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G Protein-Coupled Receptor Dependent NF-κB Signaling in Atherogenesis

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1. Introduction

Over the past decade we have witnessed an explosion of information regarding the molecular mechanisms underlying atherogenesis. While at one time atherosclerosis was viewed as a passive process of lipid deposition within muscular arteries, resulting in progressive luminal stenosis, we now understand that the process is much more complex. In particular, there is a growing appreciation for the role of both adaptive and innate immunity in atherogenesis, and for the contribution of other, non-traditional inflammatory stimuli. Indeed, atherogenesis is now understood primarily as an inflammatory disorder and much of the therapeutic focus has turned to devising approaches for reducing systemic levels of pro-inflammatory mediators and/or preventing these mediators from altering the biochemistry and physiology of the cells that make up the vessel wall. The inflammatory component of atherogenesis is particularly important from a clinical standpoint since it appears that atherosclerotic lesions characterized by on-going inflammation are those that are most unstable and susceptible to rupture, possibly leading to luminal thrombosis and acute myocardial infarction.

In this chapter, we provide a brief overview of the mechanisms underlying atherogenesis, highlighting known pro-inflammatory influences. We then focus on activation of the NF-κB family of transcription factors as a major molecular mediator of inflammation and summarize recent work that has provided new insights into how a diverse set of G protein-coupled receptors (GPCRs) may use a common mechanism to communicate NF-κB activation in cells native to the vessel wall, particularly endothelial cells. These discoveries may provide novel avenues for therapeutic intervention as we refine our approach to treating patients at risk for atherosclerosis.

2. Basic concepts and mechanisms in atherogenesis

Atherosclerosis is a chronic, progressive process through which lipid deposition, extracellular matrix production, immune cell infiltration, and smooth muscle cell proliferation all conspire to produce arterial obstruction and to disrupt normal arterial vasoreactivity (Hansson, 2005). Atherosclerosis and its related diseases account for nearly a third of all deaths, making it the

most common cause of disease-related death in the world (Hansson, 2005; Murray and Lopez, 1997). Although the process is gradual, often taking decades to proceed to a life-threatening stage, it is useful to think of atherogenesis as a series of distinct stages (Fig. 1). We will review these only briefly, as they are discussed in more detail elsewhere in this book.

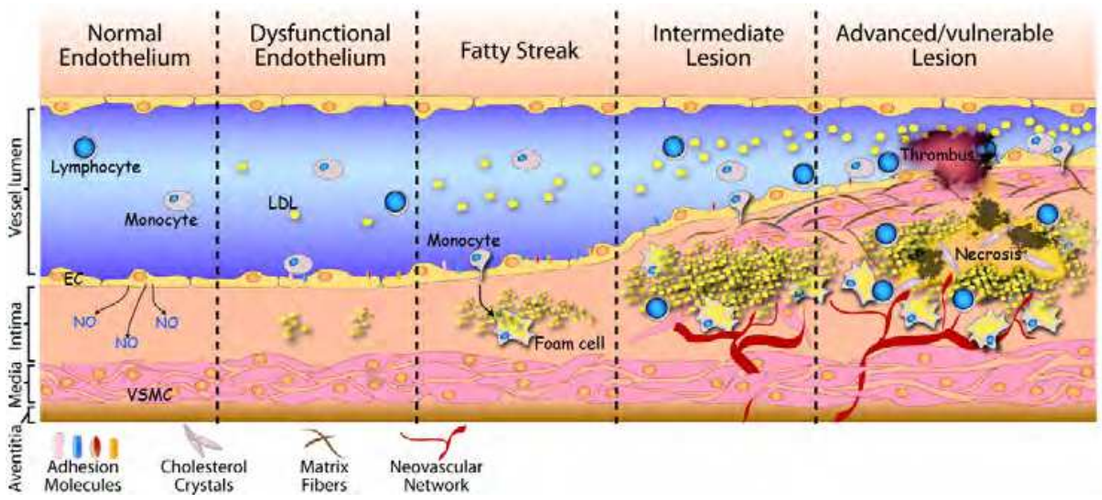

Fig. 1. The role of the endothelium through the stages of plaque formation. It should be noted that while endothelial dysfunction represents the first stage of plaque formation, it continues through all other stages as well. See text for a detailed description of each stage.

2.1 Endothelial dysfunction

The earliest recognizable stage in the development of atherosclerosis is characterized by changes in the cellular physiology of endothelial cells, referred to as endothelial dysfunction (Sitia et al., 2010). Endothelial cells form a single cell-thick, selectively permeable barrier, separating circulating blood components from the vessel wall. Aside from their barrier function, these cells also influence overall vessel function, in particular by regulating levels of nitric oxide (NO) which influences vascular contractility and tone (Jin and Loscalzo, 2010). Endothelial dysfunction ensues when these cells are exposed to injurious stimuli, resulting in a disruption in their ability to maintain a proper barrier and to promote vascular relaxation. As will be discussed, many features of endothelial dysfunction can be linked to the stimulation of signal transduction pathways culminating in NF-κB activation (de Winther et al., 2005). In particular, NF-κB activation induces expression of chemokines such as monocyte chemotactic factor (MCP-1), and adhesion molecules such as vascular and intercellular adhesion molecules (VCAM-1 and ICAM-1), which serve to recruit circulating monocytes and facilitate their process of transmigration through the endothelial barrier into the subendothelial space (de Winther et al., 2005) (Fig. 1). In addition, NF-κB activation plays a role in reorganizing tight and adherens junctions, which represent the glue connecting one endothelial cell to the next (Aveleira et al., 2010). Alteration in tight junctions can then influence the permeability of the endothelial layer to serum proteins and lipids. Finally, NF-κB activation has a complex role in controlling various aspects of NO production, and vice versa (Csiszar et al., 2008; Farmer and Kennedy, 2009; Laroux et al., 2001). As such, factors that act on endothelial cells to induce NF-κB represent important players in the initiation of atherogenesis.

2.2 Fatty streak

The fatty streak is a waxy yellow deposit in the subendothelial space that represents the first grossly visual evidence of atherogenesis (Packard and Libby, 2008). The fatty streak is formed by the accumulation of lipid and lipid-laden macrophages, also known as foam cells, which are recruited during endothelial dysfunction to the intimal space. While these streaks typically develop into more advanced lesions, they do have the potential to involute and resolve, so that at least at this stage, the process of atherogenesis is a reversible one. Lipid residing in the intima, particularly when in an oxidized form (Ox-LDL), can act to further endothelial dysfunction, initiating a vicious cycle that perpetuates fatty streak formation and can lead to more advanced lesions (Packard and Libby, 2008). This occurs in part through activation of scavenger receptors on the basolateral surface of endothelial cells, including the lectin-like oxidized LDL receptor-1 (LOX-1) (Mitra et al., 2011). Among other effects, LOX-1 activation upregulates VCAM-1 and ICAM-1 leading to further monocyte recruitment, upregulates the receptor for Angiotensin II (AGTR1), and increases release of reactive oxygen species (ROS) which cause further oxidation of LDL particles (Mitra et al., 2011).

2.3 Intermediate lesion

As the plaque progresses, there is further expansion of the intimal space with lipid and macrophages. Other leukocytes, including lymphocytes and mast cells, begin to accumulate and play key regulatory roles (Hansson, 2005; Packard and Libby, 2008). In this stage, vascular smooth muscle cells (VSMCs) begin to proliferate and some migrate into the superficial intima, leaving their usual position in the media. This occurs in response to increasing concentrations of growth factors released from endothelial cells and inflammatory cells in the developing lesion. The VSMCs in turn contribute to plaque size through their proliferation and through production of extracellular matrix proteins (collagen, elastin, proteoglycans). However, it is these matrix proteins and VSMCs that together form a protective fibrous cap separating the inflammatory core of the plaque from the endothelial layer (Libby et al., 2011). Thus, the integrity of the fibrous cap is essential for maintaining a stable lesion. Also during this stage, the vessel undergoes compensatory remodelling in an effort to maintain luminal patency, although there is invariably a progressive stenosis (Rader and Daugherty, 2008). Part of the remodelling process includes the ingrowth of a neovascular network, extending from the vasa vasorum of the outer adventitial layer of the vessel into the central portion of plaque (Libby et al., 2011) (Fig. 1). While this neovascularization serves a stabilizing function by providing for adequate blood supply to the plaque, and preventing cellular hypoxia in this region, these newly formed vessels are also leaky, delicate, and prone to rupture. In this way, neovascularization represents a double-edged sword, simultaneously promoting and risking lesion stability.

2.4 Advanced/vulnerable lesion

With increasing cycles of lipid deposition and inflammation, the plaque becomes progressively unstable and prone to rupture due to a multitude of factors. The lipid core may become necrotic, leading to release of cytotoxic substances and cellular debris. Hemorrhage of the lipid core microvasculature may occur, leading to intra-lesional thrombosis and production of pro-inflammatory molecules including thrombin. These effects attract more leukocytes to further weaken the plaque (Libby et al., 2011).

Ultimately, it is the integrity of the surface endothelial lining of the plaque that represents the greatest clinical concern. Any damage to these endothelial cells may then lead to exposure of the extracellular matrix to circulating blood. This includes the exposure of tissue factor (TF), which triggers the coagulation cascade and can lead to life-threatening local thrombosis. Damage to the endothelium may occur via processes occurring in the vessel lumen, for example as a consequence of shear stresses induced by hypertension and plasma turbulence. Alternatively, damage may come from below, as a consequence of inflammation in the plaque. Ongoing inflammation within the lipid core weakens the fibrous cap, in part because inflammatory cells release proteases that degrade the extracellular matrix. For example, macrophages release matrix metalloproteinase 9 (MMP-9), which alone is sufficient to induce rupture of advanced lesions in mice (Gough et al., 2006). T lymphocytes uniquely contribute to plaque instability via the production of IFN-γ which then downregulates VSMC matrix production (Packard and Libby, 2008). Thus, lesions that are most susceptible to rupture are those that have been weakened over time by the action of matrix-degrading proteases and have a paucity of VSMCs to provide a protective barrier separating the lipid core from surface endothelial cells (Libby et al., 2011).

3. Specific GPCR agonists as contributors to vascular inflammation

The GPCR family represents the largest family of cell surface receptors, and includes over 800 known members (Dorsam and Gutkind, 2007; Lappano and Maggiolini, 2011). 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 (Strader et al., 1994). Members of this receptor family respond to a diverse array of ligands including peptides, amines, glycoproteins and enzymes. The receptors relay extracellular signals by activating multiple intracellular signaling pathways which include those for ERK, Akt, JNK, p38MAPK, STAT, and NF-κB activation, to name only a few (Dorsam and Gutkind, 2007; Lappano and Maggiolini, 2011). Several GPCR agonists have been identified as key regulators of both endothelial cell function and atherogenesis. Below we discuss three such agonists that play particularly prominent roles in the pathophysiologic stages of atherogenesis.

3.1 Angiotensin II

Angiotensin II (Ang II) is a GPCR agonist, long known for its classic role in controlling blood pressure through regulating vascular smooth muscle tension, influencing renal reabsorption of sodium and water, and through stimulating aldosterone release from the adrenal. However, in recent years our understanding of this peptide hormone has broadened, and it is now appreciated that Ang II exerts a much wider spectrum of responses (Luft, 2001). In particular, Ang II is now appreciated for its profound pro-inflammatory effects, exerted on both endothelial and smooth muscle cells of the vasculature (Phillips and Kagiyama, 2002). Through this role, Ang II is thought to promote atherogenesis via mechanisms that are independent from its impact on blood pressure. Consistent with this notion, animal models of Ang II-dependent atherosclerosis, as well as large clinical trials investigating angiotensin converting enzyme (ACE) inhibitors or AGTR1 blockers (eg, HOPE, EUROPA, and LIFE), have demonstrated that the contribution of Ang II to atherogenesis cannot be explained solely by its ability to promote hypertension (Bertrand, 2004; Ferrario and Strawn, 2006; Kintscher et al., 2004). Instead, there is emerging evidence that perhaps the greatest impact of Ang II lies in its ability to directly induce pro-inflammatory signal transduction.

AGTR1 is expressed on both endothelial cells and VSMCs (Brasier et al., 2002). In addition, all the components of a local renin-angiotensin system (RAS) exist within the vasculature, so that Ang II can be locally produced and act in an autocrine or paracrine fashion, supplementing the effects of systemically circulating Ang II (Sata and Fukuda, 2010). Although Ang II influences numerous aspects of endothelial cell physiology, at least four categories of genes are induced that contribute to atherogenesis (Fig. 2). These include **1)** genes whose products promote recruitment and activation of monocytes and other inflammatory cells (eg, chemokines, cytokines, and adhesion molecules such as MCP-1, IL-6, IL-8, ICAM-1, VCAM-1, and E-selectin), **2)** genes whose products destabilize plaque and promote both proliferation and migration of underlying VSMCs (eg, MMP-9, PAI-1, and IGF-1R), **3)** genes whose products mediate endothelial dysfunction, particularly in the presence of oxidized LDL (eg, LOX-1), and **4)** genes encoding secondary cytokines that can feed back to ECs and VSMCs, further enhancing the pro-inflammatory milieu (eg, TNFα, Il-1β) (Fig. 2) (Brasier et al., 2002; de Winther et al., 2005; Pober and Sessa, 2007). Importantly, activation of LOX-1 sets in motion a destructive feed-forward cycle, whereby it enhances the expression of both AGTR1 and angiotensin converting enzyme (ACE) (Li et al., 2000; Li et al., 2003). This in turn results in enhanced local production of Ang II (Fig. 2). As will be discussed, all the above are NF-κB regulated genes, highlighting NF-κB activation as a key pro-atherogenic signaling event.

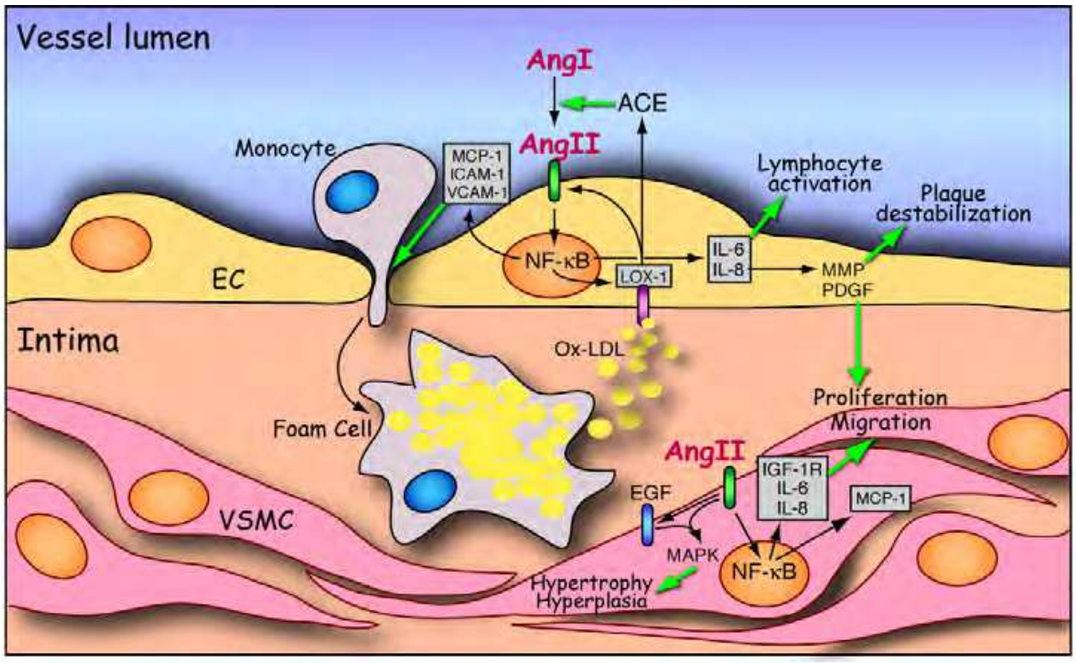

Fig. 2. Pleiotropic effects of Ang II on vascular pathophysiology.

Ang II, either made locally within the vessel wall or present following diffusion from the vessel lumen, can also act on VSMCs. Here, AGTR1 activation results in many of the same pro-inflammatory responses that are seen in endothelial cells. Additionally, MAPK pathways are activated, partly through transactivation of EGF receptors, thereby promoting the hypertrophy and hyperplasia of VSMCs that is characteristic of atherosclerotic lesions (Fig. 2) (Eguchi et al., 1998; Ohtsu et al., 2006; Saito and Berk, 2001).

3.2 Thrombin

Thrombin is known historically for its role in the clotting cascade. Active thrombin is generated from the inactive precursor, prothrombin, via cleavage by a complex consisting of factor Xa and factor Va, assembled through the actions of tissue factor (TF) (Borissoff et al., 2011). Thrombin then plays a role in generating a stable clot, in part through cleaving fibrinogen to produce fibrin, and through its actions on platelets. However, as is the case for Ang II, there is increasing appreciation for the receptor-mediated, pro-inflammatory effects of thrombin. Unlike most GPCR agonists, thrombin is a serine protease and acts on its cognate receptors through an unusual mechanism. The best studied thrombin receptor on endothelial cells is perhaps the protease activated receptor-1 (PAR-1) (Borissoff et al., 2009). In this case, thrombin binds to an extracellular hirudin-like domain on PAR-1 and cleaves the receptor at a specific site, exposing a cryptic ligand, SFLLRN, present near the N-terminal tail of the receptor. The newly exposed amino acid sequence acts as a tethered ligand by binding to a pocket on extracellular loop 2 and permanently activating the receptor (Borissoff et al., 2009). A synthetic peptide with the same SFLLRN sequence, also known as TRAP-6, can be used to induce the same response from PAR-1 as thrombin (Coughlin, 2005).

Both PAR-1 and its agonist thrombin are major participants in the regulation of endothelial cell biology and atherogenesis, affecting cell signaling, gene expression, endothelial permeability, angiogenesis, and vascular tone (Hirano, 2007). Indeed, the importance of direct pro-atherogenic effects of thrombin on cells of the vessel wall were recently highlighted by a study in mice showing that atherosclerosis can proceed independently of thrombin-induced platelet activation (Hamilton et al., 2009). As with Ang II, many of the effects of thrombin on endothelial cells can be mechanistically linked to NF-κB activation.

3.3 IL-8

A vast array of chemokines and associated GPCRs exist that influence vascular biology (Rosenkilde and Schwartz, 2004). For the purposes of this review, we highlight only one, IL-8 (CXCL8), because of the recent work demonstrating parallels between the molecular signaling pathways activated by IL-8 and those activated by both Ang II and thrombin (Martin et al., 2009). IL-8 is a CXC chemokine with many immunomodulatory functions and a broad range of biological effects. The effects of IL-8 are mediated primarily through CXCR2, a GPCR that is expressed on a broad range of cells, including endothelial cells (Rosenkilde and Schwartz, 2004). IL-8 is upregulated within developing atherosclerotic lesions, in part due to the stimulatory effect of Ox-LDL (Braunersreuther et al., 2007). Among its many effects, IL-8 induces expression of vascular endothelial growth factor (VEGF), which is synthesized and released by endothelial cells and can act in an autocrine/paracrine fashion to induce angiogenesis within the lipid core and to increase vascular permeability (Gavard et al., 2009). As with Ang II and thrombin, many of the pro-inflammatory effects of IL-8 can be attributed to the activation of NF-κB. In particular, IL-8 induction of the *VEGF* gene occurs through NF-κB binding sites in its promoter (Martin et al., 2009). See Table 1 for a summary of several pro-atherogenic effects of IL-8, and other GPCR agonists, that have been ascribed to NF-κB activation.

GPCR	Affected cell type in plaque	NF-κB related pro-atherogenic effects
Angiotensin II Type 1 receptor	Endothelial cells	• Release of pro-inflammatory cytokines • Recruitment and activation of inflammatory cells • Destabilization of plaque
	VSMCs	• Proliferation and migration into plaque
CXCR2 (IL-8 receptor)	Endothelial cells	• Expression of VEGF • Promotion of plaque neovascularization • Enhancement of vascular permeability
Protease Activated Receptor-1 (thrombin receptor)	Endothelial cells	• Recruitment of inflammatory cells • Enhancement of vascular permeability • Promotion of plaque neovascularization • Stimulation of vasoconstriction

Table 1. Selected effects of three GPCRs and their ligands on atherogenesis.

4. GPCR connectivity to NF-κB

For ligand activated GPCRs, many, but not all, signaling events are initiated through the activation of heterotrimeric G proteins (Dorsam and Gutkind, 2007; Lappano and Maggiolini, 2011). The cytoplasmic loops within GPCRs serve to recruit these G proteins, consisting of α, β, and γ subunits. Upon agonist binding, the receptors promote exchange of GDP for GTP on the Gα subunit, leading to its dissociation from the Gβγ subunits. Both GTP-bound Gα and Gβγ subunits are then able to stimulate a range of downstream effectors. At least part of the specificity in receptor signaling stems from the fact that there are numerous Gα subtypes, broadly grouped into four classes ($G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$), and GPCRs will preferentially couple to certain subtypes (Dorsam and Gutkind, 2007; Lappano and Maggiolini, 2011).

Receptors that couple to $G\alpha_{q/11}$ are known to activate protein kinase C (PKC) isoforms through G protein-dependent stimulation of phospholipase C, Ca^{2+} mobilization, and DAG generation. These include receptors for agonists described above (Ang II, thrombin, IL-8) as well as others that potentially influence endothelial biology in the context of atherogenesis, including endothelin-1, lysophosphatidic acid (LPA), and SDF-1/CXCL12 . For some time, it has been clear that PKC activation by select GPCRs is a prerequisite for subsequent NF-κB activation, but we are only now beginning to unravel the specific mechanistic links between PKC and the NF-κB machinery. In order to discuss these links, we will first briefly review the salient features of the NF-κB family of transcription factors, and their regulation. For a more nuanced treatment, the reader is referred to one of the more complete reviews of the topic (Hayden and Ghosh, 2008; Oeckinghaus and Ghosh, 2009; Vallabhapurapu and Karin, 2009).

4.1 The NF-κB family

The NF-κB family denotes a group of five transcription factors and includes the proteins RelA (p65), RelB, c-Rel, NF-κB1 (p105/50), and NF-κB2 (p100/52) (Oeckinghaus and Ghosh, 2009). All share a highly conserved Rel homology domain (RHD) which directs their dimerization, nuclear localization, and DNA binding activities (Oeckinghaus and Ghosh, 2009). Upon entering the nucleus, NF-κB subunits bind to the NF-κB consensus sequence, GGPuNNPyPyCC, present within the regulatory regions of target genes. Along with an array of co-factors, NF-κB transcription factors are able to induce or repress transcription of a wide variety of genes. Several pathways exist for activating NF-κB, depending upon the specific cellular stimulus, and this affects which NF-κB subunits are recruited into action.

4.2 The canonical NF-κB signaling pathway

In the unstimulated cell, the canonical NF-κB subunits, RelA and p50, stand at the ready in the cytoplasm, retained there by a family of regulatory proteins termed inhibitors of κB (IκBs). These IκB proteins conceal the nuclear localization sequences on RelA and p50, preventing their nuclear translocation. Various stimuli, including ligands for the TNF, interleukin, Toll-like, and antigen receptor families, act to induce intracellular signaling pathways that culminate in activation of the chief canonical regulatory complex, termed the IκB kinase (IKK) complex (Fig. 3A). The IKK complex is composed of three principal subunits, one regulatory subunit (NEMO/IKKγ), and two catalytic subunits (IKKα and IKKβ) (Oeckinghaus and Ghosh, 2009). Once activated, the catalytic subunits, particularly IKKβ, phosphorylate IκBs, leading to their ubiquitination and proteosomal degradation. This frees the RelA/p50 complex for nuclear translocation and transcriptional regulation.

While the steps leading from IKK activation to IκB phosphorylation and degradation are well-conserved, no matter what the stimulus, specificity is built into the system in that different receptors use vastly different signaling mechanisms for communicating with the IKK complex (dotted lines in Fig. 3A). It is in dissecting these "private pathways" for IKK activation that much of the recent progress in NF-κB research has been made. This is a crucial area of discovery, since identifying molecules that specifically mediate IKK activation in response to selected receptor ligands may allow for development of pharmaceuticals that interrupt (or enhance) the response to those ligands and not others. This could be a critically important area of discovery since general inhibition of NF-κB can have substantial negative side-effects including the initiation of a generalized state of immunodeficiency, or impairment of growth/development.

4.3 The non-canonical (alternative) NF-κB signaling pathway

A distinct set of stimuli, including CD40 ligand, BAFF, and lymphotoxin-β, work to activate a second set of NF-κB subunits (Oeckinghaus and Ghosh, 2009). Activation of their cognate receptors causes phosphorylation of p100, in complex with its partner, RelB (Fig. 3B). This occurs through the kinase activity of IKKα, but does not require the other components of the IKK complex. Instead, IKKα activation requires the upstream activation of NF-κB inducing kinase (NIK), which serves not only to phosphorylate and activate IKKα, but also appears to assist in recruiting p100. Once phosphorylated, p100 undergoes partial proteolysis, producing p52, and it is the RelB/p52 complex that is active as a regulator of transcription. Thus, in essence the p100 precursor acts much the same as

an IκB, preventing non-canonical NF-κB activity. An area of intense interest relates to the question of whether the non-canonical NF-κB complex of RelB/p52 regulates a distinct set of genes from those regulated by RelA/p50. Importantly, while GPCRs are known to stimulate the canonical pathway, their potential for activating non-canonical signaling remains to be elucidated.

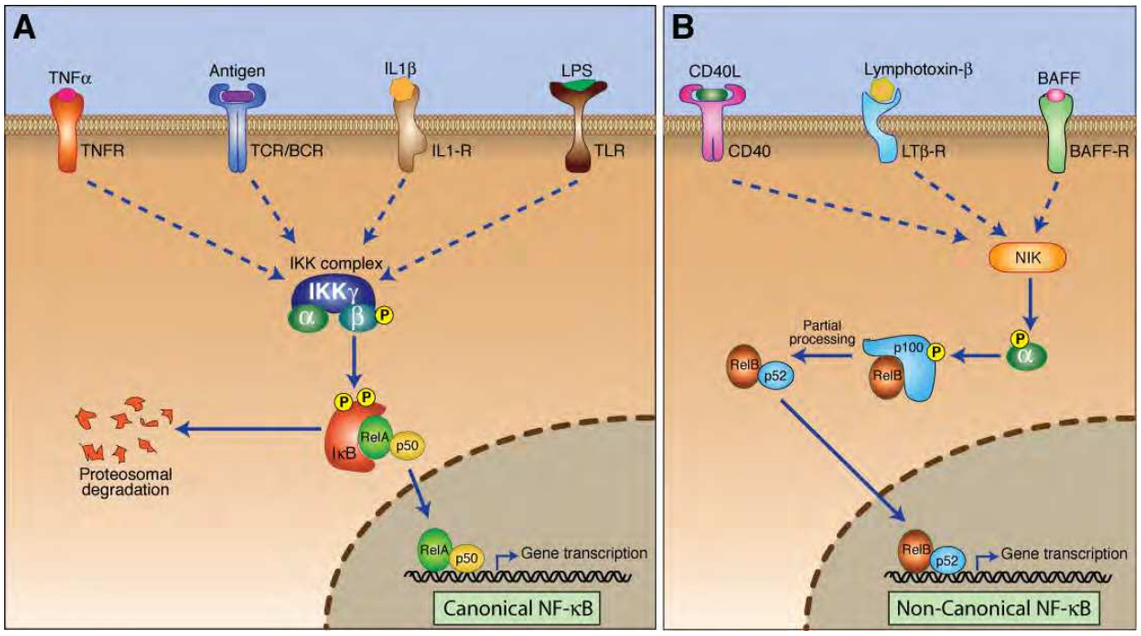

Fig. 3. Distinct pathways for canonical and non-canonical NF-κB activation.

4.4 Unique/emerging NF-κB signaling pathways

Although the canonical and non-canonical pathways for NF-κB activation have historically received the most attention, it is clear that several other mechanisms are in place for activating NF-κB subunits. Bearing in mind that several different NF-κB heterodimer complexes have been identified, it is likely that our understanding of alternative routes for NF-κB activation will only grow as the regulation of these complexes is explored in more detail. For example, recent work in B cells has demonstrated that c-Rel/p50 heterodimers can be regulated by a unique, non-proteosome dependent pathway for IκB degradation (O'Connor et al., 2004). Further, kinases other than IKKβ have been identified that can act to phosphorylate IκB proteins, leading to their degradation (McElhinny et al., 1996; Schwarz et al., 1996). Finally, other levels of control exist beyond the simple degradation of IκB proteins. Several groups have shown that NF-κB subunits are targets of secondary modification, including phosphorylation and acetylation, alterations that can affect their ability to interact with both DNA consensus sites and transcriptional co-regulators. Thus, as the body of experimental data grows, it will no doubt become obvious that the concepts of canonical and non-canonical activation, outlined above and in Fig. 3, represent only a framework for a much more complicated system of regulation.

4.5 GPCR dependent NF-κB signaling

Activation of certain GPCRs expressed on vascular cells, including the receptors for Ang II, thrombin, and IL-8, leads to all the hallmarks of canonical NF-κB activation. Although the precise mechanisms underlying this response have been unclear, it has long been appreciated that canonical activation requires proximal stimulation of PKC (Fraser, 2008). For example, Ang II induction of NF-κB in both endothelial cells, VSMCs, and cardiomyocytes is tightly linked to activation of PKC, although the specific PKC isoform responsible may differ depending on the cell type (Brasier et al., 2000; Hiroki et al., 2004; Kalra et al., 2002; Liao et al., 1997; Parmentier et al., 2006; Rouet-Benzineb et al., 2000). For PAR-1, PKCδ is known as the primary PKC mediating NF-κB activation in endothelial cells (Minami et al., 2004; Rahman et al., 2001). PKCα and δ have both been implicated in LPA-dependent NF-κB activation, in ovarian cancer cells and in airway epithelial cells, respectively (Cummings et al., 2004; Mahanivong et al., 2008). Finally, several PKC isoforms, including PKCα and PKCβ, have been shown to mediate IL-8/CXCR2-dependent signaling, but these studies have not been performed in endothelial cells or VSMCs (Waugh and Wilson, 2008).

In that upstream PKC activation is a prerequisite for GPCR-responsive NF-κB signaling, we and others recognized a parallel theme with the antigen-responsive activation of NF-κB in lymphocytes. In B lymphocytes, antigen receptor ligation induces PKCβ, and this is critical for subsequent NF-κB activation, while in T lymphocytes it is PKCθ that is crucial (Lucas et al., 2004). Over the past decade, a tremendous volume of data has been generated to define the precise molecular steps linking PKC activation with the NF-κB machinery in lymphocytes. This work revealed that a multi-protein signaling complex, termed the CARMA1/Bcl10/MALT1 (CBM) signalosome serves as a molecular bridge between the two, and is necessary for lymphocytes to mount a normal, NF-κB-dependent immune response to antigenic challenge (Lucas et al., 2004; Thome, 2004; Wegener and Krappmann, 2007). Taking cues from the lymphocyte field, we and others worked to define a novel molecular pathway that explains how GPCR-dependent PKC activation can result in NF-κB signaling. This pathway utilizes an analogous CBM signalosome, present in cells outside of the immune system, and is detailed in the next section.

It is important to note, however, that the discovery of a GPCR-responsive CBM signalosome must be viewed in the larger context of GPCR signaling, with the realization that other signaling pathways are active, some of which may influence NF-κB through independent mechanisms. For example, Brasier and colleagues have uncovered a distinct mechanism by which ligand-activated AGTR1 induces RelA in VSMCs (Brasier, 2010). In these cells, substantial levels of RelA are found inactive in the nucleus under resting conditions. Ang II stimulation induces a pathway of RhoA and NIK activation, culminating in NIK-dependent phosphorylation of the nuclear RelA species on serine 536 (Choudhary et al., 2007; Cui et al., 2006). This phosphorylated pool of RelA is free from IκB regulation and dynamically cycles through the nucleus, interacting with target genes (Bosisio et al., 2006; Sasaki et al., 2005). This mechanism of regulation has been shown to impact the NF-κB responsive, *IL-6* gene. Consistent with this observation, we have seen only a partial effect of blocking the CBM signalosome on Ang II-dependent IL-6 induction, underscoring the concept that different NF-κB responsive genes may respond to different NF-κB transcription factor complexes and/or different modes of NF-κB regulation.

5. The CARMA3/Bcl10/MALT1 signalosome; missing link for GPCR activity

5.1 Lessons learned from lymphocytes

CARMA1 (also known as Bimp3/CARD11) is a member of the membrane-associated guanylate kinase (MAGUK) superfamily of molecular scaffolds that each utilize multiple discrete protein interaction domains to cluster receptors and cytosolic signaling molecules at the cell membrane (Dimitratos et al., 1999; Fanning and Anderson, 1999). As such, all MAGUKS contain three defining interactions domains: the PSD-95/Dlg/ZO-1 homologous (PDZ) domain, the Src-homology (SH3) domain, and the guanylate kinase (GUK)-like domain (Fig. 4A). CARMA1 is expressed exclusively in lymphocytes, and a few related cells of the immune system, and is one of three known members of the CARMA subfamily. This subfamily is distinguished from members of other MAGUK subfamilies by the presence of additional coiled-coil and caspase recruitment (CARD) domains.

Numerous biochemical and genetic studies have now definitively established that CARMA1 is an essential component of the antigen-induced NF-κB signaling pathway in T cells. Data indicate that CARMA1 acts as a molecular bridge, linking PKC activation with stimulation of the downstream signaling proteins, Bcl10 and MALT1 (Fig. 4B) (Lucas et al., 2004). Together, CARMA1, Bcl10, and MALT1 form a complex (referred to as the CBM signalosome) that is recruited to the lymphocyte immunological synapse following receptor engagement. In this complex, the small Bcl10 protein appears to function as an adaptor, capable of oligomerizing MALT1. Finally, MALT1 acts as an effector subunit by stimulating IKKγ, the IKK regulatory subunit, in part through promoting its K63-linked ubiquitination (Sun et al., 2004; Zhou et al., 2004). This subsequently leads to activation of the catalytic subunits, IKKα and IKKβ, thereby allowing them to phosphorylate IκB and free NF-κB for nuclear transport. The details as to how MALT1 achieves regulation of the IKK complex are still unfolding, with the current dogma suggesting that the process includes a coordinated series of K63-linked ubiquitination events, not only of IKKγ but of several other proteins in the complex, as well as IKKβ phosphorylation, probably via the kinase TAK1.

A second mechanism of action for MALT1 has recently emerged and gained considerable attention. This relates to the discovery that MALT1 is a substrate-specific protease (McAllister-Lucas and Lucas, 2008). Although such enzymatic activity has long been postulated, based on recognition of a "caspase-like" active site in the C-terminus of MALT1 (Uren et al., 2000), it wasn't until only recently that substrates for MALT1 cleavage were identified. So far, three have been identified, and their cleavage sites mapped (Coornaert et al., 2008; Rebeaud et al., 2008; Staal et al., 2011). Two of the substrates, A20 and CYLD, are deubiquitinases, known for their ability to dampen NF-κB signaling through their ubiquitin editing functions, affecting various players in the NF-κB machinery. Thus, by targeting these two proteins for cleavage, it is thought that MALT1 proteolytic activity serves to maximize the level of NF-κB activation, following antigen stimulation of lymphocytes. Indeed, cleavage of A20 leads to loss of its inhibitory effect and magnified antigen-dependent NF-κB activation (Coornaert et al., 2008). In theory, the same could occur with cleavage of CYLD, although initial work has only shown an impact on the related JNK pathway (Staal et al., 2011).

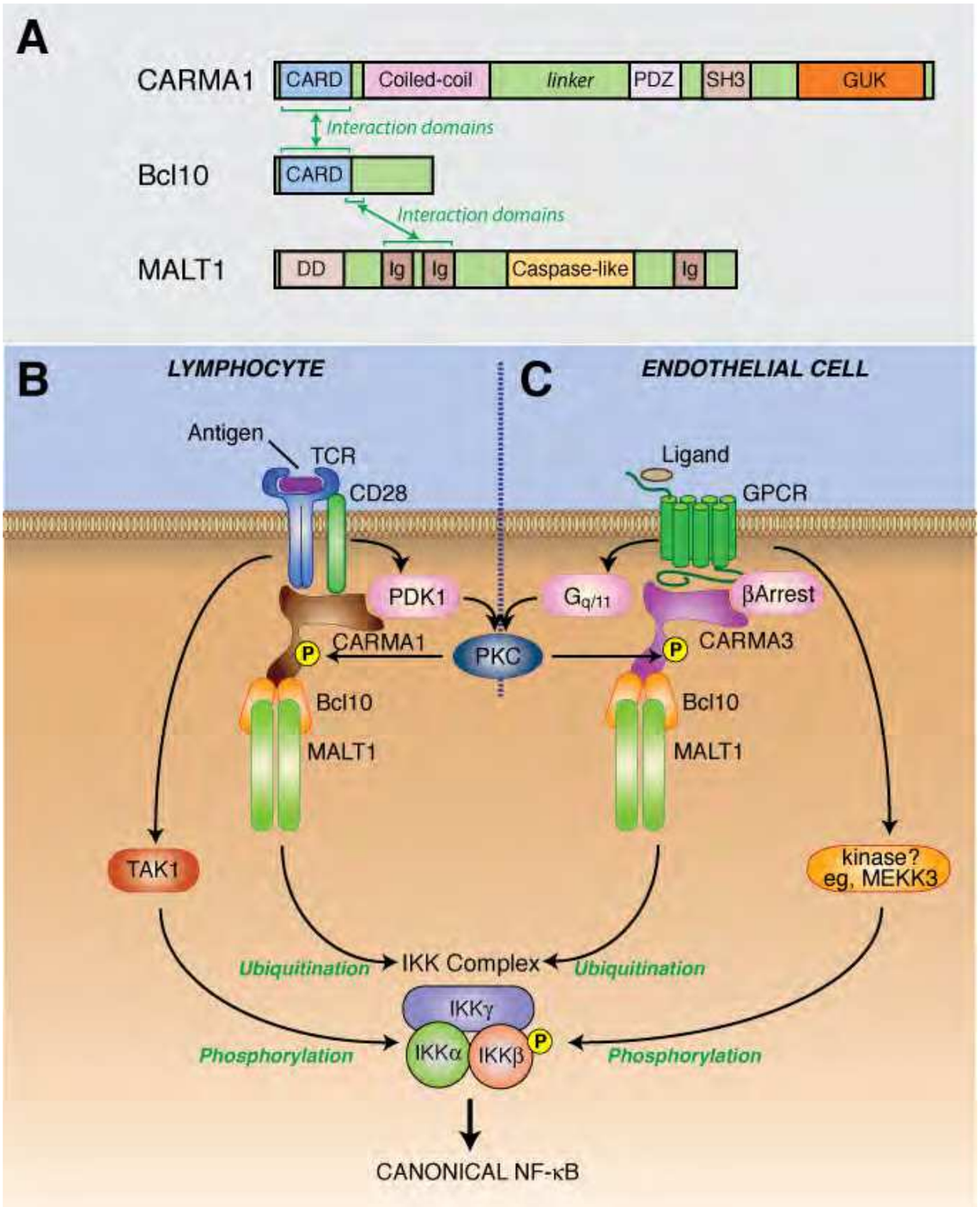

Fig. 4. A, Schematic diagram of proteins that make up the CBM signalosome. B and C, Similarities and differences exist between the mechanisms through which the CARMA1- and CARMA3-containing signalosomes act to stimulate the IKK complex; see text for description.

Many of the details concerning how the CBM complex is recruited and activated at the T cell immunological synapse have become clear only recently. First, Ghosh and co-workers demonstrated that, following T cell receptor stimulation, the enzyme 3-phosphoinositide-dependent kinase 1 (PDK1) serves to anchor both activated PKCθ and CARMA1 within close proximity to one another, at the immunological synapse (Fig. 4B) (Lee et al., 2005). PDK1 is a kinase known to phosphorylate PKC at a specific site within its "activation loop" (Belham et al., 1999; Mora et al., 2004; Newton, 2001, 2003). This phosphorylation appears to serve as a priming reaction, allowing PKC to then respond to activating signals, such as Ca^{2+} or DAG, depending on the specific isoform. However, the ability of PDK1 to interact with CARMA1 represents a newly defined role for this kinase. Although PDK1 plays a key role in recruiting the CBM signalosome to the T cell receptor, it may not represent the only molecular link; for example, recent work has demonstrated that the protein ADAP is also crucial in this regard (Medeiros et al., 2007).

A second major finding was that PKC acts to phosphorylate specific sites within the linker region of CARMA1, which resides between the CARD/coiled-coil domains and the domains present in all MAGUK proteins (PDZ/SH3/GUK) (Matsumoto et al., 2005; Sommer et al., 2005). This appears to result in a conformational change in CARMA1, allowing for exposure of the CARD domain (Sommer et al., 2005). Consequently, Bcl10 and MALT1 can then be effectively recruited to the immunological synapse because their recruitment depends primarily on a CARD-CARD interaction between CARMA1 and Bcl10. Finally, the IKK complex is recruited and can thereby be activated by the fully assembled CBM complex (Shinohara et al., 2005; Stilo et al., 2004; Weil et al., 2003). It should be noted that the majority of the work defining the mechanism of action of the CBM signalosome has been carried out using T cell models. Interestingly, not all of the concepts are likely to hold true for B cells. In particular, based on phenotypic differences between *MALT1*$^{-/-}$ mice and *BCL10*$^{-/-}$ or *CARMA1*$^{-/-}$ mice, there is some debate as to whether MALT1 is an obligate player in B cell receptor-dependent NF-κB activation.

5.2 CARMA3, a CARMA homologue expressed in cells outside the immune system

Except for CARMA1, the key molecules mediating antigen-dependent NF-κB activation in lymphocytes are ubiquitously expressed in a diverse array of cells. However, we and others had noted that a highly related protein, CARMA3 (Bimp1/CARD10), is expressed more broadly than the immune cell-specific CARMA1 (McAllister-Lucas et al., 2001; Wang et al., 2001b). The *CARMA1* and *CARMA3* genes encode proteins that are highly similar; the CARDS and coiled-coil domains share approximately 60% and 50% sequence identity with one another, respectively, while the PDZ, SH3 and GUK domains share approximately 20-30% identity. The functional similarities between CARMA3 and CARMA1 are illustrated by the fact that CARMA3 can rescue antigen-induced NF-κB activation in CARMA1-deficient T cells (Matsumoto et al., 2005). Of potential importance to cardiovascular pathophysiology, all three proteins of a putative CARMA3-containing CBM complex are abundant in heart and aorta, and western blotting confirms their presence in those tissues at the protein level (McAllister-Lucas et al., 2010; McAllister-Lucas et al., 2007). As a result, we wondered if CARMA3 might scaffold an analogous CBM signalosome in cells outside the immune system. The known dependence on PKC activation for GPCR-responsive NF-κB stimulation led to the hypothesis that specific GPCRs might represent candidates for receptors that could communicate with CARMA3 and it associated signaling molecules.

Using distinct systems and approaches, we and two other groups simultaneously demonstrated that two GPCRs could harness a CARMA3-containing CBM signalosome for the purposes of NF-κB activation (Klemm et al., 2007; McAllister-Lucas et al., 2007; Wang et al., 2007) (Fig. 4C). Our group demonstrated the essential role of CARMA3, Bcl10, and MALT1 in the Ang II-dependent activation of canonical NF-κB (McAllister-Lucas et al., 2007). This initial work focused on hepatocytes as a model, but subsequent work demonstrated that the same CBM machinery is active in endothelial cells following Ang II stimulation (McAllister-Lucas et al., 2010). Individually knocking down each component of the putative CARMA3/Bcl10/MALT1 complex completely blocked Ang II-dependent IκB phosphorylation, a marker of canonical NF-κB activation, or induction of an NF-κB responsive reporter gene. In addition, expression of a dominant negative mutant of CARMA3 was sufficient to impair Ang II-dependent K63-linked IKKγ polyubiquitination.

The other two studies focused on mouse embryonic fibroblasts (MEFs) from *BCL10*$^{-/-}$ and *MALT1*$^{-/-}$ mouse strains. In contrast to wild-type MEFs, these knockout cells showed a complete lack of NF-κB activation when stimulated with lysophosphatidic acid (LPA) (Klemm et al., 2007; Wang et al., 2007). Follow-up work revealed the same phenomenon with *CARMA3*$^{-/-}$ MEFs (Grabiner et al., 2007). Like the receptor for Ang II (AGTR1), receptors for LPA ($LPA_{1\text{-}4}$) are prototypical GPCRs, coupled primarily with $G\alpha_{q/11}$ subunits. With regard to pathophysiology, LPA receptor-induced NF-κB activation has been linked to a variety of consequences, depending upon the cell type affected, which include the promotion of carcinoma cell survival and spread, as well as endothelial dysfunction. However, despite the potential importance of the CBM complex in mediating LPA-dependent effects in endothelial cells or VSMCs, studies have yet to be published that specifically link LPA receptors to the CBM components in the vasculature.

Following these initial studies, several groups have added to the list of GPCRs capable of harnessing the CARMA3-containing CBM signalosome. To date, however, the only receptors that have been specifically shown to utilize the signalosome in endothelial cells are those for Ang II (AGTR1), thrombin (PAR-1) and IL-8 (CXCR2) (Delekta et al., 2010; Martin et al., 2009; McAllister-Lucas et al., 2010).

5.3 Distinct mechanisms for recruiting CARMA1- and CARMA3-containing signalosomes

Although there are strong parallels between the mechanisms underlying recruitment and activation of the CARMA1-containing CBM signalosome of lymphocytes and the CARMA3-containing CBM signalosome of endothelial cells, there are also notable differences. Most striking are the differences in how the signalosomes communicate with their cognate receptors. We have already described work demonstrating a crucial role for PDK1 in scaffolding an interaction with antigen receptors in the T cell. In contrast, PDK1 may have no role in coordinating GPCR-dependent CBM assembly; at least for PAR-1, knockdown of PDK1 in endothelial cells has no effect on thrombin-dependent NF-κB activation (Delekta et al., 2010). Instead, we and others have implicated β-arrestin 2 as a protein that could serve the function of scaffolding CARMA3 to select GPCRs (Delekta et al., 2010; Sun and Lin, 2008).

Traditionally, the β-arrestin proteins have been known for their role in down-regulating activated GPCRs through receptor endocytosis, leading to their recycling or degradation. Activated GPCRs are phosphorylated by various G protein receptor kinases (GRKs),

which then allows recruitment of arrestins to the receptors. This helps to uncouple G proteins from the receptors and assists in receptor internalization through clathrin-coated pits. But more recently, arrestins have become known as scaffold proteins that facilitate the recruitment and activation of a number of distinct secondary signaling molecules (DeFea, 2011). In this way, arrestins are now appreciated not just for their ability to terminate GPCR signaling, but also for their ability to promote a second layer of GPCR-dependent responses.

Lin and colleagues originally showed that β-arrestin 2 deficient MEFs were unable to respond to LPA treatment with an NF-κB signal (Sun and Lin, 2008). They then determined that β-arrestin 2 bound to CARMA3. Further, co-immunoprecipitation experiments demonstrated that CARMA3 could interact with the LPA receptor only when β-arrestin 2 was present to act as a bridge. Subsequent work showed that β-arrestin 2 deficient MEFs are also defective in thrombin-dependent NF-κB activation (Delekta et al., 2010). Thus, it is likely that for GPCR signaling, β-arrestin 2 serves a scaffolding role, analogous to what has been shown for PDK1 in the lymphocyte system (Fig. 4C).

Despite the progress that has been made in understanding the regulation of CARMA3-containing CBM signalosomes, much remains to be learned. The CARMA1-containing complex has been studied for a much longer period of time, and many of the finer details have been explored in more detail. For example, the sites of PKC-dependent CARMA1 phosphorylation, which allow for unfolding of CARMA1 and exposure of the CARD domain, have been mapped (Matsumoto et al., 2005; Sommer et al., 2005). In addition, other kinases have been identified that can positively or negatively regulate CARMA1 (Bidere et al., 2009; Brenner et al., 2009; Ishiguro et al., 2006; Shinohara et al., 2007). In contrast, mechanisms for regulation of CARMA3 remain mostly speculative at this time.

5.4 Distinct mechanisms for CARMA1- and CARMA3-dependent IKK activation

Another area of potential distinction, differentiating CARMA1- and CARMA3-containing signalosomes, relates to their ability to facilitate IKK complex phosphorylation, a necessary step for full IKK activation. As described previously, MALT1 is thought to play a major role as an "effector" protein in the CBM complex, coordinating the activation of the IKK complex. This occurs at least in part through the ability of MALT1 to direct multiple K63-linked ubiquitin modifications, targeting IKKγ, Bcl10, and even MALT1 itself. In the lymphocyte, these ubiquitin chains may then serve as a scaffold to recruit the kinase, TAK1, which completes the activation of the IKK complex through phosphorylation of IKKβ at specific residues within its activation loop (Shinohara et al., 2005; Wang et al., 2001a). As for the activation of TAK1, this appears to occur through a parallel pathway, initiated by the antigen receptor, that does not depend upon the CBM complex (Shambharkar et al., 2007) (Fig. 4B).

Interestingly, recent work has shown that TAK1 is dispensable for LPA-dependent NF-κB activation. Instead, another mitogen-activated protein kinase, MEKK3, takes its place (Sun et al., 2009). Thus, the ligand-activated LPA receptor induces a parallel pathway, independent of the CARMA3-containing CBM complex, that causes MEKK3 activation, subsequently leading to IKKβ phosphorylation (Fig. 4C). It remains an open question as to whether MEKK3 will be involved in IKK complex activation downstream of all GPCRs, or whether distinct kinases will act in concert with the CARMA3-containing CBM complex, depending on the specific GPCR being induced.

6. The CARMA3/Bcl10/MALT1 signalosome and endothelial phenotype

6.1 Role for endothelial NF-κB activation in atherogenesis

As discussed earlier, many pieces of evidence implicate endothelial cell NF-κB activation as an important GPCR-mediated signaling event favoring atherogenesis. Recently, this concept was reinforced by elegant studies using two related mouse models (Gareus et al., 2008). These researchers first generated an endothelial-specific *IKKγ*$^{-/-}$ mouse, to disrupt any NF-κB signaling in this cell type. These *IKKγ*$^{-/-}$ mice were crossed with a mouse model of atherosclerosis (*ApoE*$^{-/-}$) and fed a cholesterol-rich diet. After ten weeks, these mice showed a 30% reduction in plaque size, 40% reduction of T cells in plaques, and an overall retardation in the progression to advanced plaques as compared to *IKKγ*$^{+/+}$/*ApoE*$^{-/-}$ mice under the same diet. To further demonstrate the specific role of endothelial NF-κB in atherogenesis, an additional transgenic mouse model was created, expressing dominant negative IκBα (IκB-SR) under control of the Tie2 promoter. This effectively targeted expression of the dominant negative mutant to endothelial cells. Since the dominant negative mutant lacks phosphorylation acceptor sites for IKKβ, its expression effectively keeps NF-κB subunits sequestered in an inactive state, regardless of whether or not the cell is being stimulated by any of the classic NF-κB inducers. These mice were once again backcrossed with *ApoE*$^{-/-}$ mice and placed on a high cholesterol diet for ten weeks, after which they showed a 60% reduction in plaque size and a significant reduction in plaque progression as compared to *ApoE*$^{-/-}$ mice with normally functioning NF-κB (Gareus et al., 2008). The endothelium of IκB-SR/*ApoE*$^{-/-}$ mice was almost completely free from expression of most cytokines, chemokines and adhesion molecules. Taken together, this study provides exceptionally strong evidence that NF-κB activation within the endothelium alone is necessary to drive a significant atherogenesis response.

6.2 The GPCR-CBM-NF-κB axis in endothelial dysfunction and atherogenesis

To date, three GPCRs have been linked to the CBM signalosome and NF-κB activation, specifically in endothelial cells. These are the receptors for Ang II, thrombin, and IL-8. Others are sure to follow; for example, clear evidence exists for an important role for LPA receptor-dependent NF-κB activation in endothelial biology, but to date the connections between this receptor and the CBM signalosome have been explored only in cell models outside vascular biology. In the following sections, we describe the specific work that has been done to investigate the GPCR-CBM-NF-κB signaling axis in endothelial cells, focusing on the cellular and pathophysiologic consequences of activating this signaling axis.

6.2.1 IL-8 and VEGF induction

Vascular endothelial growth factor (VEGF) is a key endothelial-specific growth factor that is induced in response to tissue damage. VEGF modulates endothelial cell phenotypes by inducing cell proliferation, promoting cell migration, and inhibiting apoptosis, and is regarded as a key regulator of angiogenesis (Ferrara et al., 2003). In atherogenesis, VEGF may play a role in promoting the pathologic ingrowth of the neovascular network from the vasa vasorum, into plaque developing in the subintimal space. These effects are mediated through VEGF binding to its own receptors, VEGFR-1/2 (Ferrara et al., 2003).

Under certain conditions such as tissue hypoxia, VEGF synthesis and secretion is regulated via activation of the transcription factor, hypoxia inducible factor-1 (HIF-1) (Semenza, 2007). Although hypoxia is a potent stimulus for VEGF expression, inflammatory cytokines have also been reported to stimulate VEGF expression through mechanisms that have not been fully delineated. One recent study, however, revealed that regulation of VEGF expression in endothelial cells can occur via CBM signalosome-mediated activation of NF-κB (Martin et al., 2009). These investigators showed that the pro-inflammatory cytokine IL-8 (CXCL8) stimulated VEGF production and secretion through activation of its cognate GPCR, CXCR2. This receptor, in turn, was linked to the CBM signaling complex and NF-κB activation. Importantly, the effect of IL-8 on VEGF induction was independent of HIF-1 but entirely dependent on NF-κB. Inhibition of any of the CBM components by siRNA was effective at reducing NF-κB activation and resulted in a marked inhibition of VEGF mRNA expression and protein secretion. Further, this was accompanied by decreased autocrine VEGFR2 activation. These results suggest that the CBM signalosome is necessary for regulating VEGF production in endothelial cells in response to certain inflammatory cytokines, and might indirectly contribute to the VEGF-dependent transition of the endothelium into a pro-angiogenic phenotype.

6.2.2 Thrombin and endothelial cell/monocyte adhesion

As discussed earlier, thrombin and its receptor, PAR-1, are thought to induce a variety of pro-inflammatory responses that may also contribute to endothelial phenotype changes (Hirano, 2007). Thrombin levels are increased at sites of vascular injury and thrombosis, where the persistent stimulation of its receptor leads to endothelial dysfunction, thereby increasing inflammatory responses leading to further vessel wall damage and atherosclerotic lesion progression. One of the key responses to local thrombin production is the increase in endothelial cell expression of adhesion molecules, allowing for firm adhesion of circulating monocytes and other leukocytes (Minami et al., 2004). Our lab has specifically shown that thrombin-dependent activation of the CBM-NF-κB signaling axis in endothelial cells results in upregulation of two such adhesion molecules, VCAM-1 and ICAM-1, at both the mRNA and protein levels (Delekta et al., 2010). Further, we showed that thrombin-induced adhesion of monocytes to endothelial cells requires the intact CBM signalosome; siRNA-mediated knockdown of Bcl10 in endothelial cells altered their phenotype to completely abolish thrombin-dependent monocyte adherence. Further work will be required to test the role of the CBM proteins in modulating other aspects of thrombin-dependent endothelial dysfunction.

6.2.3 Ang II and *in vivo* atherogenesis

Similar to thrombin, Ang II produced locally in the vasculature has been reported to induce a number of inflammatory responses, including the expression of NF-κB-sensitive adhesion molecules and cytokines in endothelial cells, and the recruitment of inflammatory cells to the vessel wall (Daugherty and Cassis, 2004). Our group recently showed that Ang II activation of its receptor, present on endothelial cells and on VSMCs, stimulates NF-κB through the CBM signalosome (McAllister-Lucas et al., 2010).

Further, we tested the effects of manipulating the signalosome *in vivo*. In these studies, we utilized the *ApoE*$^{-/-}$ mouse strain described earlier, which is hyperlipidemic and prone to developing atherosclerosis. The development of lesions, however, can be dramatically

accelerated through infusion with Ang II, even at subpressor doses. When infusions are carried out for as little as 4 weeks, the mice develop prominent and premature atherosclerotic lesions, even in the absence of a high-fat diet; these can be visualized grossly by staining the intimal surfaces of the aorta with Oil-red-O, a stain that reacts to lipid-laden lesions (Fig. 5). This effect of Ang II infusion is generally accepted to be the result of its direct pro-inflammatory effects on the vessel wall, which conspires with hyperlipidemia to cause accelerated atherogenesis. We tested the role of the CBM signalosome by crossing *ApoE*$^{-/-}$ and *Bcl10*$^{-/-}$ mice to generate a double knock-out line (McAllister-Lucas et al., 2010). The absence of Bcl10 in the ApoE-deficient strain revealed a dramatic phenotype in which the mice were protected from developing Ang II-induced atherosclerosis and aortic aneurysms (Fig. 5). Additionally, the reduction in atherosclerotic lesions in aortas from *ApoE*$^{-/-}$*/Bcl10*$^{-/-}$ mice was associated with reduced aortic gene expression of several pro-inflammatory molecules, as compared to *ApoE*$^{-/-}$ mice infused with Ang II in the same way.

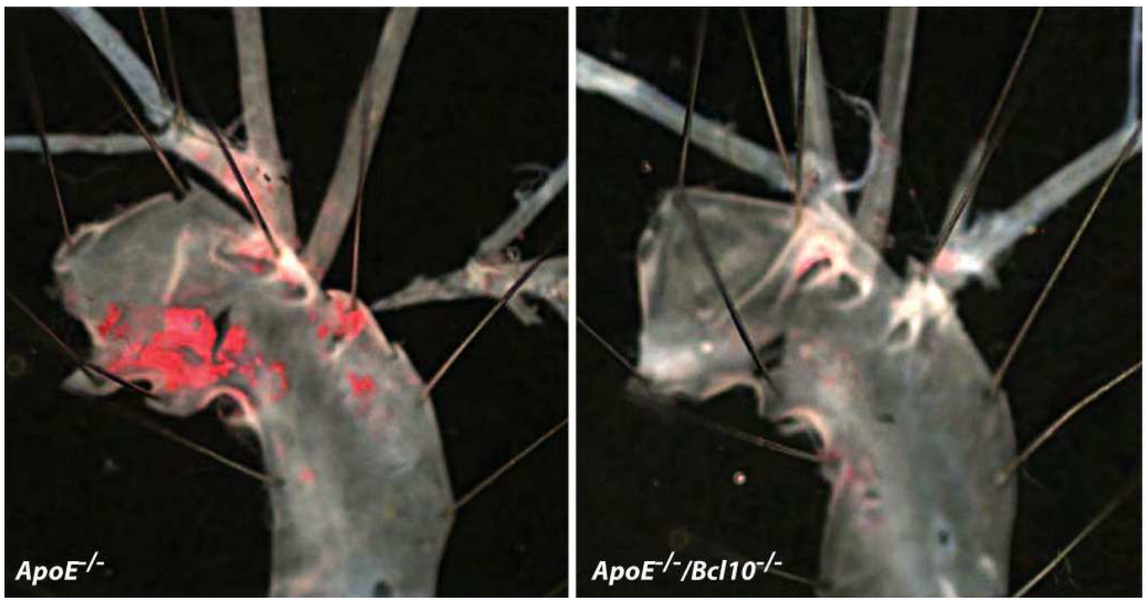

Fig. 5. Representative aortic arches from mice infused with Ang II for 4 weeks. Genotypes are as indicated. Aortas are stained with Oil-red-O to highlight lipid-laden intimal lesions (fatty streaks-advanced lesions). See text and McAllister-Lucas et al., 2010 for details.

6.2.4 LPA and the CBM signalosome in atherogenesis?

As mentioned, it is likely that other GPCRs will be linked to the CBM-NF-κB signaling axis in endothelial cells, since several have already been linked in this way through work on other cell types. In particular, the receptors for LPA are likely to harness the CBM proteins in endothelial cells, considering their prominent role in affecting endothelial cell biology. A new study by Schober and colleagues demonstrates that LPA, produced via oxidation of LDL particles, enhances atherosclerotic lesion formation in *ApoE*$^{-/-}$ mice (Zhou et al., 2011). This effect is mediated largely via LPA receptor-dependent elaboration of CXCL1 (GRO-α) on the surface of endothelial cells. CXCL1 is a chemokine that acts to promote monocyte recruitment to the endothelial wall, and thus plays a role in promoting atherogenesis. The authors showed that CXCL1 expression was in part NF-κB dependent. Thus, since LPA-

stimulated NF-κB activation has been shown to require the CBM complex in MEFs and in ovarian cancer cells (Grabiner et al., 2007; Klemm et al., 2007; Mahanivong et al., 2008; Wang et al., 2007), it is likely that the same will hold true for endothelial cells.

7. Therapeutic opportunities

Identification of the CBM signalosome as a critical mediator of GPCR-dependent pro-inflammatory effects suggests that pharmaceutical targeting of the CBM proteins could represent a new strategy for preventing or treating atherosclerosis. Since disruption of the CBM signalosome blocks NF-κB activation and inflammatory signaling downstream of AGTR1, PAR-1, CXCR2 and probably other GPCRs within the vessel wall that contribute to endothelial dysfunction and atherogenesis, inhibiting vascular CBM activity may prove beneficial. Potential pharmaceutical approaches include: **1)** preventing specific upstream events that link GPCR stimulation to CBM activation, **2)** blocking key post-translational modifications of CBM components, and **3)** directly targeting the activity of CBM components themselves.

7.1 Upstream targets

The specific upstream molecular mechanisms by which GPCR stimulation promotes assembly and activation of the CBM have not yet been extensively investigated, and these mechanisms likely vary significantly depending on ligand, GPCR and cell type. However, there are some clues to potential therapeutic targets that could be critical for GPCR-induced CBM activation in the vasculature. For example, β–arrestin 2 associates with CARMA3, and studies thus far demonstrate that β–arrestin 2 is required for both LPA and thrombin to induce GPCR-dependent NF-κB activation (Delekta et al., 2010; Sun and Lin, 2008). Intriguingly, recent studies demonstrate that in VSMCs, β–arrestin 2 mediates AGTR1-dependent prevention of apoptosis and is required for both LPA and thrombin-induced vascular smooth muscle cell proliferation (Ahn et al., 2009; Kim et al., 2008). Furthermore, deficiency of β–arrestin 2 protects LDL receptor knockout (*ldlr*$^{-/-}$) mice from aortic atherosclerosis (Kim et al., 2008). Together, these studies suggest that somehow targeting β–arrestin 2 could represent a rational therapeutic strategy for preventing GPCR-dependent CBM activation and combating atherosclerosis. Precisely how to inhibit β–arrestin 2-dependent CBM activation remains to be investigated, but one potential approach would be to block GRK-mediated phosphorylation of GPCRs, thus preventing phosphorylation-dependent recruitment of β–arrestin 2 to the GPCR (DeFea, 2011). In addition, "G-protein-biased ligands" which selectively activate G-protein-mediated signaling downstream of specific GPCRs, while inhibiting β–arrestin-mediated signaling, are currently under development, and such agents may prove to be useful in modulating β–arrestin 2/CBM-dependent vascular inflammatory disease (Whalen et al., 2011).

7.2 Targets involved in CBM modification

In addition to β–arrestin 2-mediated recruitment of CARMA3, another critical step in GPCR-induced CBM activation that represents a potential therapeutic target is the PKC-mediated phosphorylation of CARMA3. As discussed above, T-cell receptor (TCR) or B-cell receptor (BCR) stimulation induces PKC-dependent phosphorylation of CARMA1, thus causing a conformational change that allows CARMA1 to recruit Bcl10/MALT1 to the receptor and

form the CARMA1-Bcl10-MALT1 (CBM) complex (Matsumoto et al., 2005; Sommer et al., 2005). It is not yet known if a similar mechanism of PKC-induced CARMA3 phosphorylation occurs downstream of GPCRs, although it is well established that GPCR stimulation leads to phosphorylation and activation of various PKC isoforms and treatment with broad-spectrum PKC inhibitors can block GPCR-dependent NF-κB activation. However, a pharmaceutical approach targeting PKC in atherogenesis is likely to be complex (Ding et al., 2011), since as we described earlier, each GPCR may utilize distinct PKC isoforms to communicate with the CBM complex. Nevertheless, progress is being made on this front; a recent report demonstrated that treatment with the DAG/calcium-dependent PKC inhibitor, Go6976, and siRNA-mediated silencing of PKCα□both blocked AGTR1-dependent NF-κB signaling in VSMCs (Doyon and Servant, 2010). Likewise, PKC inhibitors RO318220 and GF109203X have been shown to abrogate thrombin-dependent pro-inflammatory signaling in human aortic VSMCs (Chung et al., 2010). Perhaps the PKCβ isoform has been most thoroughly studied in the context of atherosclerosis. For example, genetic knockdown of PKCβ□or treatment with the PKCβ inhibitor, ruboxistaurin, results in decreased atherosclerosis in ApoE-deficient mice (Harja et al., 2009), and this same PKC inhibitor has also been shown to reduce endothelial dysfunction in human patients (Mehta et al., 2009). Because there is much evidence supporting a critical role for PKCs in atherogenesis and there are multiple isoform-specific PKC inhibitors already available, it will be of great interest to determine whether inhibition of particular PKCs blocks CBM activation by specific GPCRs within the vasculature and whether these effects are associated with a pharmaceutical benefit in the setting of atherosclerosis.

Like PKC-mediated phosphorylation of CARMA3, other post-translational modifications of CBM components may be critical to GPCR-induced CBM activity and could therefore represent potential targets for pharmaceutical intervention in GPCR-driven atherosclerosis. In lymphocytes, several kinases and phosphatases have been implicated in regulating the phosphorylation status of CARMA1 and Bcl10, and similarly, several ubiquitin ligases and deubiquitinases have been implicated in regulating the ubiquitination status of all three components of the CBM complex. In contrast to antigen receptor-dependent CBM activation in lymphocytes, GPCR-dependent regulation of the phosphorylation and ubiquitination of CARMA3, Bcl10 and MALT1 has not yet been investigated, although it seems likely that at least some of the same processes that regulate CBM activity in response to antigen receptor stimulation will also play a role in GPCR-dependent CBM regulation. Future studies may identify specific kinases, phosphatases, ubiquitin ligases and/or deubiquitinases that could be targeted in an effort to treat atherosclerosis by inhibiting GPCR/CBM pro-inflammatory activity in the vasculature.

7.3 Targeting the enzymatic activity of the CBM signalosome itself

MALT1, recently discovered to be a protease, is the only component of the CBM complex that is known to possess intrinsic enzymatic activity, and inhibition of MALT1 proteolytic activity may indeed represent a promising new therapeutic target for the treatment of atherosclerosis. As described in section 5.1, three proteolytic substrates for MALT1 have been identified so far: the MALT1 binding partner Bcl10, and the NF-κB-inhibiting deubiquitinases A20 and CYLD (Coornaert et al., 2008; Rebeaud et al., 2008; Staal et al., 2011). In T-cells, MALT1-dependent cleavage of Bcl10 is induced by TCR stimulation and may play a role in integrin-mediated T-cell adhesion (Rebeaud et al., 2008). Whether

GPCR stimulation can induce MALT1-dependent cleavage of Bcl10 has not yet been investigated, and how Bcl10 cleavage might impact endothelial or VSMC function is totally unknown. TCR stimulation also induces the cleavage of A20 by MALT1, and this results in loss of A20's ability to inhibit TCR-dependent NF-κB activation. It is speculated that A20 cleavage, which separates the N-terminal deubiquitination domain from the C-terminal substrate interaction domain, prevents the removal of activating K63-linked polyubiquitin from A20's substrates, which include TRAFs 2 and 6, Bcl10, IKKγ and MALT1 itself, and that preserving these activating ubiquitination events promotes NF-κB activity. In this way, MALT1-dependent A20 cleavage can amplify the degree of NF-κB dependent gene expression (Coornaert et al., 2008). Whether GPCRs within the vasculature such as AGTR1, PAR-1, CXCR2 or LPA receptors induce MALT1-dependent A20 cleavage is not known. Intriguingly, A20 appears to have a protective effect against atherosclerosis in both mice and humans. In *ApoE*$^{-/-}$ mice, A20 haploinsufficiency results in a significant increase in atherosclerosis compared to normal A20 controls, whereas transgenic overexpression of A20 results in decreased atherosclerosis (Wolfrum et al., 2007). Moreover, in human diabetic patients, polymorphisms at the *A20* locus leading to reduced levels of A20 expression are associated with increased coronary artery disease (Boonyasrisawat et al., 2007). Whether inhibition of MALT1-mediated cleavage of A20 might impact GPCR-driven atherogenesis remains to be investigated, but based on these studies, one might predict that preventing A20 cleavage could be protective. Initial studies suggest that MALT1-induced cleavage of CYLD is required for TCR-induced JNK activation (Staal et al., 2011), but MALT1-dependent CYLD cleavage has not been studied in the vasculature. Interestingly, CYLD overexpression attenuates neointimal formation in a rat model of carotid artery injury (Takami et al., 2008). How these studies of CYLD relate to GPCR/CBM-mediated atherogenesis remain to be investigated. Clearly much remains to be learned about the role of MALT1 proteolytic activity and its biologic affects. Future studies will hopefully elucidate whether MALT1 proteolytic activity contributes to vascular pathobiology and whether inhibiting this activity represents a rational therapeutic approach to atherogenesis.

8. Conclusion

There is a pressing need to understand the molecular mechanisms underlying cardiovascular disease, as it is the leading cause of disease-related deaths worldwide. Atherosclerosis is a chronic inflammatory disease of the vasculature in which the pro-inflammatory transcription factor, NF-κB, is a chief driving force. NF-κB-dependent plaque formation is mediated via the expression of cytokines, chemokines, and adhesion molecules, and via changes in endothelial biology including a reduction in NO production. While many inducers of NF-κB have been identified that act on endothelial cells, selective GPCR ligands clearly represent major players in the process of atherogenesis. Up until recently, little was known about the precise molecular mechanisms through which GPCRs communicate NF-κB activation in endothelial cells. However, dramatic progress has now been made in understanding this process, and we have outlined much of this work here in this chapter. It is hoped that delineating the molecules mediating GPCR-dependent NF-κB activation will provide new avenues for pharmaceutical development, adding a new layer of therapeutic opportunity in our efforts to combat atherogenesis.

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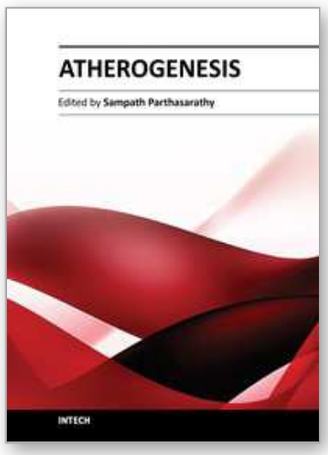

Atherogenesis

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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