

As described in chapter 2, β-arrestin 2 has been revealed to recruit CARMA3 to the stimulated LPA and thrombin receptors to induce the NF-κB pathway (13,14). When *β-arrestin 1*$^{-/-}$ MEFs and *β-arrestin 2*$^{-/-}$ MEFs are treated with LPA, only the loss of β-arrestin 2, but not β-arrestin 1, inhibits NF-κB activation (14). CARMA3 functions as a scaffold protein that, through its CARD domain, binds to the CARD domain of Bcl10 (15). Bcl10 then acts as an adaptor between CARMA3 and the Ig-like domains of MALT1 (16). MALT1 binds to TRAF6 which in turn causes the K63-linked polyubiquitination of the entire CBM complex (17,18). The IKK complex binds to ubiquitin chains of the CBM complex, allowing TRAF6 to induce K63-linked polyubiquitination of the IKK complex. The IKK complex is then phosphorylated by a signaling pathway parallel to the CBM complex, such as MEKK3 in the case of the LPA receptor. This completes the activation of the IKK complex, allowing it to phosphorylate IκBα, leading to proteasomal degradation of the latter and the release of the NF-κB transcription factors (19-21).

Here we report that not only is PAR-1 recruitment of β-arrestin 2 necessary for NF-κB activation via the CBM complex, but β-arrestin 1 is equally necessary. β-arrestin 1 appears to act as a signaling node, possibly activating the RhoA/ROCK and PI3K/Akt kinase pathways, which we show is necessary for PAR-1-dependent NF-κB activation. Finally, while investigating the role of G protein receptor kinases (GRKs) in recruiting the β-arrestins to PAR-1, we identify a new role of GRK2 as a negative regulator of NF-κB activation.

Experimental Procedures

Reagents

A polyclonal antibody to p-ERK1/2 (Thr^{202}/Tyr^{204}; cat. No. 9101) was from Cell Signaling Technology (Danvers, MA). Polyclonal antibody to GRK2 (sc-562) was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody GAPDH (sc-32233) was from Santa Cruz, Biotechnology. Monoclonal antibody to VCAM-1 was from R&D Systems. Monoclonal antibodies to p-IκBα (Ser32/34; cat. No. 9246) and p-Akt (Ser^{473}; cat. No. 4060) were from Cell Signaling Technology. Antibody to tubulin (cat. No. T5168) was from Sigma (St. Louis, MO). Thrombin (cat No. T4393) and TNFα (cat. No. T6674) were purchased from Sigma. IL-1β was from R&D Systems (Minneapolis, MN). Y-27632 (cat No. 10005583) was from Cayman Chemical (Ann Arbor, MI). Gene-specific siRNA oligos were obtained from Dharmacon (Lafayette, CO), utilizing the ON-TARGET*plus* smartpool format.

Plasmids

The NF-κB-responsive luciferase reporter plasmid (pNF-κB-luciferase) was purchased from Stratagene (La Jolla, CA). Control renilla plasmid (phRL-TK) was from Promega (Madison, WI).

Cell culture

SVEC4-10 mouse endothelial cells were obtained from ATCC and cultured in DMEM with 10% FBS. The generation of SVEC4-10 cells stably expressing an NF-κB-responsive luciferase reporter plasmid has been described previously (22). Briefly, the reporter plasmid (pNF-κB-luciferase; Stratagene) was transfected along with a pSV2neo helper plasmid from Clontech (Palo Alto, CA). Cells were then cultured in the presence

of G418 at a concentration of 500µg/ml. Approximately 3 weeks after transfection, G418-resistant clones were isolated and analyzed for luciferase activity to obtain an optimally responsive line. The resulting line was then maintained in media containing 300µg/ml G418. These SVEC4-10 reporter cells were a kind gift of Dr. Yoshiyuki Hattori. Characterization and culturing of *β-arrestin 1*$^{-/-}$ and *β-arrestin 2*$^{-/-}$ MEFs were described previously by Kohout *et al.* (23). These MEFs were a kind gift of Dr. Robert Lefkowitz.

Western analysis

After lysing cells with RIPA buffer containing protease and phosphatase inhibitors, immunoblotting was performed as described (24). When analyzing transient induction of phosphorylated proteins (p-IkBα, p-Akt, p-ERK), cells were starved overnight in serum-free media and then treated for the indicated periods of time with either 0.2U/ml thrombin in SVEC4-10 and 1U/ml in MEFs, 0.4ng/ml IL-1β, or 10ng/ml TNFα in serum-free DMEM before harvesting for western analysis.

Transfection and luciferase reporter assay

For transient transfection experiments, cells were transfected with pcDNA3-GRK2, pNF-κB-luciferase and the phRL-TK control renilla plasmid (Promega) using FuGene6 (Roche). 36 hours later, cells were treated for an additional 5 hours with or without media containing either 5U/ml thrombin or 0.125ng/ml IL-1β. Cells were harvested and luciferase activity in lysates was measured with a dual luciferase reporter assay system (Promega) and LMax II^{384} luminometer (Molecular Devices), as described (24).

RNA interference

Individual proteins were targeted with the use of siRNA pools from Dharmacon. In brief, cells were transfected with gene-specific siRNA, or non-targeting siRNA, at a concentration of 10nM using Lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's instructions. Cells were allowed to recover for 48 hours in order to achieve maximal knock-down.

Results

Thrombin-dependent NF-κB signaling requires both β-arrestin 1 and β-arrestin 2

PAR-1 is a well-established activator of multiple cell signaling pathways that play key roles in endothelial biology. PAR-1-dependent NF-κB activity is reliant on several receptor-proximal proteins being activated: $G\alpha_{q,}$ Gβγ, PKC-δ and PI3K (25-28). Additionally, β-arrestin 1 and β-arrestin 2 function in the role of scaffold proteins for GPCR-dependent cell signaling. This scaffold function has been well described for GPCR-dependent ERK, Akt and JNK signaling (Table 3-1) (29). Using ***β-arrestin 2***$^{-/-}$ MEFs, we recently reported that PAR-1-dependent NF-κB signaling was blocked and Sun *et al.* demonstrated that β-arrestin 2 binds to CARMA3 to act as a bridge to the active LPA receptor for NF-κB signaling (13,14).

While β-arrestin 1 is not required for LPA receptor-dependent NF-κB signaling, β-arrestin 1 is necessary for numerous GPCRs to activate several signaling pathways such as ERK (Table 3-1) (30-38). We have previously shown that PAR-responsive ERK activation is dependent on PAR-1 cross-activation of the EGFR in endothelial cells, and inhibiting ERK activation did not impact PAR-1-dependent NF-κB signaling (13). It has been reported that β-arrestins play a role in receptor cross-activation by some GPCRs through an interaction with Src (13,32). Similarly, we found that both ***β-arrestin 1***$^{-/-}$ and ***β-***

arrestin 2$^{-/-}$ MEFs were unable to activate ERK signaling after thrombin challenge (Figure 3-1).

Given the great variability among GPCRs in their use of β-arrestins for signaling, we wanted to evaluate whether PAR-1 may differ from the LPA receptor and utilize β-arrestin 1 to activate NF-κB signaling (Table 3-1). We used well-characterized *β-arrestin 1*$^{-/-}$ MEFs and challenged them with thrombin to test the necessity of β-arrestin 1 in PAR-1-dependent NF-κB signaling (23). Surprisingly, we demonstrated that β-arrestin 1 and 2 are both required for PAR-1-dependent phosphorylation of IκBα after thrombin treatment. This is in contrast with the LPA receptor, which requires only β-arrestin 2 for NF-κB signaling (Figure 3-1). This illustrates a previously unknown essential role for both β-arrestins in PAR-1-dependent NF-κB signaling, and differentiates PAR-1 from LPA receptors in their mechanisms of NF-κB induction.

Several GPCRs have biphasic activation of ERK signaling, with an early G protein phase that peaks at 5' post-stimulation and a β-arrestin phase that peaks at 10' post-stimulation of the receptor [40, 41]. Since PAR-1-dependent NF-κB signaling is known to be dependent on both G proteins and β-arrestins, we wanted to test for any possible biphasic characteristics in *β-arrestin 1*$^{-/-}$ MEFs. Even with longer time points, we still did not identify any induction of PAR-1-dependent NF-κB signaling in *β-arrestin 1*$^{-/-}$ MEFs (Figure 3-2). There do not appear to be independent G protein or β-arrestin phases to PAR-1-dependent NF-κB signaling, indicating that G proteins and β-arrestins work together to activate signaling (13,28).

β-Arrestin 1's role in thrombin-induced NF-κB signaling is to recruit parallel pathways that induce NF-κB signaling

We next wanted to explore possible mechanisms to explain PAR-1 dependence on β-arrestin 1 in addition to β-arrestin 2's interaction with the CBM complex. β-arrestin 1

has been reported to be a necessary scaffold protein in the PAR-1-activated PI3K/Akt signaling pathway in Chinese hamster embryo fibroblasts (39). We and others have shown that the PAR-1-dependent PI3K/Akt pathway modulates the activation of NF-κB signaling (Figure 2-4, 2-5) (13,26,28). We sought to explore whether either *β-arrestin 1*$^{-/-}$ or *β-arrestin 2*$^{-/-}$ MEFs would be unable to activate the PI3K/Akt pathway, which could explain their inability to activate the NF-κB pathway. Each MEF line was grown to near confluence alongside its respective wild-type control line, serum-starved and treated with thrombin. Cells were then lysed for western blot and evaluated for Akt and NF-κB activation. Interestingly, *β-arrestin 2*$^{-/-}$ MEFs maintained Akt activation while *β-arrestin 1*$^{-/-}$ MEFs lost their ability to activate Akt. Both cells were unable to activate the NF-κB pathway (Figure 3-1).

These results, along with our previously reported data that chemical inhibition of PI3K leads to a complete blockade of Akt and a partial blockade of IκBα phosphorylation, implicate Akt as partially responsible for the activation of the NF-κB pathway (Figure 2-4, 2-5). This provides a model for the role of PAR-1-dependent NF-κB signaling, in which each β-arrestin forms a separate but equally necessary signaling node with PAR-1. In this model, β-arrestin 2/CBM complex facilitates the polyubiquitination of IKKγ. In a parallel pathway, β-arrestin 1/PI3K/Akt contributes to NF-κB signaling through a poorly defined mechanism.

An additional kinase pathway that may co-regulate PAR-1 activation with the PI3K/Akt pathway is the RhoA/ROCK pathway. PAR-1 is known to signal via G proteins to activate the RhoA/ROCK pathway, which then induces actin cytoskeleton rearrangements (40,41). Additionally, RhoA/ROCK pathway-dependent actin cytoskeleton rearrangements, through an unclear mechanism, are required for NF-κB induction (42). Lastly, another GPCR, AGTR1, uses both β-arrestin 1 and $G\alpha_{q/11}$ to initiate RhoA/ROCK-dependent permeability, while β-arrestin 2 functions independently

to activate ERK (43,44). These data are intriguing because they propose a model in which both β-arrestin 1 and G proteins work together to activate the RhoA/ROCK pathway similarly to PAR-1 activated NF-κB signaling, indicating that these pathways may be related.

Based on the above, we sought to explore the concept that PAR-1 recruits β-arrestin 1 to activate the RhoA/ROCK pathway. We found this pathway, along with the β-arrestin 2/CBM complex, co-activates the NF-κB pathway in human and mouse endothelial cell lines. To do this, we used Y-27632, a specific chemical inhibitor of ROCK, which inhibits the kinase's ability to bind ATP, thus blocking its activation (45). Endothelial cells were grown to confluence and then medium was changed to serum-free twelve hours before inhibitor treatment. The inhibitor was added one hour before thrombin challenge and cell lysates were then collected for western blot. Y-27632-inhibition of the ROCK pathway completely blocked the thrombin-induced phosphorylation of IκBα ($Ser^{32/36}$) when compared to vehicle control (DMSO) in two distinct endothelial cell lines (Figure 3-3). The blockade of the RhoA/ROCK pathway was specific to the PAR-1-dependent NF-κB pathway, as PAR-1's ability to activate the ERK signaling pathway was unaffected. This inhibition was not a general effect on all NF-κB signaling as TNFα induction of NF-κB remained unchanged (Figure 3-3). The most notable differences between PAR-1 and TNFα induction of the NF-κB pathway are related to how the IKK complex becomes activated; it seems probable that this is where the RhoA/ROCK pathway feeds into PAR-1-dependent NF-κB activation.

These data, when taken together, reveal a possible mechanism for both β-arrestins in PAR-1-dependent NF-κB signaling (Figure 3-4). Both β-arrestin 1 and β-arrestin 2 are recruited to thrombin stimulated PAR-1, then act as independent signaling nodes to recruit their own signaling pathways which feed into NF-κB activation. β-arrestin 2 is established as being necessary for the recruitment of CARMA3 to the

receptor, leading IKKγ to activate ubiquitination by the CBM/TRAF6 complex. β-arrestin 1 acts as a node for two kinase pathways, PI3K/Akt and RhoA/ROCK. Each of these pathways can alter NF-κB induction, possibly through the phosphorylation of IKKβ, which is known to be independent of β-arrestin 2 (14). Both polyubiquitination and phosphorylation of the IKK complex are needed for NF-κB activation. The loss of either β-arrestin would thus disrupt NF-κB activation (Figure 3-4).

The hunt for the GRK necessary for arrestin recruitment to PAR-1

G protein receptor kinases (GRKs) are a seven-member family of serine/threonine kinases. GRKs phosphorylate the C-terminal tail of ligand-activated GPCRs, and recruit β-arrestins to the receptor for desensitization and signaling (46). GRKs are categorized into three subfamilies based on homology: the GRK1 subfamily includes GRK1 and GRK7, the GRK2 subfamily includes GRK2 and GRK3, and the GRK4 subfamily includes GRK4, GRK5 and GRK6. GRK1, GRK7 and GRK4 have restricted tissue expression, but the remaining GRKs are ubiquitously expressed, including in endothelial cells (46-48). Structurally, GRKs share a highly conserved kinase domain in the center. The N-terminus of the enzyme contains protein-binding domains for substrate specificity and kinase regulation while the C-terminus is needed for membrane localization (47). Having characterized a necessary role for β-arrestin 1 and β-arrestin 2 for PAR-1-dependent NF-κB signaling, we sought to next identify the GRKs that are required for the recruitment of β-arrestins to PAR-1.

We hypothesized that depleting the endothelial cells of the GRKs needed for PAR-1 phosphorylation would inhibit β-arrestin recruitment and thus block PAR-1-dependent NF-κB signaling. We started with GRK2 as it is the best functionally defined GRK and is known to be expressed in endothelial cells (48). To test this, we used GRK2-specific siRNA to knockdown GRK2 expression in the endothelial cell cultures for 36

hours followed by 12 hours of serum-free media before treatment with thrombin. Western blot showed that GRK2 siRNA treatment of endothelial cell cultures caused the depletion of GRK2 proteins after transfection, while the GRK2 levels of control siRNA-treated cells remained unaffected. Surprisingly, knocking down GRK2 had the opposite effect on PAR-1-dependent NF-κB activation in endothelial cells from what we had hypothesized (Figure 3-5). GRK2 knockdown caused a notable increase in the level of phosphorylation of IκBα ($Ser^{32/36}$) after thrombin challenge, indicating an increase in the activation of the NF-κB pathway. The effect of increasing phosphorylation of IκBα with GRK2 depletion was specific to PAR-1-dependent NF-κB activation, as IL-1β induction of the NF-κB pathway did not increase. This is the first report to demonstrate that GRK2 can act as a negative regulator of the GPCR-dependent canonical NF-κB signaling pathway.

Several *in vivo* models of endothelial dysfunction demonstrate that both GRK2 expression levels and kinase activity were increased versus normal control. GRK2 was able to co-immunoprecipitate with Akt and this was associated with reduced Akt activity. Loss of Akt activity resulted in the reduction of nitric oxide production, which contributed to endothelial dysfunction (48,49). We theorized that GRK2's negative regulation of Akt activity could, in turn, down-regulate NF-κB signaling. We then performed western blots for three signaling pathways that we previously established as being dependent on PAR-1 activation: NF-κB, ERK and Akt. The western blot for phosphorylation of Akt (Ser^{473}), as we predicted, showed an increase in the activation of the Akt protein (Figure 3-5). Meanwhile, phosphorylation of ERK remained unaffected, thereby displaying specificity to this pathway. Correlative increases in both the phosphorylation of Akt and IκBα added to speculation that the increased NF-κB activation is at least partly due to an increase in the PI3K/Akt pathway (13,28).

GRK2 regulates PAR-1-dependent NF-κB transcriptional activity and gene expression

Having defined GRK2 as a negative regulator of the NF-κB pathway at the upstream cytosolic point of the phosphorylation of IκBα, we sought to measure the effect of GRK2 depletion in the terminal steps of the NF-κB pathway. We used an endothelial cell line stably transfected with an NF-κB reporter plasmid to gauge the effect of transient GRK2 depletion on the transcription potential of the NF-κB pathway. These cells were transfected with GRK2-specific siRNA or control for 36 hours and then changed to serum-free media for an additional 12 hours. GRK2 depletion resulted in an increase in NF-κB transcriptional potential after thrombin treatment (Figure 3-6). This increase in transcriptional activity was specific to thrombin stimulation of the NF-κB pathway, as IL-1β activation of NF-κB transcription did not change.

In a similar experiment, NF-κB reporter plasmids were co-transfected with a construct expressing wild-type GRK2 to test if overexpressing the GRK2 proteins in endothelial cells could inhibit PAR-1-dependent NF-κB activation. Results showed a 40% decrease specific to thrombin activation of the NF-κB transcriptional potential, as IL-1β activation of NF-κB remained relatively unchanged (Figure 3-7). GRK2-specific regulation of thrombin-dependent but not IL-1β-dependent NF-κB signaling indicates that GRK2 is likely to be involved upstream of where these two receptor pathways meet at the IKK complex.

We have previously published that thrombin can stimulate VCAM-1 expression through PAR-1-dependent CBM complex activation of the NF-κB signaling pathway in endothelial cells (13). VCAM-1 binds to mononuclear leukocytes, particularly monocytes, when expressed on inflamed endothelial cells, which contributes significantly to endothelial dysfunction (50). We tested whether the increases in phosphorylation of IκBα

and activation of the NF-κB reporter plasmid seen in GRK2-depleted cells would also correspond with an increase in NF-κB-dependent gene expression. We used GRK2 and control siRNA to knock down GRK2 expression in endothelial cells for 36 hours, then put the cultures in serum-free media overnight. The next day, cells were challenged with thrombin for either four or six hours to test the effects of GRK2 depletion on the ability of endothelial cells to up-regulate VCAM-1 expression. Thrombin treatment induced VCAM-1 expression in control siRNA-treated endothelial cells at both time points, but GRK2 depletion caused a more robust induction of VCAM-1 at both time points (Figure 3-8).

This demonstrates that GRK2 can down-regulate PAR-1-dependent gene expression and GRK2 may help to protect the endothelium from the pro-atherogenic effects of GPCR-dependent NF-κB activation. Thus, it appears that the reported increases in GRK2 expression levels of dysfunctional endothelium may act as a double-edged sword: increased GRK2 may reduce Akt-dependent vascular relaxation, a pro-endothelial dysfunction property, but also down-regulate GPCR-dependent NF-κB activation, an anti-endothelial dysfunction property. This, therefore, complicates speculation that GRK2 would make a good pharmaceutical target in endothelial dysfunction (48,49). Future studies on the effects of GRK2 in endothelial dysfunction should be broadened to include multiple signaling pathways besides Akt and additional endothelial properties besides relaxation. This would enable us to better understand the total impact of GRK2 in the endothelium.

Discussion

Thrombin activation of PAR-1 induces the NF-κB signaling pathway in endothelial cells which contributes to atherogenesis by modulating gene expression, endothelial

permeability, and vascular tone. Here we provide evidence for the complex roles of β-arrestin and the GRKs in regulation of PAR-1-dependent NF-κB signaling. In an apparent difference with the LPA receptor, we reported here that PAR-1-dependent NF-κB stimulation was inhibited with a loss of either β-arrestin 1 or β-arrestin 2. This led us to define a previously unknown role for β-arrestin 1 in PAR-1 induction of the NF-κB pathway. We then searched the literature for signaling pathways that had the following criteria: 1) activation by a GPCR in a β-arrestin 1-dependent fashion and 2) modulation of the NF-κB signaling pathway at a point before IκBα phosphorylation. We identified two kinase pathways, the PI3K/Akt and RhoA/ROCK pathways, that fit this criteria (28,39,51,52).

The PI3K/Akt pathway has been illustrated to participate in PAR-1-dependent NF-κB signaling (13,25,28). We previously explored the possible role of PI3K/Akt in PAR-1-dependent NF-κB signaling and found that blocking Akt activation correlated with a reduction of phosphorylation of IκBα. Phosphorylation of IκBα was never completely blocked with Akt inhibition, possibly pointing to the involvement of an additional pathway for NF-κB activation. (Figure 2-4, 2-5) (13). These data, along with the fact that some GPCRs use β-arrestin 1 to activate Akt signaling, made us wonder what impact the loss of β-arrestins would have on PAR-1-dependent Akt. PAR-1-dependent Akt activation was blocked in the *β-arrestin 1*$^{-/-}$ MEFs but was still functional *in β-arrestin 2*$^{-/-}$ MEFs. This points to the parallel pathways of the β-arrestin 1/PI3K/Akt and β-arrestin 2/CBM complexes, which merge to facilitate PAR-1-dependent NF-κB activation.

The RhoA/ROCK pathway relies on β-arrestin 1 and G protein activation, and has been implicated in NF-κB induction through an unclear mechanism (51,52). These data, in which both β-arrestin 1 and G proteins work together to activate the RhoA/ROCK pathway, suggest a mechanism similar to one that PAR-1 uses to activate NF-κB signaling, indicating that these pathways could be interdependent. Using a

ROCK-specific chemical inhibitor, we demonstrated a complete blockade of NF-κB activation after thrombin challenge. We demonstrated that both β-arrestin 1 and activation of ROCK are necessary for PAR-1-dependent NF-κB signaling, raising the possibility that β-arrestin 1 and RhoA/ROCK operate in a single pathway parallel to the β-arrestin 2/CBM complex pathway. However, further testing is needed to validate this fully.

GRKs are a family of serine/threonine kinases that phosphorylate the ligand-activated GPCR and recruit β-arrestins that both desensitize the receptor and induce signaling pathways [65]. Having established the necessity of β-arrestin 1 and β-arrestin 2 for PAR-1-dependent NF-κB signaling, we sought to determine which GRKs were required for NF-κB induction. Instead, we discovered that GRK2 negatively regulated PAR-1-dependent NF-κB signaling. When GRK2 was depleted, we observed an increase in PAR-1-dependent NF-κB signaling which did not affect IL-1β-dependent NF-κB signaling. This increase in PAR-1-dependent NF-κB activity resulted in increased expression of VCAM-1, a mediator of endothelial dysfunction. We are the first to establish that GRK2 is negatively regulating GPCR-dependent canonical NF-κB signaling.

GRK2 is a recognized negative regulator of the Akt pathway. In endothelial dysfunction, increases in GRK2 were linked with decreased Akt function. This inhibition in Akt activation blocked NO production, which contributes to endothelial dysfunction (48,49). We hypothesized that GRK2's negative regulation of Akt activity could, in turn, down-regulate NF-κB induction. Depletion of GRK2 caused an increase in phosphorylation of both Akt and IκBα, providing evidence that these pathways are both regulated by GRK2. This data, combined with our results that loss of β-arrestin 1 and chemical inhibition of PI3K inhibit both NF-κB and Akt activation, makes a strong

circumstantial argument that these pathways are interdependent, with Akt activation needed for PAR-1-dependent NF-κB signaling (Figure 3-9).

Additionally, it is interesting to note that several studies on the role of GRK2 in endothelial dysfunction concluded that GRK2 was an ideal drug candidate since GRK2 inhibited Akt-regulated NO production, which leads to a loss of vascular tone regulation. While they showed an improvement in vascular tone with an inhibitor or RNAi knockdown of the GRK2 in the endothelium, they did not address the effects on pro-endothelial dysfunction NF-κB signaling or other endothelial phenotypes (48,49). Our data show that inhibition of GRK2 leads to an increase in NF-κB activation and cell adhesion molecule expression, both of which are classic markers of endothelial dysfunction. These effects induced by GRK2 inhibition should be weighed when considering GRK2 as a pharmaceutical target for treatment of endothelial dysfunction.

An alternative or additional potential model for how GRKs and β-arrestins can both induce and inhibit GPCR-dependent NF-κB signaling comes from multiple studies of how different GPCRs activate the ERK pathway (53-58). ERK activation is biphasic, consisting of an overlapping G protein-dependent early phase and a β-arrestin-dependent late phase. In several studies, knockdown of GRK2 family members caused a significant increase in the G protein-dependent phase of ERK signaling, which is consistent with GRK2's role of uncoupling G proteins, and this increase lasted through the β-arrestin-dependent phase. These results were strikingly similar to our PAR-1-dependent NF-κB activation in GRK2-depleted cells, possibly indicating that GRK2 is involved in the uncoupling of the G proteins needed for PAR-1-dependent NF-κB activation.

Next, researchers evaluated the role of GRK4 family members in GPCR-dependent ERK activation. Knockdown of GRK4 family members resulted in the reduction of later β-arrestin phases of ERK activation and, when combined with a

general inhibitor of PKC, which is activated by the G protein phase, all activation was completely blocked. This illustrates that the GRK4 family is responsible for the β-arrestin recruitment that leads to signaling and not to desensitization. Also, both G protein- and β-arrestin-dependent signaling work together for the activation of the ERK pathway in certain GPCRs. This is similar to the need for both β-arrestins and G proteins for the co-activation of PAR-1-dependent NF-κB signaling by the β-arrestin 1/ROCK, β-arrestin1/Akt and β-arrestin 2/CBM complex pathways in conjunction with G protein activation (52,56,58).

Interestingly, it was discovered that only the GRK2 subfamily was responsible for signal terminating receptor internalization and only the GRK5 subfamily was responsible for ERK signaling despite both subfamilies participating in GPCR phosphorylation and β-arrestin recruitment (56,58). The authors theorize that the functional differences between the GRK subfamilies could be due to still unknown variances in their phosphorylation sites on the receptor. These differences may form a phosphorylation “bar code” which is read by the β-arrestins, causing them to take on different conformational changes depending on which GRK’s phosphorylation they are binding to. This would allow β-arrestins to either induce internalization to end signaling or act as scaffolds for signaling (56,58). In support of this, it has been shown that β-arrestin 1 and β-arrestin 2 do take on different active conformations when bound to phosphorylated GPCRs (59).

Building upon our β-arrestin model and our GRK2 data, a possible hypothesis for PAR-1/β-arrestin-dependent NF-κB signaling is that GRK2 subfamily members are responsible for the signal terminating phosphorylation that desensitizes PAR-1 (Figure 3-9). This would lead to the recruitment of β-arrestin in a conformation that terminates G protein signaling and thus down-regulates NF-κB signaling. In contrast, GRK4 subfamily members would be responsible for the phosphorylation of PAR-1 and recruitment of β-arrestins in a conformation needed for signaling nodes as described above. How the

GRK2 subfamily and GRK4 subfamily differently phosphorylate GPCRs remains an important but unclear mechanism.

In summary, we show that multiple parallel pathways connect PAR-1 to the activation of the NF-κB pathway, in addition to the CBM complex which we previously reported. These parallel pathways originate from the recruitment of β-arrestins by GRK-mediated phosphorylation of the receptor. β-arrestin 2 serves as the node for the CBM complex which leads to IKK complex polyubiquitination. In addition, we theorize that β-arrestin 1 serves as a node to the activation of the RhoA/ROCK and PI3K/Akt pathways. These co-stimulate the NF-κB pathway, possibly contributing to the phosphorylation of the IKK complex. Lastly, based on our data and the literature, GRK2 and GRK4 subfamily members may work in opposition to each other, potentially due to the fact that their different target phosphorylation sites alter β-arrestin function (56,58). GRK2 is now identified as a negative regulator of PAR-1-dependent NF-κB activation through receptor desensitization and/or by inhibiting Akt's ability to participate in NF-κB signaling. All of these pathways represent additional complex levels of PAR-1- dependent NF-κB activation. This level of complexity might be needed to control the powerful NF-κB pathway from causing endothelial dysfunction, yet still allowing the endothelium the flexibility to respond to various stimuli properly. This work provides us with additional insights into the complex mechanism that initiates and terminates PAR-1-dependent NF-κB signaling.

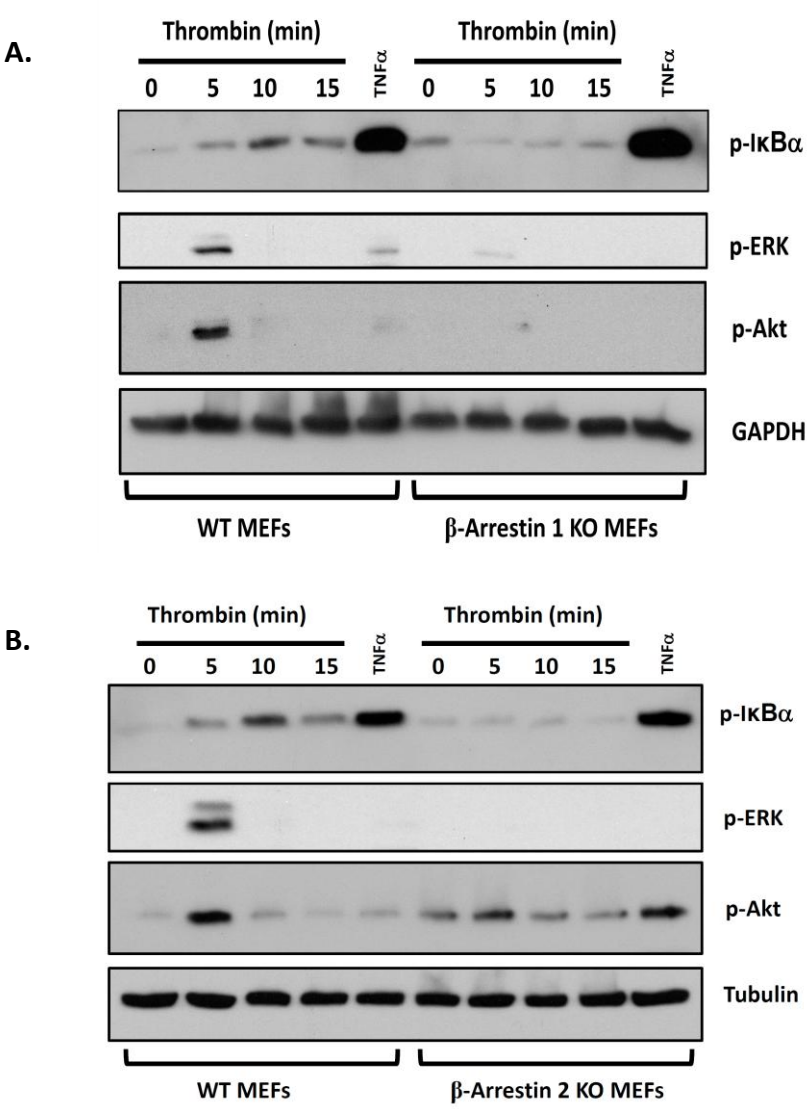

Figure 3-1. Loss of either β-arrestin 1 or β-arrestin 2 inhibits PAR-1-dependent NF-κB signaling. β-arrestin 1 knock-out, β-arrestin 2 knock-out and their matched wild-type MEFs were treated with thrombin for the indicated periods of time or with TNFα for 5 min. The cells were harvested and analyzed for IκBα phosphorylation. The results demonstrated that wild-type MEFs respond to both thrombin and TNFα with p-IκBα generation, but both β-arrestin 1 and β-arrestin 2 knock-out (KO) MEFs are completely unresponsive to thrombin while they maintain their responsiveness to TNFα. Wild-type MEFs respond to thrombin with p-Akt generation, while β-arrestin 2 knock-out MEFs maintained p-Akt generation and β-arrestin 1 knock-out MEFs are unresponsive to thrombin-dependent Akt signaling. Meanwhile, wild-type MEFs respond to thrombin with p-ERK generation, but both β-arrestin 1 and β-arrestin 2 knock-out MEFs are unresponsive to thrombin for ERK signaling.

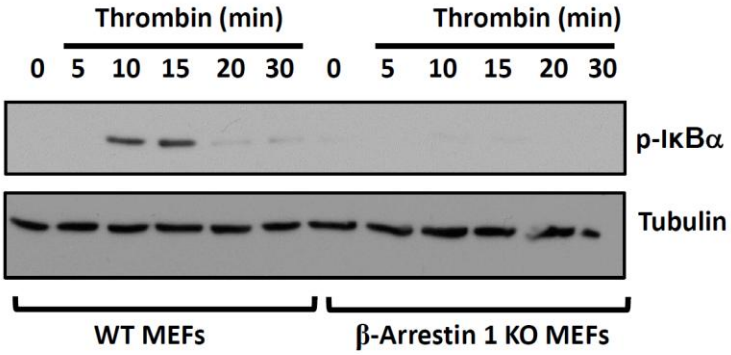

Figure 3-2. PAR-1-dependent NF-κB signaling is through a single phase of activation. β-arrestin 1 knock-out and their matched wild-type MEFs were treated with thrombin for the indicated periods of time. The cells were harvested and analyzed for IκBα phosphorylation. The results demonstrated that wild-type MEFs respond to thrombin with p-IκBα generation which peaked between 10-15' and then tapers off. Meanwhile, β-arrestin 1 knock-out MEFs are completely unresponsive to thrombin, demonstrating induction consists of a single phase of induction which was totally dependent on arrestin activity.

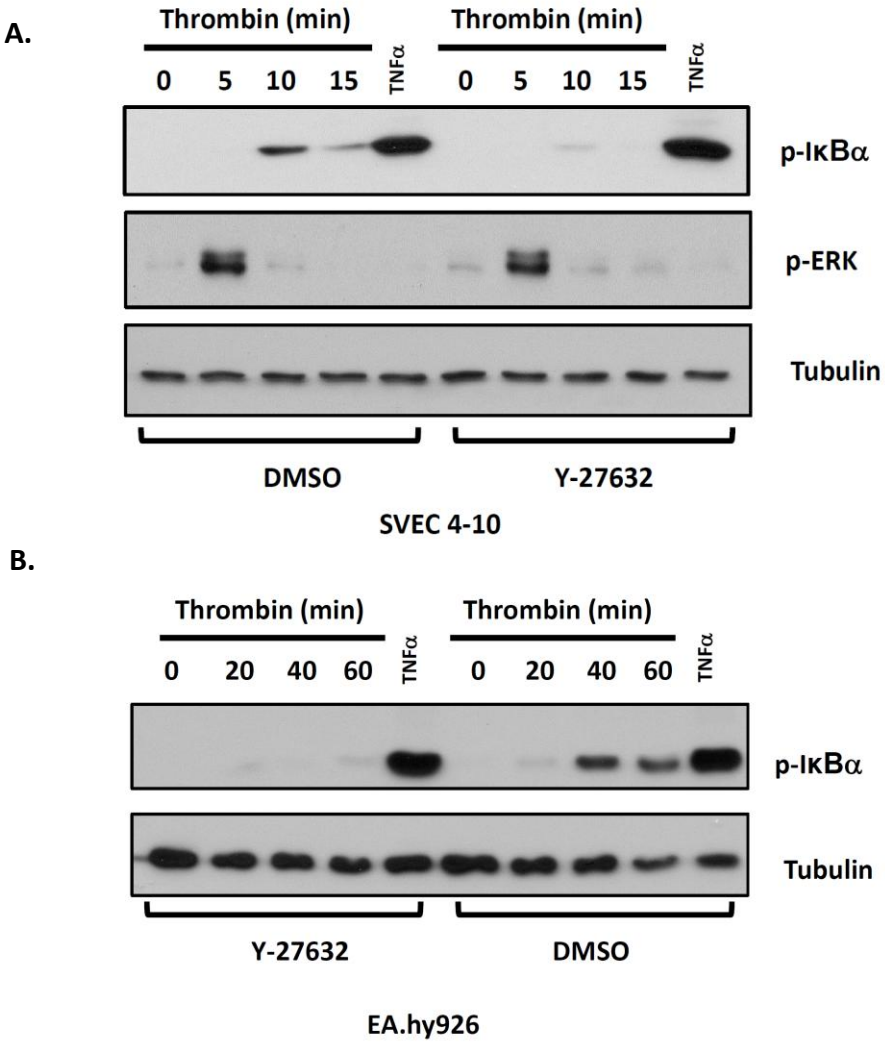

Figure 3-3. Thrombin-induced NF-κB signaling is dependent on RhoA/ROCK activation. A, SVEC4–10 mouse endothelial cells were pretreated for 1 hour with dimethyl sulfoxide (DMSO) vehicle or with 10μM Y-27632, a specific chemical inhibitor of ROCK. The cells were then treated with thrombin for the indicated periods of time. The cells were harvested and analyzed by western blotting with the indicated antibodies. The inhibitor had a significant effect on thrombin-dependent phosphorylation of IκBα. As expected, TNFα-dependent IκBα phosphorylation and thrombin-dependent ERK phosphorylation were not affected by Y-27632, showing specificity for the effects of the inhibitor at the10μM dose. B, EA.hy926, human endothelial cells, were treated the same as in 3-3A. The inhibitor had a significant inhibition of thrombin-dependent phosphorylation of IκBα, showing that this is a common pathway of PAR-1 signaling across species. **Please note that the DMSO and Y-27632 wells are reversed compared to figure 3-3A.**

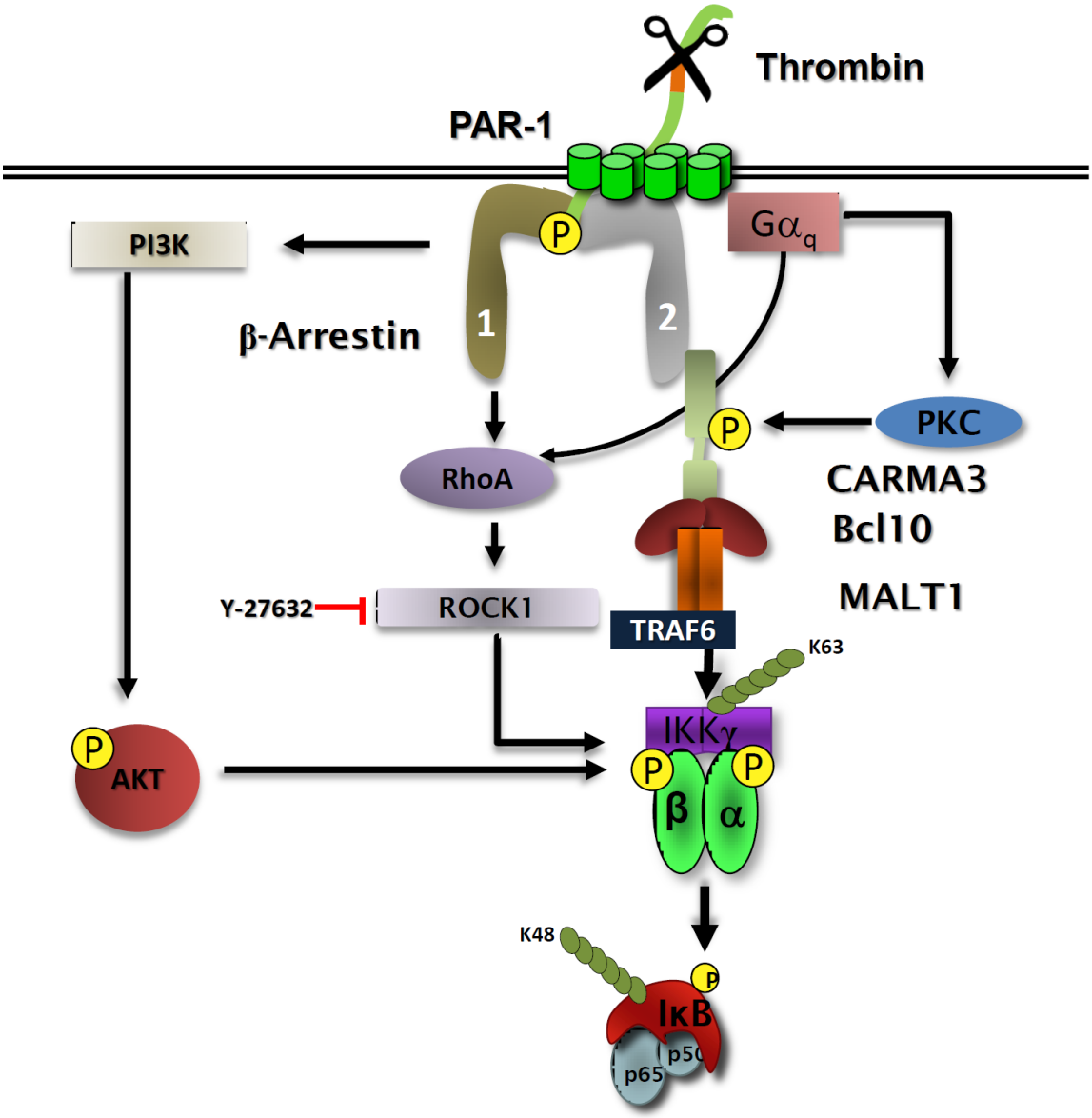

Figure 3-4. A role for both β-arrestin 1 and β-arrestin 2 in PAR-1-dependent NF-κB signaling. Both β-arrestin 1 and β-arrestin 2 are recruited by thrombin-activated PAR-1, then act as independent signaling nodes to recruit their own signaling pathways which input into NF-κB activation. β-arrestin 2 is recognized as being necessary for the recruitment of CARMA3 to the receptor, leading to IKKγ activating ubiquitination by the CBM/TRAF6 complex. β-arrestin 1 acts as a node for two kinase pathways, PI3K/Akt and RhoA/ROCK. Each of these pathways can alter NF-κB induction, possibly through the phosphorylation of IKKβ, which is known to be independent of β-arrestin 2/CBM complex activity. Both polyubiquitination and phosphorylation of the IKK complex are needed for NF-κB activation. The loss of either β-arrestin disrupts NF-κB activation by thrombin-stimulated PAR-1.

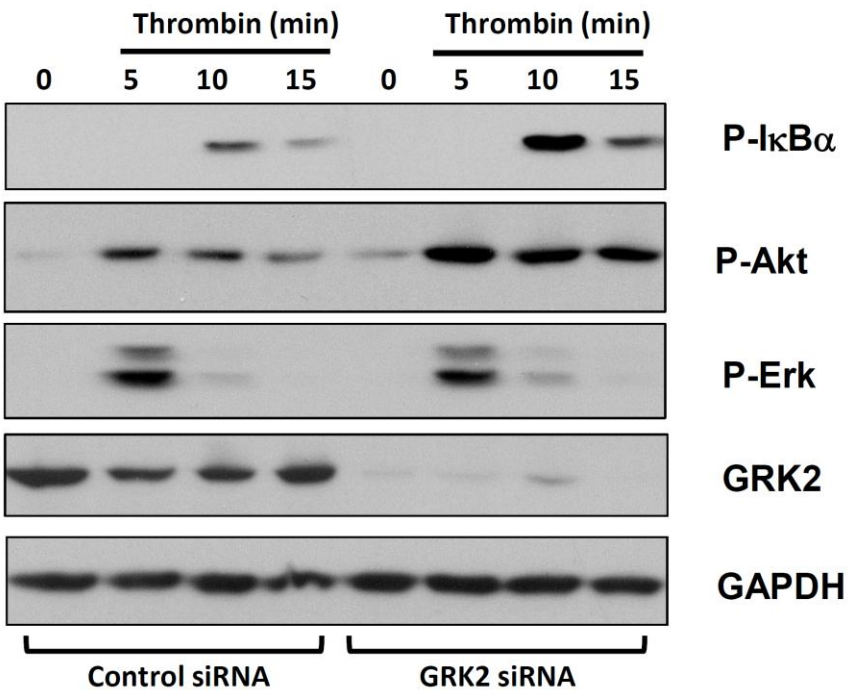

Figure 3-5. GRK2 is an inhibitor of PAR-1-dependent NF-κB and Akt signaling. SVEC4–10 cells were transfected with siRNA pools targeting GRK2, designed by Dharmacon at a concentration of 10nM. 48 hours following transfection, the cells were treated with thrombin for the indicated periods of time. The cells were harvested and analyzed by western blotting with the indicated antibodies. GRK2 knockdown increased both p-IκBα and p-Akt generation following thrombin treatment. The impact of GRK2 on thrombin signaling was specific to the canonical NF-κB and Akt pathways, because GRK2 knockdown had no effect on the ability of thrombin to induce p-ERK. GAPDH was used as a loading control.

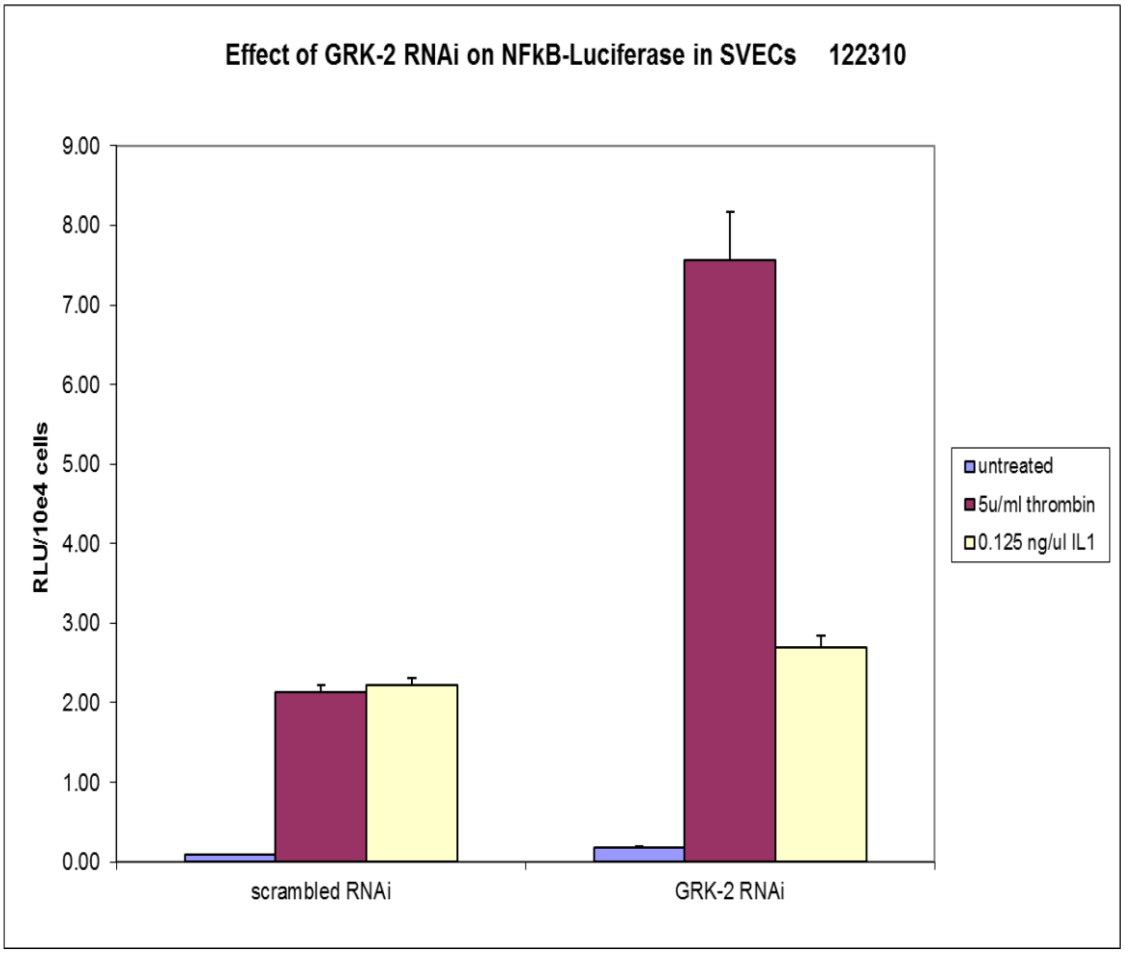

Figure 3-6. GRK2 knockdown increases thrombin-responsive NF-KB reporter activity. SVEC4–10 endothelial cells stably expressing the NF-κB-luciferase reporter were transfected with either a control siRNA pool or with a siRNA pool targeting GRK2. After 48 hours, the cells were treated for another 5 hours with either thrombin (5U/ml) or IL-1β (0.125ng/ml). The cells were harvested and NF-κB induction was evaluated by measuring luciferase levels, corrected for cell number. The outcomes indicated that GRK2 knockdown increased NF-κB activation by thrombin but had no effect on the activation achieved with IL-1β. This result is representative of three independent experiments.

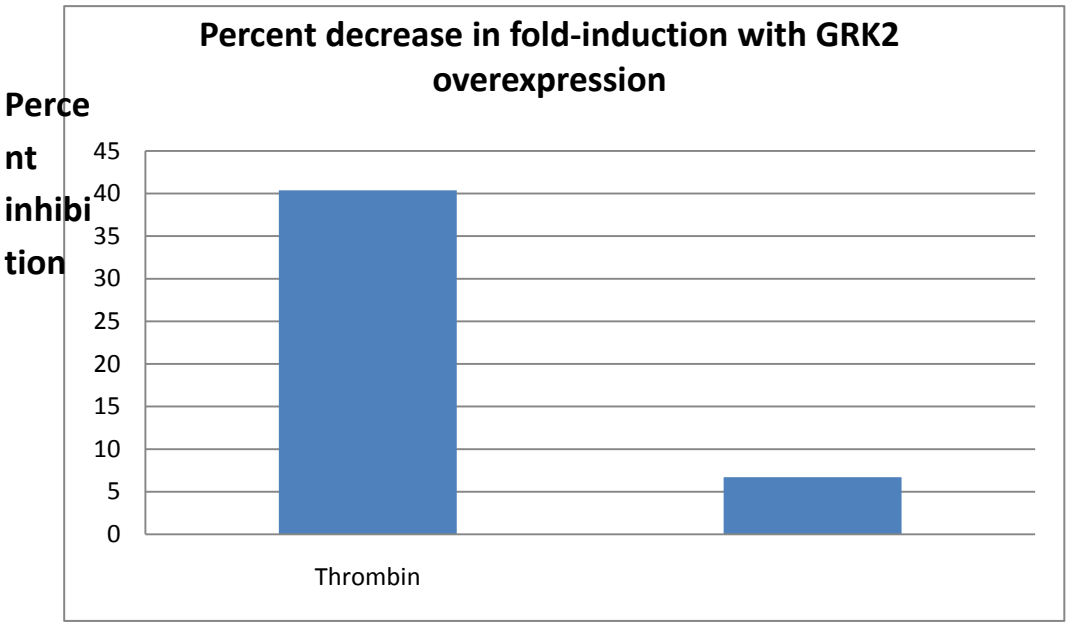

Figure 3-7. GRK2 overexpression decreases thrombin-responsive NF-KB reporter activity. SVEC4-10 cells were co-transfected with NF-κB-luciferase and control Renilla reporter plasmids, and either GRK2 expression vector or empty vector control. The cells were then stimulated for 5 hours with or without thrombin. The cells were harvested, and the subsequent NF-κB induction was measured by calculating the luciferase/Renilla ratio. GRK2 overexpression was able to block thrombin-induced NF-κB activation under these conditions. The results are expressed as percentage inhibition of fold NF-κB induction.

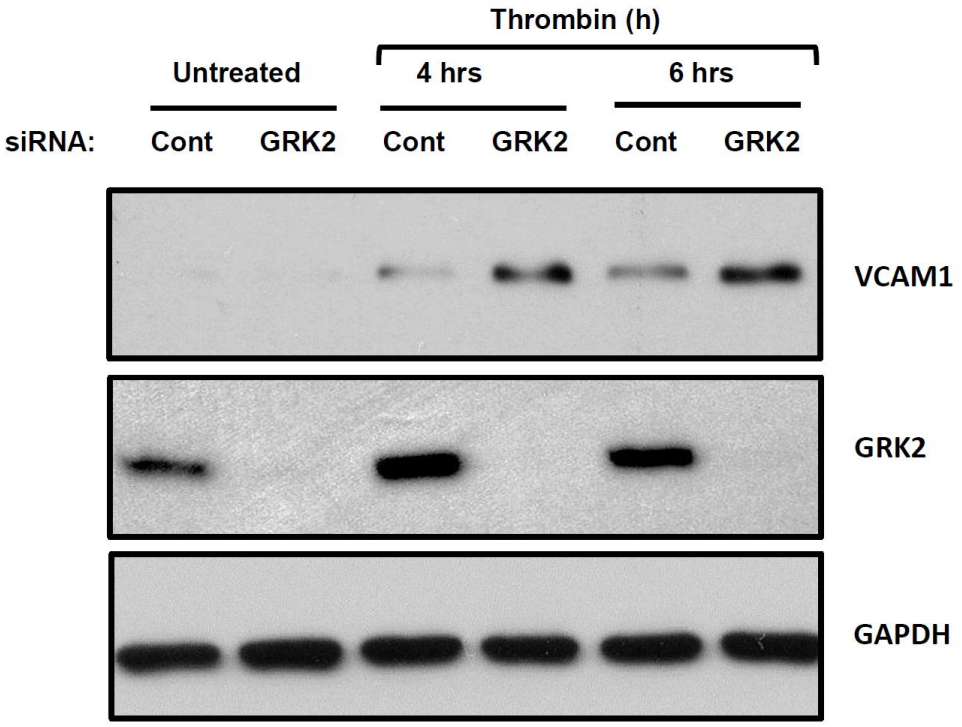

Figure 3-8. GRK2 knockdown increases thrombin-dependent induction of cellular adhesion molecules. SVEC4-10 cells were transfected with control or GRK2 siRNA and allowed to recover for 48 hours. The cells were then treated with thrombin for the indicated periods of time before harvest and western analysis. GRK2 knockdown not only increased NF-κB signaling but also increased expression of an NF-κB responsive gene, VCAM-1.

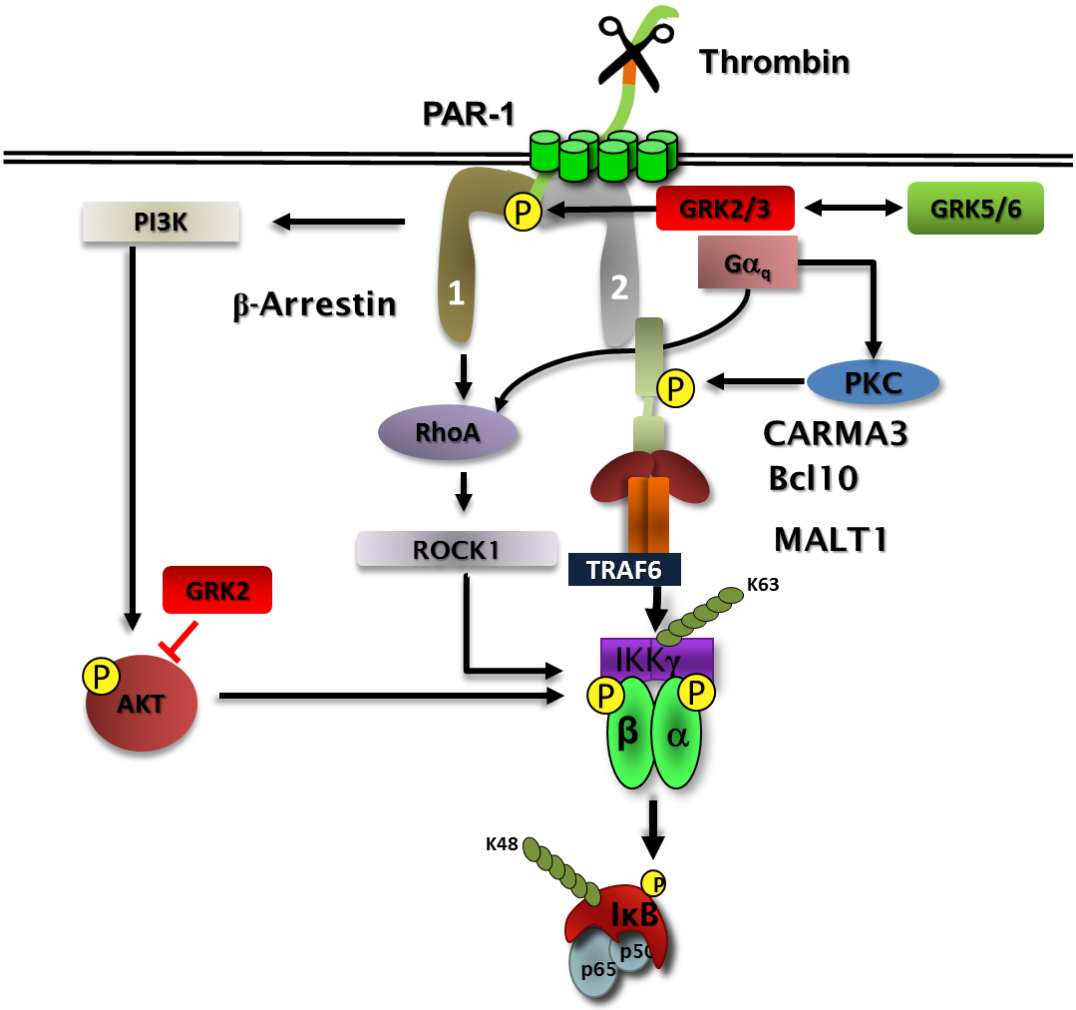

Figure 3-9. A role for G protein receptor kinases in the regulation of PAR-1 signaling. The role of GRK2 in the inhibition of PAR-1-dependent NF-κB signaling could occur through the down-regulation of Akt kinase activity that would reduce the phosphorylation of the IKK complex. An additional or alternative mechanism for GRK2 inhibition of PAR-1-dependent NF-κB signaling may be caused by desensitizing phosphorylation of PAR-1. This would lead to the recruitment of β- arrestins in a conformation that terminates G protein signaling and causes receptor internalization, thus down-regulating NF-κB activation. GRK5 subfamily members may be responsible for the phosphorylation of PAR-1 that recruits β-arrestins in a conformation to act as signaling nodes. In this model GRK5 and GRK2 subfamilies are opposing forces in PAR-1-dependent signaling.

GPCR	Pathway	β-Arrestin	Reference
Adenosine A 1 receptor	ERK, JNK	β-arrestin 1	(56)
Ang II type 1 receptors	ERK, JNK	β-arrestin 2	(60,61)
β2-adrenergic receptor	ERK	β-arrestin 1	(62) (63)
Growth hormone secretagogue receptor type 1a	ERK	β-arrestin 1 and 2	(34)
Metabotropic glutamate receptor 1	ERK	β-arrestin 1	(33)
Parathyroid hormone type 1 receptor	ERK	β-arrestin 1 and 2	(54) (37)
Prostaglandin E2 receptor	Akt, ERK, STAT3, JNK	β-arrestin 1	(32,64)
Protease-activated receptor-1	Akt, NF-κB	β-arrestin 1, β-arrestin 2	(13,65)
Protease-activated receptor-2	ERK	β-arrestin 1 and 2	(38)

Table 3-1. The requirement of β-arrestins for GPCR-dependent cell signaling.

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Chapter IV

Summary and Future Directions

Summary of Results

There is a pressing need globally to understand the molecular mechanisms of cardiovascular disease as it is the leading cause of disease-related deaths in the world (1). Atherosclerotic plaques narrow the vessel lumen, decreasing blood flow to the organs, and plaque rupture can result in thrombosis, causing stroke or myocardial infarction (Figure 1-3) (2). Atherosclerosis is a chronic inflammation of the vasculature in which the pro-inflammatory pathway, NF-κB, is a chief driving force (3,4). NF-κB-dependent plaque formation is mediated via the expression of cytokines, chemokines and CAMs by the dysfunctional endothelium, causing leukocyte recruitment and VSMC proliferation (Figure 1-1, 1-3) (4).

GPCRs have been implicated as mediators of endothelial dysfunction for a number of diseases such as atherosclerosis, diabetes and infectious diseases (5-7). While it was known that GPCRs were facilitating atherosclerosis via NF-κB signaling, little was known prior to our work about the molecular mechanism of this signaling (8,9). The purpose of this work has been to expand our understanding of the regulation of GPCR-dependent NF-κB signaling in endothelial dysfunction through the use of PAR-1 and AGTR1 as models with the expectation that this will lead to the development of novel treatments for atherosclerosis and other inflammatory diseases (Figure 4-1).

Protease Activated Receptor-1 is a player in endothelial dysfunction

Here we explored PAR-1's signaling pathways, their regulation, and phenotypic effects as a model for GPCR-dependent endothelial dysfunction. PAR-1 is a major participant in atherogenesis, regulating cell gene expression, endothelial permeability, and vascular tone (6,10). We demonstrated for the first time that PAR-1 activates ERK, Akt, and NF-κB signaling pathways in SVEC4-10, an immortalized mouse endothelial cell line (Figure 2-1, 2-3). These results are consistent with previously published data from HUVEC, a primary endothelial cell line (11,12). The robust and consistent nature of PAR-1-dependent signaling in SVEC4-10 cells makes them an ideal cell model to test the mechanisms of GPCR signaling and the effects of PAR-1 in endothelial biology.

Until recently, GPCR-dependent canonical NF-κB signaling was poorly defined (Figure 1-2). However, it was noted by our group and others to have a key similarity with T-cell receptor-dependent CARMA1/Bcl10/MALT1 complex-induced NF-κB signaling: dependence on PKC activation (Figure 1-4) (13,14). This led us to discover that the CARMA3/Bcl10/MALT1 complex was required for GPCR-dependent NF-κB signaling through the activation of PKCs and G proteins (Figure 1-6) (13-15). Prior to our work, Rahman *et al.* and Minami *et al.* reported that thrombin treatment of HUVEC led to PAR-1-dependent p65 nuclear localization and NF-κB-regulated gene transcription in a PKC-, G protein- and PI3K-dependent manner. But they did not elucidate the intermediate steps between receptor proximal events and the phosphorylation of IκBα (Figure 1-2) (11,12).

We sought to bridge this critical gap in PAR-1 biology, and established that the CBM complex is necessary for PAR-1-dependent NF-κB activation (Figure 2-2). This finding is particularly exciting in the larger context of NF-κB-dependent atherogenesis, as we also demonstrated that AGTR1 uses the CBM complex to induce atherogenesis *in vivo* (Figure 2-9) (9). Our *in vitro* studies found similar potential for atherogenesis by

PAR-1 activation of the CBM complex, as its disruption inhibited thrombin-induced CAM expression (Figure 2-7), and reduced the adhesion of monocytes to the endothelial cell (Figure 2-8). This resulted in the disruption of a key step in plaque progression. It now appears that the CBM complex is at the crossroads of multiple GPCRs that contribute to atherogenesis (Figure 4-1). It is our belief that GPCRs involved in inflammatory diseases are likely to use CBM complex-dependent NF-κB signaling to advance the disease. Therefore, understanding the mechanisms of this protein complex is key to understanding inflammatory disease progression and potential treatments.

Differences in GPCR and T-cell receptor CBM complex recruitment

Once we established that PAR-1 signals through the CBM complex, we sought to use PAR-1 to compare regulation of the CBM complex by GPCRs to that of the well-defined regulation by the T-cell receptor. The activation of the T-cell receptor stimulates PI3K, which recruits PDK1 that serves as a scaffold for both PKCθ and CARMA1 (Figure 1-4) (16). It has been previously reported that PAR-1-dependent NF-κB activation is reliant on both PI3K and PKC, making PAR-1 an attractive model to test if CARMA3 is also recruited to the receptor by PDK1 (Figure 1-6) (12,17). Through co-immunoprecipitation, we were able to show that CARMA3 could bind to PDK1 when co-expressed in 293T cells (Figure 2-3). Based on the ability of exogenously expressed Carma3 to rescue T-cell receptor-dependent NF-κB signaling in *Carma1*$^{-/-}$ MEFs, this was the predicted result (18,19).

Despite this, the PDK1-CARMA3 interaction had no biological consequences within the context of PAR-1-dependent NF-κB activation, as depletion of PDK1 did not inhibit signaling (Figure 2-4). This is the first demonstrated difference between GPCR- and T-cell receptor activation of the CBM complex. In many ways, this is not surprising since the potential differences between GPCR- and T-cell receptor-dependent CBM

complexes recruitment start with 53% amino acid sequence differences between the CARMAs. Additionally, the receptors themselves and the pathways that they stimulate significantly differ. Understanding the differences between how these different receptor families interact with their respective CBM complexes will provide insight into how each complex functions (Figure 1-6). These differences may prove invaluable for the development of pharmaceutical inhibition of CBM complex recruitment where it could be advantageous to inhibit the ability of one receptor family to activate the CBM complex over the other for disease treatment. Certainly future studies should continue to investigate differences between how these receptors activate the CBM complex.

β-arrestin 1 and β-arrestin 2 act as scaffold proteins for GPCR-dependent cell signaling (Table 3-1) (20). Sun *et al.* illustrated that β-arrestin 2 binds to CARMA3 to act as a bridge to the active LPA receptor for NF-κB signaling while β-arrestin 1 was dispensable for this (21). Our PAR-1-dependent NF-κB signaling results agree, showing that *β-arrestin 2*$^{-/-}$ MEFs were defective in thrombin-dependent NF-κB signaling (Figure 2-4, 3-1). Thus, the role of β-arrestin 2 in GPCR signaling appears to be analogous to the role of PDK1 in the T-cell receptor recruitment of CARMA1 (Figure 2-10). It is interesting to speculate that β-arrestin 2 may bind to PKCs to orientate them to phosphorylate CARMA3 since PDK1 does this in CARMA1 recruitment (22).

We demonstrated a previously unknown critical role for β-arrestin 1 in PAR-1-dependent NF-κB signaling (Figure 3-1), which differentiates PAR-1 from LPA receptor-dependent NF-κB activation. To understand the role of β-arrestin 1 in PAR-1-dependent NF-κB signaling, we focused on published differences between the mechanisms of LPA receptor- versus PAR-1-dependent NF-κB signaling. LPA receptor-dependent NF-κB activation required two separate parallel pathways that merged at the IKK complex for NF-κB activation: the β-arrestin 2/CBM complex for polyubiquitination and the MEKK3 pathway for phosphorylation of the IKK complex (21,23). We searched for kinase

pathways that are implicated in GPCR-dependent NF-κB signaling and whose components are recruited to GPCRs by β-arrestin 1.

The RhoA/ROCK and PI3K/Akt kinase pathways have been implicated in GPCR-dependent NF-κB activation and have been shown to require β-arrestin 1 for their activation by GPCRs (Figure 3-4) (17,24-26). While both of these pathways have been demonstrated to modulate PAR-1-dependent NF-κB signaling, how they do this remains largely unknown. A possible mechanism for how both these pathways modulate PAR-1-dependent NF-κB signaling comes from research into IL-1β-dependent NF-κB activation, in which IL-1β-activated Akt and ROCK were able to phosphorylate the IKK complex (27,28). Similarly, we found that both the PI3K/Akt (Figure 2-4, 2-5) and RhoA/ROCK (Figure 3-3) kinase pathways function downstream of PAR-1 to activate NF-κB signaling. Additionally, Watts *et al*. show PAR-1-dependent RhoA/ROCK activation down regulates the activity of eNOS and thus NO levels, and others have demonstrated that NO is an inhibitor of NF-κB signaling (29-31). Thus, we believe that β-arrestin 1 acts as a parallel signaling node to the β-arrestin 2/CBM complex to activate both the PI3K/Akt and RhoA/ROCK pathways. These two pathways are necessary for activation of the NF-κB pathway, possibly through the phosphorylation of the IKK complex and/or, in the case of ROCK, by reducing the NO levels in the cell (Figure 4-2). A possible reason for the need for multiple parallel pathways for the activation of NF-κB signaling may be to allow the GPCRs to fine tune the induction of the NF-κB pathway. The differences in these parallel pathways between GPCRs may allow them to individualize their induction of NF-κB signaling with respect to their biological function in the cell.

GRK2: a novel regulator of PAR-1-dependent NF-κB signaling

GRKs are a family of serine/threonine kinases which phosphorylate ligand-activated GPCRs to recruit β-arrestins (32). We hypothesized that GRKs are positive

regulators of PAR-1-dependent NF-κB signaling via their recruitment of β-arrestins to GPCRs. To our surprise, GRK2 depletion noticeably increased the ability of thrombin to activate NF-κB signaling (Figure 3-5, 3-6). This effect was specific to PAR-1-dependent NF-κB signaling, as the IL-1β-dependent NF-κB pathways did not increase. This is the first report of GRK2 as a negative regulator of the GPCR-dependent canonical NF-κB pathway.

Our results are analogous to the role of GRKs in GPCR-dependent ERK activation, in which depletion of GRK2 family members caused an increase in ERK signaling, due to a loss of G protein uncoupling by GRK2. This increase in G protein activation could cause the change in NF-κB signaling we observed. Another possible mechanism for GRK2-mediated negative regulation of PAR-1-dependent NF-κB signaling may be in GRK2's ability to bind to Akt (Figure 4-2). Studies have shown that depletion of GRK2 results in an increase in Akt phosphorylation and kinase activity (33,34). Given that PI3K/Akt signaling modulates PAR-1-dependent NF-κB signaling, we predicted that the increase in NF-κB activation may be due to an increase in Akt kinase activity in the absence of GRK2. Depletion of GRK2 leads to an increase in the phosphorylation of both Akt and IκBα after thrombin challenge (Figure 3-5). While this data is correlative, based on the literature, we believe that GRK2's inhibition of the PI3K/Akt pathway is at least in part responsible for the inhibition of PAR-1-dependent NF-κB signaling by GRK2 (Figure 4-2). Parsing out which of these models of GRK2 inhibition is correct, if not both of them, will be difficult but important. This is because GRK2 has been recognized as a promoter of endothelial dysfunction which seems to contradict our finding that GRK2 can down regulate the pro-inflammatory signals of PAR-1-dependent NF-κB signaling (discussed in future directions) (34,35).

Future Directions

1) Which GRKs are responsible for PAR-1-dependent NF-κB signaling?

β-arrestin 1 and β-arrestin 2 are essential for PAR-1 to activate the NF-κB pathway. Which GRKs are responsible for the recruitment of β-arrestins to PAR-1 for NF-κB activation remains unknown (Figure 4-2). Addressing this question provides an opportunity to identify a new kinase family involved in GPCR-CBM complex signaling, and could reveal a pharmaceutical target that would not affect T-cell receptor-CBM complex recruitment. Since we ruled out GRK2 at the beginning of our siRNA screening (Figure 3-5), future efforts will focus on GRK5 and GRK6 as the most likely candidates to recruit the CBM complex to PAR-1.

SVEC4-10 cells will be transfected with Dharmacon smartpool siRNA for individual GRKs and knockdown will be monitored by western blot using commercially available antibodies. Additionally, GRK2 family members will also be evaluated to assure there are no off-target effects of the siRNA. Once knockdown is achieved, effects of depleting each GRK on the NF-κB pathway will be initially measured by western blot for p-IκBα ($Ser^{32/36}$) and p65 nuclear localization to assure the whole pathway is equally affected. To determine the effect of GRK depletion on PAR-1's ability to recruit β-arrestins, cells will be stimulated with thrombin and then treated with a membrane-permeable hydrolysable protein crosslinker Dithiobis[succinimidylpropionate], after which PAR-1 will be immunoprecipitated and western blot will be performed to determine changes to β-arrestin 1 and β-arrestin 2 receptor binding (36).

The physiological relevance of depleting the target GRKs will be assessed by measuring thrombin-induced monocyte adhesion to endothelial cell cultures as described in chapter 2. An alternative to using siRNA depletion of GRKs would be to carry out these experiments with kinase dead GRKs (GRK5-K215R and GRK6-K215R)

which inhibit GPCR β-arrestin recruitment. Due to the potentially redundant nature within the GRK subfamily, both members may need to be depleted to achieve maximal effect (37). We expect GRK5 and/or GRK6 will be responsible for PAR-1-dependent NF-κB signaling in the endothelium (Figure 4-2).

2) What is the mechanism for GRK2-mediated down regulation of NF-κB signaling in the endothelium?

We demonstrated that GRK2 acts as an inhibitor of PAR-1-dependent canonical NF-κB signaling through an unclear mechanism that may involve the need for PI3K/Akt activation (Figure 3-5) (8,17,38). It has been published that *in vivo* models of vascular injury cause an increase in GRK2 expression which contributes to endothelial dysfunction through inhibition of the Akt/eNOS/NO pathway, decreasing vasodilation (33,34,39). It was recently found that insulin-dependent Akt activation was also inhibited by GRK2 in an *in vivo* model of endothelial dysfunction [32]. This supports the theory that GRK2 inhibition of Akt signaling is a universal function of GRK2, unlike its role in GPCR desensitization (Figure 4-2). These results seem contradictory since GRK2 inhibition of NF-κB signaling is considered to protect against endothelial dysfunction (Figure 4-1), while GRK2's inhibition of Akt/eNOS/NO signaling is known to promote certain aspects of endothelial dysfunction. Liu *et al.* illustrates that the Akt inhibition due to GRK2 can be overcome with the expression of the constitutively active Akt mutant, myr.Akt (34). If GRK2 inhibition of PAR-1-dependent NF-κB signaling occurs through the Akt pathway (Figure 4-2), then transfecting the endothelial cells (SVEC4-10) with myr.Akt followed by thrombin stimulation should overcome GRK2's inhibition. Phosphorylation of GSK-3, a known Akt kinase substrate, will be used to monitor Akt activity. If it appears that GRK2-Akt interaction is responsible for PAR-1-dependent NF-

κB inhibition, we will use co-immunoprecipitation to confirm that GRK2 is binding to Akt and also test if this interaction is affected by thrombin stimulation.

To evaluate the possible contributions of GRK2-dependent phosphorylation and desensitization of PAR-1, we will perform the following experiments. PAR-1 will be co-immunoprecipitated as described in aim 1 and evaluated by western blotting for alterations in PAR-1's ability to bind to β-arrestins when GRK2 is depleted as compared to control siRNA cells (36,40). Additionally, PAR-1 phosphorylation status will be measured in control and GRK2 siRNA transfected cells pre-incubated with [^{32}P]Pi and then stimulated with thrombin. PAR-1 will be immunoprecipitated and alterations in phosphorylation due to GRK2 depletion or expression of a kinase dead GRK2 mutant (GRK2-KD) will be analyzed by autoradiography (34,36,40). By evaluating both GRK2's effects on PAR-1-dependent NF-κB and Akt signaling in the same model, we should gain insight into the potential contribution of each mechanism in regulating PAR-1-dependent endothelial dysfunction (Figure 4-2).

3) An *in vivo* model of PAR-1-dependent NF-κB activation and endothelial dysfunction

Vascular injury increases GRK2 expression *in vivo* which is a contributing factor to post-injury endothelial dysfunction. Decreasing GRK2 levels leads to a partial restoration of normal endothelial function through increased NO production (Figure 4-2). Also, *Grk2*$^{+/-}$ mice had a less severe endothelial dysfunction phenotype after insult (33,34). In contrast, we showed that increasing GRK2 protects against PAR-1-dependent NF-κB activation, a phenomenon that should prevent certain aspects of endothelial dysfunction (Figure 3-5, 3-8). It should be noted that PAR-1-dependent Akt activation does not lead to NO production, but instead PAR-1-dependent activation of ROCK causes inhibitory phosphorylation of eNOS, thus causing a rapid decrease in NO

levels (29,41). The seemingly bipolar nature of GRK2 in endothelial dysfunction raises some complex questions: 1) If increasing GRK2 levels has both pro- and anti-endothelial dysfunction properties, which is more important in a disease state? 2) To what degree is PAR-1 contributing to endothelial dysfunction once GRK2 expression levels rise?

In the following experiments, we will evaluate the role of PAR-1 and determine its pathophysiologic contributions following the initiation of endothelial dysfunction. We will use the well-characterized *in vivo* model of endothelial dysfunction induced by liver portal hypertension that is surgically induced by common bile duct ligation (CBDL) in adult male rats (34,42-44). Portal hypertension is a restriction of the blood flow through the portal vein due to liver injury, such as cirrhosis, causing hypertension in the portal vein and its branches. This model will allow us to extend our research into a new disease state, portal hypertension, which shares similarities with atherosclerosis, such as decreased vasodilation, dysfunctional endothelium, and vascular remodeling (34,45). Additionally, PAR-1 is relevant to this disease as the severity of human liver cirrhosis correlates with increasing PAR-1 expression (46).

3a) If portal hypertension inhibits Akt signaling *in vivo* does this mean NF-κB signaling is also inhibited? First, we will evaluate the level of NF-κB signaling induced by CBDL surgery in primary sinusoidal endothelial cells since Liu *et al*. demonstrate down regulation in Akt signaling was innate due to the surgery itself (34). Protocols for CBDL surgery, liver perfusion, and isolation of primary endothelial cells from male Sprague-Dawley will be as described by Liu *et al.* (34,47). Primary endothelial cells will be collected ten days after CBDL operation, as this has been reported to be the peak of injury-induced increase in GRK2 expression (34). Via western blot, we will gather data on downstream markers of endothelial health such as pro-inflammatory markers (Figure 4-2): p-IκBα ($Ser^{32/26}$), p-eNOS (Thr^{495}an inhibitor phosphorylation), p65

nuclear localization, CAM induction (VCAM-1, ICAM-1), and anti-inflammatory markers such as p-eNOS (Ser^{1177}an activating phosphorylation), p-AKT (Ser^{473}), and p-GSK-3 to compare samples from CBDL versus sham operation (4,29,34). Also, phosphorylation of ERK will be assessed as a negative control which, based on our *in vitro* data, will not change due to alterations in GRK2 expression. GRK2 will also be probed to make sure that injury has induced an increase in GRK2 expression.

Additionally, we will compare transcription levels of pro-inflammatory cytokines (*IL-6, IL-18*) and chemokines (*MCP-1, MIP-1α*), CAM (*VCAM-1, ICAM-1*) and anti-inflammatory cytokines (*IL-10*) by quantitative real-time PCR from CBDL versus sham operation (4). We will also monitor the transcription levels of *GRK2*. As part of the liver perfusion process, we will measure portal vein pressure as described by Rockey *et al*. to collect data on changes in vascular tone to add another measure of endothelial dysfunction (48). Previous studies of the role of GRK2 in endothelial dysfunction were narrow in scope; our experiments here will provide a more complete picture of the impact an increase in GRK2 has on the functioning of the endothelium (33,34). We predict that the increase in GRK2 levels will reduce both NF-κB activity and NO production in these cells. However, this reduction in NF- κB signaling may not be significant enough to inhibit NF- κB signaling from continuing to contribute to ongoing endothelial dysfunction.

3b) Since GRK2, an inhibitor of PAR-1-dependent NF-κB signaling, is increased during endothelial dysfunction, does PAR-1 continue to have a role in the endothelial dysfunction beyond initiation? In order to evaluate the ability of thrombin to induce NF-κB signaling in endothelial cells that are already dysfunctional, we will use the method described above to isolate and culture primary sinusoidal endothelial cells from CBDL and sham-operation rats. Lui *et al.* demonstrated that these cells, even after four days in culture, maintained the dysfunction phenotype with inhibition of the

Akt/eNOS/NO signaling pathway, and had significantly increased GRK2 expression when compared to control cells (34). Primary endothelial cells will be grown to a monolayer then treated with thrombin. Protein and mRNA extracts will be collected and evaluated as described in question 3a. These cells can also be readily transfected with a GRK2 siRNA pool, challenged with thrombin, and screened for NF-κB induction as previously described in question 3a to confirm that GRK2 is responsible for any inhibition of PAR-1/NF-κB signaling. Based on our studies in immortalized endothelial cells (Figure 3-7), we would predict an overall decrease but possibly not a complete inhibition in NF-κB signaling in cells with elevated GRK2 expression levels. This would mean that increased GRK2 expression is associated with both anti- and pro- endothelial dysfunction influences in the cell (Figure 4-2). If so, it becomes a question of which GRK2-mediated signaling pathway has a stronger influence on endothelial cell dysfunction: CBM complex/NF-κB or Akt/NO signaling? Is PAR-1 signaling more important to the initial stages of endothelial dysfunction than to maintaining it?

3c) Can PAR-1-dependent NF-κB signaling continue to contribute to endothelial dysfunction once GRK2 levels increase *in vivo*? Unlike in humans, PAR-1 is not the receptor for platelet activation and aggregation in rats (49). This allows for the PAR-1-specific agonist, TRAP-6 (Figure 2-1), to be administered *in vivo* without causing clotting in the vasculature (49-52). We will exploit this difference to test PAR-1 signaling in this *in vivo* endothelial dysfunction model. The adult male rats will receive CBDL or sham surgery and be allowed to recover for ten days by which time Liu *et al.* reported that the increase in GRK2 expression will have peaked and the portal vein endothelium will be dysfunctional. At this point we will subcutaneously implant osmotic minipumps (Alzet) filled with either saline (negative control) or TRAP-6 peptide diluted in saline. This method has previously been shown to be able to successfully deliver peptide hormones for GPCR activation to the liver after CBDL surgery (53,54). These will

continuously release TRAP-6 for two weeks before sinusoidal endothelial cells are isolated and protein and mRNA extracts are made and evaluated as described in question 3a. Any reduction in PAR-1 activity in the dysfunctional rat endothelium can be confirmed to be caused by increased GRK2 expression by using one of these methods of *in vivo* GRK2 inhibition. Taguchi *et al.* used a specific chemical inhibitor of GRK2 kinase activity (βARK1 inhibitor) to restore aortic endothelial function in male *ob/ob* C57BL/6J mice, which we could administer via minipump along with TRAP-6 or saline (33,55). Additionally, adenovirus-expressing GRK2 shRNA could be injected into the femoral vein for successful knock down in the liver [32, 33, 51]. Any comparison between treatment groups will be carried out as described in question 3a. These experiments, along with our previous data, are likely to show that PAR-1-dependent NF-κB signaling is most robust in the early steps of endothelial dysfunction but is tuned down as the disease progresses. This will be due to GRK2's ability to inhibit Akt activity and this mechanism may very well apply to multiple receptors' abilities to activate NF-κB signaling in the context of established endothelial dysfunction (Figure 4-2).

Summary of chapter

In this chapter, I have outlined a series of experiments designed to explore the critical questions that come out of the work presented in this thesis and to take strides to increase the impact of our discoveries. Our findings that β-arrestins are indispensable for PAR-1-dependent NF-κB signaling raise a host of new questions which bring to light two new families of proteins, β-arrestins and GRKs, that are important for CBM complex-dependent NF-κB activity.

To determine which GRK is essential for recruiting the CBM complex to the receptor for NF-κB activation, we propose using siRNA knockdown to screen each GRK for its impact on PAR-1 signaling. To this end, we began by testing the role of GRK2, but

surprisingly we established that GRK2 acts as an inhibitor of PAR-1-dependent NF-κB signaling. The most probable mechanism by which GRK2 inhibits this response is through either promoting internalization/desensitization of PAR-1 receptors and/or causing direct inhibition of Akt activity. This chapter describes several experimental approaches to distinguish these two possibilities. Lastly, I propose here a series of *in vivo* experiments designed to explore the interplay of GRK2 and PAR-1-dependent NF-κB signaling in the initiation and progression of endothelial dysfunction, with a focus on the efficacy of GRK2 as a therapeutic target. Thus far, GRK2 has been described as a mediator of endothelial dysfunction due to its ability to inhibit the Akt/eNOS/NO pathway. Based on this observation, it has been suggested that blocking GRK2 activity may be an effective approach for the treatment of diseases in which endothelial dysfunction plays a prominent role. However, our *in vitro* findings that inhibition of GRK2 increases two important endothelial dysfunction markers, NF-κB activity and VCAM-1 expression, call this notion into question so that GRK2 inhibition as a treatment approach may be seriously flawed. The experiments outlined in this chapter should establish which is the more influential function of GRK2: inhibition of NO production or NF-κB activity. This will clarify the potentially paradoxical actions of GRK2 in vascular disease, leading to a better understanding of endothelial dysfunction and the regulation of PAR-1-dependent NF-κB signaling (Figure 4-2).

Summary of thesis

In this thesis, we provide new insights into the complex regulation of GPCR-dependent cell signaling. Our work establishes that PAR-1's recruitment of the CBM complex causes the activation of the potent pro-inflammatory NF-κB signaling pathway (Figure 2-2). The CBM complex is necessary for PAR-1's ability to induce the expression of NF-κB-dependent proteins important for endothelial dysfunction, such as VCAM-1 for

monocyte/endothelial cell adhesion (Figure 2-7, 2-8). We next explored the role of β-arrestins in PAR-1-dependent NF-κB activity. While β-arrestin 2 appears to recruit the CBM complex to the PAR-1 receptor, we found that β-arrestin 1 is also required for effective PAR-1-dependent NF-κB activation, unlike what has been shown for the LPA receptor (Figure 3-1). The requirement for β-arrestin 1 is likely due to its ability to mediate PAR-1-dependent ROCK and Akt activation, which are likely involved in IKK complex phosphorylation (Figure 2-4, 2-5, 3-3, 3-4). Lastly, while trying to discover the GRK responsible for the β-arrestin-dependent recruitment of the CBM complex to PAR-1, we discovered that GRK2 is an inhibitor of PAR-1-mediated NF-κB signaling, possibly due to GRK2's inhibition of Akt activity (Figure 3-5). This finding, along with previously published work on GRK2, suggests that GRK2 has conflicting effects on endothelial dysfunction, raising the question of which effect is more influential in endothelial biology (Figure 4-2). We believe that this research sheds new light on the poorly understood mechanism of NF-κB signaling by many, but possibly not all, GPCRs and we hope this will prove useful for the future development of treatments to combat GPCR-influenced pro-inflammatory disease.

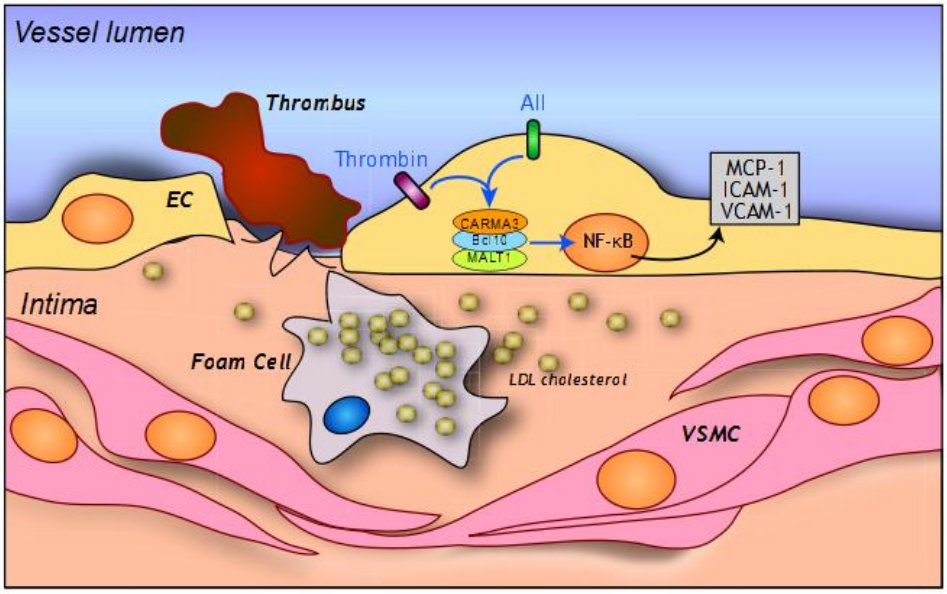

Figure 4-1. The CBM complex is at the crossroads of GPCR-dependent endothelial dysfunction. We have shown that both AGTR1 and PAR-1 use the CBM complex to induce endothelial dysfunction by activation of NF-κB signaling (8,9). See text for more detail.

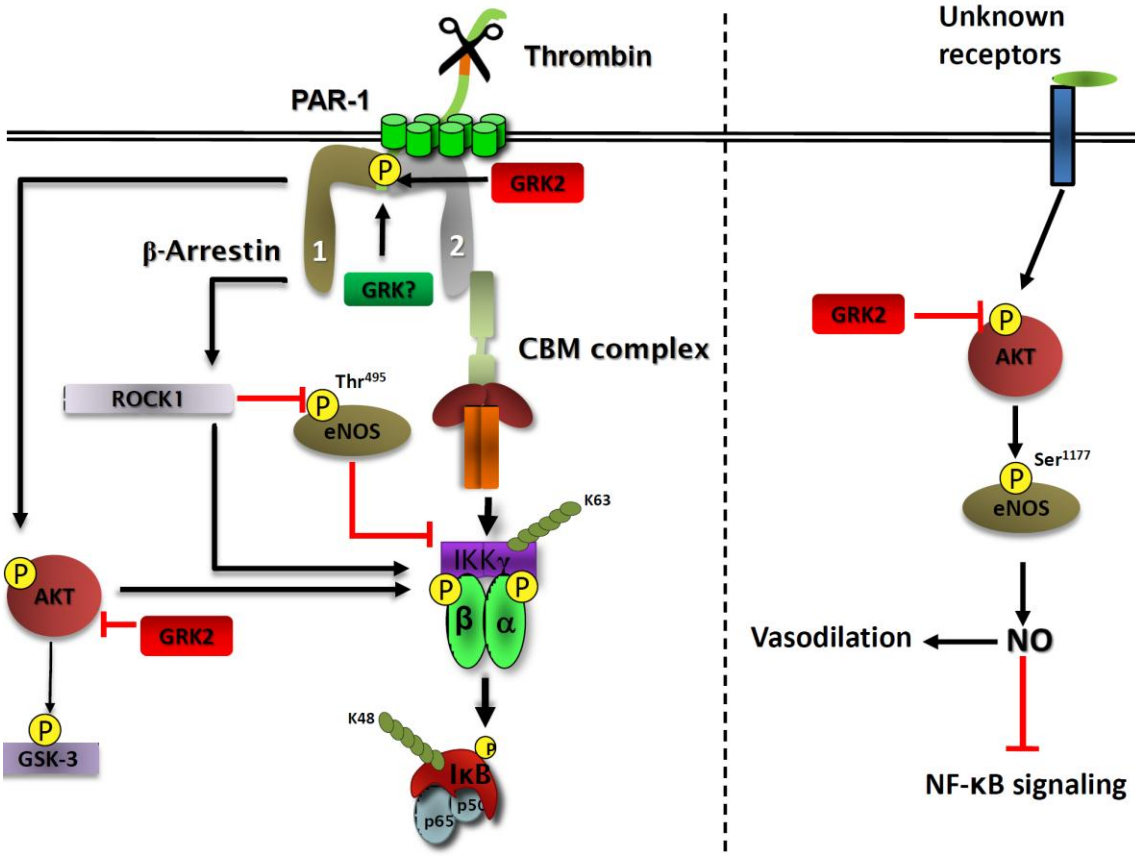

Figure 4-2. Mechanisms of regulation of PAR-1-dependent NF-κB signaling. The pathways on the left are involved in regulating PAR-1-dependent NF-κB signaling. On the right is the reported mechanism of GRK2 in inhibiting vasodilation (33,34). Others have reported that NO inhibits NF-κB signaling (30,31,56). See text for more detail.

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