

**DYSREGULATION OF NUCLEAR FACTOR KAPPA B ACTIVITY AND
OSTEOPONTIN EXPRESSION IN OXIDANT-INDUCED ATHEROGENESIS**

A Dissertation

by

EDWARD SPENCER WILLIAMS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Toxicology

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ABSTRACT

Dysregulation of Nuclear Factor Kappa B Activity and Osteopontin
Expression in Chemically-Induced Atherogenesis.

(December 2003)

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NF- κ B activity is critical in the regulation of atherosclerotic vascular smooth muscle cell (vSMC) phenotypes induced following oxidative injury by allylamine. The present studies were designed to detail dysregulation of NF- κ B activity in these altered phenotypes, and to assess the importance of NF- κ B in the regulation of osteopontin, a cytokine which modulates atherosclerosis. Increased degradation of I κ B α was observed in allylamine-induced atherosclerotic vSMC phenotypes (henceforth referred to as allylamine cells). Enhanced phosphorylation of I- κ -kinases was observed by Western immunoblotting. NF- κ B DNA binding activity as assessed by electrophoretic mobility shift assay demonstrated changes in the kinetics and magnitude of induction of binding. Enhancement of NF- κ B binding activity was evident in allylamine cells compared to controls when seeded on plastic, fibronectin, and laminin, but not collagen I. Post-transcriptional alterations in Rel protein expression and nuclear localization partly account for changes in NF- κ B DNA binding activity. Promoter-specific NF- κ B binding profiles suggest altered dimer prevalence as a consequence of the changes in Rel protein expression. The expression of NF- κ B regulated genes osteopontin and MMP-2 was

enhanced in allylamine-treated aortas, while cyclin D1 and MMP-9 were unchanged. As the importance of osteopontin in atherosclerosis has been described in several models, subsequent studies were designed to assess osteopontin promoter activity. Activity of the osteopontin promoter was significantly reduced in allylamine cells compared to controls as assessed using a luciferase reporter. Deletion analysis suggested the presence of inhibitory cis-acting elements in the regulatory region of the gene. Mutation of these elements, including VDRE, AP-1, NF- κ B, and USF1, indicated that NF- κ B and USF1 mediate suppression of osteopontin promoter activity in allylamine cells. Decreased serine phosphorylation of immunoprecipitated RelA/p65 was observed in allylamine cells, indicating decreased ability of this protein to transactivate gene promoters. NF- κ B was found to play a role in suppression of osteopontin promoter activity by collagen I-mediated integrin signaling. These findings suggest that enhancements in NF- κ B activity suppress osteopontin promoter activity in oxidant-activated vSMC cultures.

Dysregulation of NF- κ B activity occurs as a result of altered matrix and intracellular signaling upstream of the nucleus and possibly differential dimer assembly leading to cell-specific profiles of NF- κ B-dependent gene regulation.

ACKNOWLEDGEMENTS

Only thanks to the dedication, patience, and affability of many people was I able to successfully complete my graduate training at Texas A&M. My advisor, Dr. Kenneth Ramos, has been a source of most of my training. I also thank Dr. Stephen Safe, Dr. Robert Burghardt, and Dr. Emily Wilson for serving on my graduate committee, and for their meaningful and thoughtful advice on many occasions. I would be remiss if I failed to mention the tireless efforts of Kim Daniel, Kathy Mooney, Lorna Safe, Jeannie Bowman, and Mary Taylor, who continually solved any problems I had and made it look easy. I will always value the people I interacted with in Dr. Ramos' laboratory: Kevin Kerzee, Napoleon Alejandro, Kim Lu, Kimberly Miller, Marc Holderman, Charlie Johnson, Rick Metz, Charlie Partridge, Hadi Falahatpisheh, Lance Chapman, Qiang He, and Adrian Nanez. In the College of Medicine, I've had the privilege of working with Katherine Kelly and Jan Patterson, whose experience I found invaluable. In my last days as a graduate student, I benefited greatly from the hospitality of Weston Porter, Ron Tjalkens, and Emily Wilson, the expertise of Rick Metz, and the help of Jong-II (Henry) Kwak. The support of my family has been priceless. And lastly, I bow to my wife Jennifer Williams, of whom I could not possibly be more proud.

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INTRODUCTION

According to the World Health Organization (WHO), cardiovascular diseases are responsible for one third of all deaths in the world, over 16 million per year. By 2025, this number is expected to reach 25 million (1). In the United States, cardiovascular disease is listed as the cause in over 60% of deaths, more than the next five leading causes combined (cancer, chronic respiratory diseases, accidents, diabetes, and influenza/pneumonia). Medicare costs alone for cardiovascular disease were estimated at \$26.3 billion in 1999 (2).

Atherosclerosis

Atherosclerosis is a disease in which large- to medium-sized arteries develop a lesion that can occlude the lumen, blocking blood flow, or rupture, resulting in a thrombus. Either of these outcomes can result in myocardial infarction, as well as cerebral and renal damage. Occlusion of the lumen by atherosclerosis can also cause gangrene of the extremities (3).

Fatty Streak

Many hypotheses about the onset of atherosclerosis center on the importance of oxidation of LDL in the vessel wall. For many years, the prevailing hypotheses have

This dissertation is formatted in the style of the *Journal of Biological Chemistry*.

included a vital role for cholesterol in the onset of atherosclerosis (4).

There are several different types of lipoprotein particles. In order of increasing density, there are chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Each of these families of lipoproteins contain a specific subset of the nine basic apolipoproteins. These lipoprotein particles are synthesized in the liver, and then are distributed to various parts of the body through the blood. HDL is responsible for removing cholesterol from peripheral tissues and returning it to the body (5).

Generally, lesions form at sites where fatty deposits have accumulated on the wall of the vessel. These so-called “fatty streaks” result in concentration-dependent accumulation of low-density lipoproteins (LDL) in the vessel walls (6). LDL associates with the extracellular matrix in the intimal and medial layers. Oxidative modification of the LDL particles occurs either as a result of interaction with oxidative wastes of cells within the tissue, or the action of macrophages participating in an ongoing inflammatory response (7).

Increases in modified LDL levels in the wall serve to amplify the inflammatory response. The earliest stages of this response are characterized by altered expression of VCAM-1 and ICAM-1, which in turn facilitates migration of leukocytes through the endothelial layer into the intimal and medial layers. Furthermore, oxidized lipids induce transcription of MCP-1 (monocyte chemotactic protein) and M-CSF (macrophage

colony stimulating factor) (6, 8, 9). These actions may cause greater oxidation of LDL that begins and exacerbates the cycle of inflammation at this site.

Inflammation

Atherosclerosis is a disease of chronic inflammation. It is generally accepted that a prolonged inflammatory response at the vessel wall produces an environment conducive to vascular smooth muscle cell proliferation.

Many studies and hypotheses suggest that the inflammatory response occurs as a result of oxidative modification of LDL within the vessel wall (7). Indeed, oxidized LDL (oxLDL) is capable of upregulating several inflammatory molecules. oxLDL is also chemotactic for monocytes (10). The oxidation of LDL (the lipids as well as the proteins) results in a shift to recognition by the scavenger receptors of infiltrating macrophages (6). Thus, macrophages phagocytize the oxLDL in large quantities to become foam cells.

Some evidence suggests that a pre-existing inflammatory condition promotes deposition of LDL in the vessel. For example, some inflammatory cytokines including tumor necrosis factor alpha (TNF- α), interleukin 1 (IL-1), and M-CSF increase binding of LDL to endothelium (11). Further, mouse models which do not express MCP-1 or its receptor display reduced deposition of lipids in the aorta (12).

Whether lipid accumulation or another stimulus initiates the inflammatory response, the development of pathogenic lesions depends on inflammation. Disruption of macrophage function by knocking out MCP-1, M-CSF, or osteopontin attenuate

lesion formation (13). The infiltration of monocytes and T lymphocytes starts a cycle of immune cell recruitment that promotes proliferation of vascular smooth muscle cells, setting the stage for development of a complex lesion.

The upregulation of P-selectin is critical in initiation of an immune response to the inner surfaces of the vessel, as it supports monocyte rolling, and is only expressed in preatherogenic and atherosclerotic lesions (14). As noted above, the sudden appearance of vascular cell adhesion molecule (VCAM-1) and intracellular adhesion molecule (ICAM-1) on endothelial cells are important for the infiltration of immune cells through the intima and into the medial wall (3, 8). MCP-1 (monocyte chemotactic protein 1) is expressed by vSMCs, attracting monocytes into the tissue (8). Once they have infiltrated the tissue, macrophage colony stimulating factor (M-CSF) stimulates differentiation of monocytes into macrophages. These differentiated macrophages continue the cycle of inflammation by secreting more cytokines, including MCP-1, M-CSF, $\text{TNF}\alpha$, $\text{TGF-}\beta$, and interleukins (14). Macrophages also secrete matrix metalloproteinases (MMPs), which are vital to their ability to migrate through the tissue (15). The action of MMPs begins extensive remodeling of the extracellular matrix within the vessel wall. Lastly, the macrophages can secrete growth factors including PDGF and IGF-1 that stimulate the proliferation of vSMCs (14, 16-18).

Endothelial Injury

Some findings suggest that dysfunction of the endothelial layer is a primary contributor to atherogenesis, perhaps as a function of increased susceptibility to LDL

deposition (11). Causes of endothelial dysfunction include oxidative stress as a result of hypertension, smoking, high plasma homocysteine concentrations, and diabetes.

Matrix Remodeling

The extracellular matrix of the vessel wall plays a critical role in maintaining its structural integrity, but perhaps more importantly, signaling of normal matrix through cell surface receptors such as integrins helps maintain function and differentiation status of endothelial cells and vascular smooth muscle cells (vSMCs) (11). Thus, the intense matrix remodeling that occurs in atherosclerotic progression plays two pathological roles: decreased ability of the vessel to maintain its function in blood flow and pressure, and also release of vSMCs and endothelial cells from inhibitory mechanisms that check proliferation.

There are several classes of proteases that participate in degradation of matrix proteins of the vessel wall during atherosclerotic progression. These include metalloproteinases MMP-1 (collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin), MMP-9 (gelatinase B), and MMP-12 (matrilysin) (19) in human atherosclerotic plaques as well as in rabbit models, as well as cathepsins S and K (14).

Matrix metalloproteinases are members of a family of secreted enzymes that degrade ECM components. The activity of these enzymes is regulated at multiple levels, including transcription and protein synthesis of inactive zymogens, enzymatic activation, and interaction of the activated MMP with natural inhibitors, tissue inhibitors of metalloproteinase (TIMPs) (20, 21). vSMCs constitutively produce MMP-2, TIMP-1

and TIMP-2 (22, 23). In atherosclerotic lesions, a major source of metalloproteinases appears to be the infiltrating macrophages, which secrete metalloproteinases and cytokines that stimulate the vascular smooth muscle cell layer to produce more and a wider range of MMPs (24,2 5).

Subsequent to these degradative processes, there are striking changes in the composition of the extracellular matrix. Basement membrane components, generally regarded to be quiescent in regard to vSMCs, are downregulated (eg fibrillar collagen, and laminin), while interstitial proteins, including monomeric collagen I, elastin, and fibronectin, increase in predominance (26).

Type I and III fibrillar collagens are major components of the ECM in normal vessels (27). However, in atherosclerotic vessels, monomeric collagen I predominates (28). The abundance of precursor collagen I rather than polymerized collagen may be important in allowing cells to proliferate (29). Several studies have shown a role for fibrillar collagen in vSMC quiescence, possibly through enhanced expression of cyclin-dependent kinase inhibitors (30, 31). Monomeric collagen I, a major component of the remodeled matrix, is unable to arrest the growth of vascular smooth muscle cells (32). Thus, through degradation of mature collagens, and production of collagen monomers, vascular smooth muscle cells are released from an inhibitory mechanism. However, the presence of monomeric collagen in the injured vessel wall plays a role in activation of infiltrating macrophages, causing upregulation of MMP-9 and CD71 (33).

Early after balloon injury, the heparan sulfate and chondroitin sulfate proteoglycans are disrupted. Altered SMCs start to produce chondroitin sulfate anew,

but it is not quiescent for SMCs (34). Heparan sulfate proteoglycans can inhibit vSMC proliferation by interfering with PKC-mediated transcription factor activation (29). Thus, these matrix constituents may be critical to maintenance of SMC contractile phenotype.

Another protein that helps maintain SMC in their contractile state is laminin, a component of the intimal basement membrane (35). Some findings suggest that disruption of the basal lamina is vital to vSMC activation. Indeed, laminin disappears in atherosclerotic regions, specifically around vSMCs that have shifted to a more proliferative phenotype (26).

Fibronectin is a matrix protein that promotes vSMC proliferation (36). This protein is not present in healthy vessels, but appears rapidly after vessel injury (26). Not only does fibronectin promote vSMC dedifferentiation and proliferation, but subsequent to deposition, it may polymerize into fibers that provide tracks for vSMC migration into the intima (37).

Osteopontin is regarded as a key molecular target of injury within the vascular wall, as a result of its appearance in atherosclerotic plaques. Originally, this protein was described as a critical regulator of bone remodeling; later, it was described as a cytokine capable of modulating the action of inflammatory cells (38). For example, osteopontin plays a role in macrophage functions, including differentiation, migration, adhesion, and cytokine release (39).

Osteopontin has been the focus of many studies in recent years due to its dramatic elevation in atherosclerotic lesions, though it does not appear in normal vessels

(40). Several studies indicate that osteopontin plays a key role in plaque progression; antibody-mediated blockade inhibited intimal thickening in a rat model (41).

Subsequent to production, osteopontin is sequestered in the extracellular matrix. There, it binds to integrin receptors $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_9\beta_1$ on vSMCs (41, 42). Through binding to these receptors, osteopontin can modulate several functions of vSMCs including proliferation, migration, and expression of inflammatory cytokines through activation of the transcription factor nuclear factor kappa B (NF- κ B) (43).

Vascular Smooth Muscle Cell Phenotypic Modulation

Mature vascular smooth muscle cells maintain the tone of large blood vessels thanks to a highly developed contractile apparatus, which consists of smooth muscle myosin and actin isoforms, and anchoring proteins such as vinculin, metavinculin, α -actinin, and talin (44). Specific components of the contractile machinery are useful as markers of SMC identity, including SM22 α , SM-myosin heavy chains, SM α -actin, calponin, and β -tropomyosin to name a few (45, 46).

Many investigators focus on vascular smooth muscle cell activation as the critical step in atherogenesis. Fully differentiated vSMCs exhibit minimal proliferation or cell death (47). Following injury, a phenotypic shift occurs, allowing migration and proliferation to begin. This shift leads to changes in intra- and extracellular protein expression (29). Phenotypic modulation of vSMCs occurs in various physiologic processes, including fetal and post-natal development, regeneration and repair of blood vessels, and myometrial development during pregnancy (48).

vSMC dedifferentiation is signaled by a loss of expression of contractile proteins (49). The expression of these SMC-specific proteins appears to be under the control of serum response factor (SRF) (50, 51). This transcription factor regulates transcription via binding to a CarG element, which is present in the promoter of all the putative SMC marker genes. The ability of SRF to effect transcriptional activation of these smooth muscle-specific promoters is dependent on a number of signaling pathways, including interactions with a number of other proteins, including Nkx3.2, GATA-6 (52), and a member of the ternary complex factor (TCF) family of proteins, which is activated by MAPK-dependent phosphorylation (53). This relationship may account for a recent finding that Akt-mediated Raf inhibition can also inhibit the expression of smooth muscle marker proteins (54). Also, the ability of SRF and its partner proteins to bind a CarG element on smooth muscle-specific promoters may rely on hyperacetylation of these regions, a phenomenon which only occurs in differentiated SMCs (55). Lastly, recent studies have demonstrated that SRF-dependent expression of smooth muscle markers is dependent on RhoA-mediated actin polymerization, suggesting an intricate relationship between the status of contractile machinery of vSMCs and the expression of its components (56).

Dedifferentiation of vSMCs is accompanied by expression and increased activity of NAD(P)H oxidase (57). This enzyme is comprised of 5 subunits: gp91phox, p22phox, p47phox, p67phox, and Rac (58). The activity of this oxidase has been implicated in increased vSMC proliferation *in vitro* (58), and in SMC hyperplasia following bypass graft (57).

Features of the altered SMCs in atherosclerosis include morphologic changes (49), altered secretion and deposition of extracellular matrix (26, 27, 34), hyperproliferation (59), and an accompanying panoply of changes in intracellular signaling (60). A number of studies have suggested chromosomal alterations or mutations in cells isolated from atherosclerotic lesions (61-64), but no specific mutation has been implicated in atherogenesis.

Morphologic changes – As mentioned above, phenotypic modulation of vSMCs during atherogenesis leads to a distinct morphological change. The loss of smooth muscle-specific contractile proteins and the reorganization of the remaining structure have been detailed in several studies (47, 49). Differentiated vSMCs are known as “contractile”, and injured vSMCs are termed “synthetic”, due to increased visibility of the nucleolus and more extensive endoplasmic reticulum (ER). These synthetic phenotypes also appear more rounded in culture, while contractile vSMCs are more elongated and spindle-shaped (44).

Matrix signaling – The extensive remodeling of vascular ECM during atherosclerosis leads to a variety of changes in cell behavior as a result of matrix-related signaling. Degradation of interstitial fibrillar collagens and basal lamina component laminins effectively release vSMCs from stimuli that help maintain them in a differentiated phenotype (65, 66). Although the synthetic vSMCs begin to synthesize collagen I, the monomeric form is unable to effect the same regulation of phenotype as the mature form

(29). Further, the buildup of collagen I can effectively isolate vSMCs from cell-cell contacts, which can contribute to a loss of contractile phenotype (28).

Hyperproliferation - Deregulated proliferation of vSMCs is thought to be a critical event in the development of atherosclerotic lesions (67). Changes in the medial milieu both expose vSMCs to growth stimuli and release them from inhibitory influences. As a result, proliferating vSMCs and the accompanying increase in ECM deposition cause thickening of the media. vSMCs that migrate into the intima also cause neointimal thickening (29).

Several studies have verified that atherosclerotic hyperplasia of SMCs occurs through clonal expansion, either of a single altered cell (68), or of a population of clonal cells in lesion-prone areas (69).

There are a large cadre of signaling pathways that contribute to vascular smooth muscle cell growth and proliferation. Some of these are detailed in Fig. 1. Binding of growth factors to their cognate receptors, as well as attachment of integrin receptors to ECM ligands, cause activation of ras, PI-3-kinase (70), and phospholipases. Three growth factors in particular receive most of the attention as promoters of vSMC phenotypic modulation: PDGF, EGF, and IGF (67). The activities of signaling intermediates downstream of these growth factors branch out through a cellular network of signaling molecules; the combined actions of these proteins activate proteins in the mitogen-activated protein kinase (MAPK) family (71, 72).

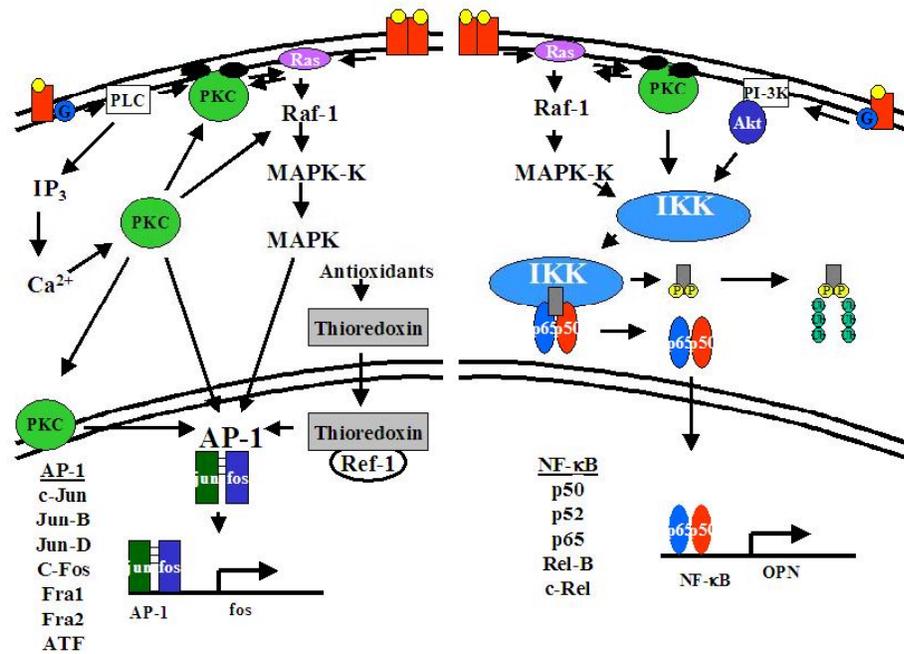


Fig. 1: **Pathways of growth and proliferation of vSMCs.** A cornucopia of signaling pathways interact to effect regulation of vSMCs growth and proliferation, including phospholipase C (PLC), growth factor receptor binding, integrin binding, ras/raf/mitogen activated protein kinase (MAPK), protein kinase C (PKC), phosphatidylinositol 3 kinase (PI3K), IκB-kinases (IKK). These pathways converge at two transcription factors, AP-1 and NF-κB, which regulate expression of many genes related to growth and proliferation.

The MAPK family includes serine/threonine kinases ERK 1 and 2, which regulate growth and differentiation, c-Jun N-terminal kinase (JNK), and p38 MAPK. The latter two kinases play roles in stress responses, specifically apoptosis and inflammation. Though many different signals feed into MAPK cascades, specificity of signal to response may be maintained by a set of so-called scaffolding proteins (reviewed in 73, 74). Two of the specific targets of the MAPK pathways include constituents of the transcription factors AP-1 and NF-κB.

Activating protein 1 (AP-1) is a dimeric transcription factor. It is composed of a combination of proteins from the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Maf (c-Maf, MafB, MafA, MafG, Nr1), and ATF (ATF2, ATF3, B-ATF, JDP1, JDP2) families. Each of these proteins are substrates for MAP kinases, and thus after JNK and ERK are activated, they translocate to the nucleus and phosphorylate the AP-1 constituent proteins (75). These proteins then dimerize and bind to the promoters of genes with roles in proliferation, inflammation, and matrix protein genes. AP-1 can bind to two elements, the TPA response element (5'-TGAG/CTCA-3') and the cAMP response element (5'-TGACGTCA-3').

The importance of AP-1 in vSMC proliferation following balloon injury was recently demonstrated using decoy oligonucleotides for AP-1 binding (76). Also, AP-1 is activated in vSMCs in response to MCP-1 and IL-1, which suggests potential activation in response to the inflammatory cascade in the vessel wall (77).

NF- κ B is a dimeric transcription factor first described in B cell activation. However, this transcription factor has been observed ubiquitously in mammalian cells. Its targets include growth genes (cyclin D1, cyclin D3), inflammatory genes (interleukins, TNF α , MCP-1), cell adhesion molecules (ICAM-1, VCAM-1), stress response genes (angiotensin II, iNOS, COX-2), apoptosis genes (Bcl-xl, Fas ligand, IAPs), growth factors (PDGF, M-CSF), and enzymes (matrix metalloproteinases, xanthine oxidase) (reviewed in 78). The DNA binding site for NF- κ B is 5' – GGRRNNYYCC –3', where R represents any purine base, Y any pyrimidine, and N any base (79).

NF- κ B is comprised of almost any hetero- or homodimer of Rel proteins, which include p50, p52, p65/RelA, RelB, and c-Rel. These proteins consist of two primary domains: a Rel homology domain (RHD), which contains the regions responsible for DNA binding, nuclear localization, and protein dimerization, and a transactivation domain (Fig. 2). However, neither p50 nor p52 contain a potent transactivation domain (80).

Each dimer has varying specificity for NF- κ B binding domains (reviewed in 79).

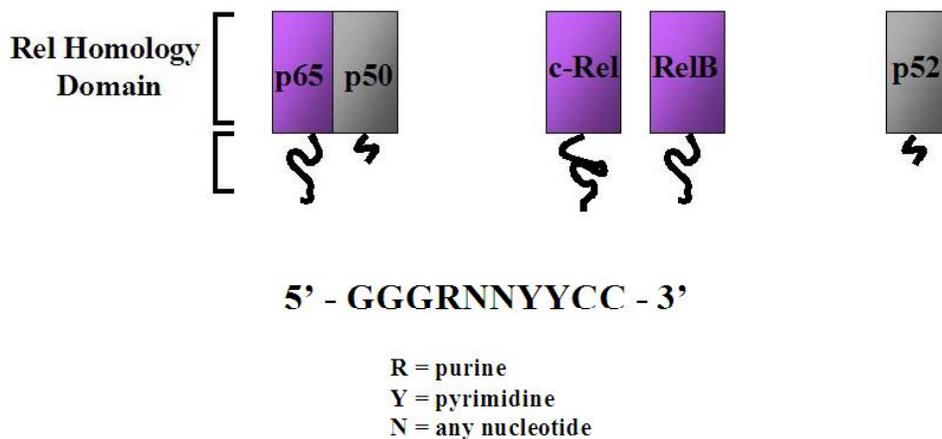


Fig. 2: **A schematic representation of the Rel proteins, the constituents of NF- κ B.** There are five members of this family, including Rel A/p65, c-Rel, RelB, p50, and p52. All of these proteins possess a Rel-homology domain, which contains the protein dimerization and DNA-binding domains. p65, c-Rel, and RelB also contain potent transactivation domains, which are lacking in p50 and p52. NF- κ B dimers bind to the depicted generalized DNA sequence and regulate gene expression.

Recently, Nijnik et al. examined the binding specificity of p50 and p52 to a wide variety of NF- κ B binding sequences, using principal coordinate analysis to predict binding affinity of these proteins for all possible NF- κ B DNA-binding sequences (81). The result of varied affinity of NF- κ B dimers for binding domains is regulation of a subset of genes that possess specific sequences. Differential dimer binding specificity plays two complementary roles in gene expression: 1) different external stimuli may lead to activation of different dimers (82), and 2) polymorphisms in promoter regions may cause altered differential binding of NF- κ B and thus transcriptional activation (83). Examinations of this phenomenon have barely scratched the surface, and it appears certain that this level of regulation will become a critical point of study in coming years.

The NF- κ B dimer is typically sequestered in the cytoplasm, through its association with a protein inhibitor of κ B activity, I κ B. Binding of I κ B masks the nuclear localization sequence of the Rel protein dimer (84, 85).

The critical point of regulation for NF- κ B activation is accomplished by phosphorylation of the I κ B by an I κ B-kinase (IKK) complex. Following phosphorylation, the I κ B is polyubiquitinated by E3 ligase, which targets it for degradation by the 26S proteasome. After the I κ B is degraded, the nuclear localization sequence on the NF- κ B dimer is exposed, allowing nuclear translocation and subsequent binding to DNA (Fig. 3) (86).

There are several varieties of I κ B, including I κ B α , I κ B β , I κ B ϵ , and Bcl-3. p50 and p52 are generated by processing of precursor proteins p105 and p110, respectively.

Before processing, these proteins contain I κ B-like domains. Each I κ B may play a role in different pathways (87). I κ B α is the strongest inhibitor of NF- κ B translocation and is rapidly degraded in response to several stimuli, while I κ B β and I κ B ϵ are degraded with slower kinetics (88). I κ B α is degraded in response to IL-1, LPS and TNF α , while I κ B β is degraded in response to IL-1 and LPS, but not TNF- α (89).

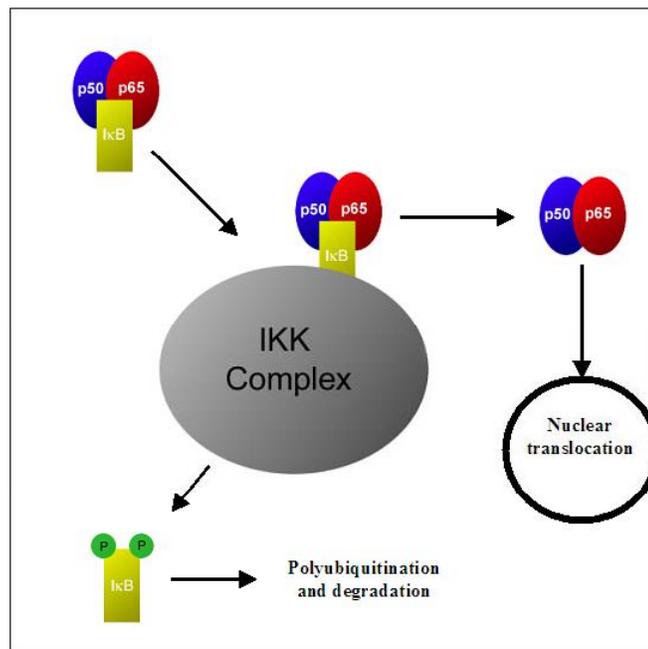


Fig. 3: **A schematic representation of NF- κ B activation.** An NF- κ B dimer, typically composed of p50 and p65, is sequestered in the cytosol via masking of the nuclear localization sequence by I κ B. The IKK complex binds the I κ B and serine phosphorylates it, which targets it for polyubiquitination and degradation. The free dimer then translocates to the nucleus where it is free to bind to DNA.

The IKK complex consists of two kinase subunits (IKK1/ α and IKK2/ β), and a regulatory subunit (IKK γ or NEMO) that facilitates regulation of kinase activity via interactions with upstream signaling molecules. A variety of upstream signaling cascades funnel into NF- κ B activation, including PKC (90), ras (91), NIK (92) and MEKK1 (MAPK kinases) (93), and PI3K/Akt (94, 95). Once activated, the IKK complex performs multiple roles in NF- κ B activation, the best studied of which is phosphorylation of I κ B. Recent studies indicate that IKK2/ β is responsible for this activity, while IKK1/ α may be responsible for phosphorylation of the Rel proteins themselves. This phosphorylation event apparently plays a role in the ability of the NF- κ B dimer to transactivate promoters and thus promote transcription of target genes, thanks to association with chromatin remodeling factors including CBP/p300 (96) and HDAC (97).

Several reports have indicated that NF- κ B activity plays a critical role in proliferation (98, 99), especially in vascular smooth muscle cells (100).

Risk factors

The American Heart Association has identified a number of risk factors for the development of atherosclerosis. Not surprisingly, most of them are inter-related. Many of them share a common endpoint: the generation of reactive oxidative species within the vessel, which suggests that oxidative stress is a crucial step in atherogenesis (101).

Hypercholesterolemia - Familial hypercholesterolemia (FH) is an inherited disorder characterized by high levels of blood cholesterol. Homozygous defects in LDL receptor usually result in atherosclerosis, which causes heart attacks at a young age. Normally, the LDL receptor is localized to a specialized region of the plasma membrane, known as a clathrin-coated pit. Normally, an LDL particle, a composite of proteins, fatty acids, and cholesterol esters, is internalized by LDL receptor-mediated endocytosis. However, in cases of high concentrations of cholesterol in blood, a concentration-dependent accumulation of cholesterol in the vessel wall can occur. Deposition of these lipid particles is quickly followed by oxidation, and an inflammatory response conducive to the formation of atherosclerotic lesions (4, 102, 103).

The sequence of events in this process is still a matter of debate; injury to the vessel wall as a result of shear stress or chemical injury and a subsequent inflammatory response may make the vessel wall more permeable to lipid particles (104). Several critical examinations of epidemiological and experimental evidence call into question the importance of cholesterol in atherosclerosis and cardiovascular diseases (reviewed in (105, 106). These reviews suggest that in most animal models, including non-human primates, extremely high doses are required to induce atherosclerosis. Also, thorough analysis of epidemiological data suggests a failure to demonstrate conclusively a correlation between dietary cholesterol and atherosclerosis. However, a role for oxidation of LDL in atherosclerotic plaque progression has a lot of experimental support (For a review, see 6, 10, 107).

High fat diet/obesity - Several studies have shown relationships between obesity and higher levels of serum cholesterol. Aside from the increased risk conferred by hypercholesterolemia, adipose tissue can secrete an array of inflammatory cytokines including TNF- α , IL-6 and angiotensinogen (13). These adipokines create a proinflammatory milieu which favors the development of atherosclerotic lesions. Also, high TNF- α can cause insulin resistance, setting up a diabetic condition. Thus obesity converges with three other risk factors: diabetes, hypercholesterolemia, and hypertension.

Hypertension - Angiotensin II is often elevated in patients with hypertension. This cytokine is known to stimulate growth of smooth muscle cells (via activation of PLC), and increase steady-state levels of hydrogen peroxide, superoxide, and hydroxyl radicals in plasma (11). The increases in ROS may occur subsequent to AngII-mediated induction of NADPH oxidase expression and activity. (108) Also, AngII enhances osteopontin expression in kidney (109).

Homocysteine - Autopsy results have suggested that defects in metabolic pathways for homocysteine degradation coincide with advanced atherosclerosis. Common defects include cystathionine β -synthase and methylenetetrahydrofolate reductase.

Homocysteine is toxic to endothelium, increases collagen production in vSMCs, and decreases the levels of nitric oxide, which plays a role in protecting the vessel wall from

atherogenesis. (11). Serum homocysteine is also elevated in patients suffering from hypertension, suggesting a link between these two risk factors (110).

Shear stress - “Oscillations” in blood flow have been described in regions where atherosclerotic lesions develop (111). Disturbances in flow can alter expression of critical genes involved in atherosclerosis, including PDGF and ICAM-1. Also, at sites of altered blood flow, eNOS expression is reduced, thus lowering the levels of NO, which is both antiatherogenic and vasodilatory (14). Further, these stresses stimulate superoxide production in endothelial cells, and a concomitant decrease in glutathione levels (108)

Diabetes mellitus - Diabetes is a disease of impaired glucose utilization. Metabolic alteration in diabetes is associated with increased formation of glucose-derived aldehydes, leading to advanced glycation endproducts (AGEs), and methylglyoxal. Protein adduction of these metabolites in blood vessels results in compromised vasomotor function. Further, nonenzymatic glycation reactions can contribute to oxidative stress (112). Increased glycation of collagen (nonenzymatic, would occur more in diabetes) can enhance entrapment of LDL (65).

Some evidence suggests a role for a circulating form of a vascular-specific enzyme semicarbazide-sensitive amine oxidase (SSAO), which may be secreted by vascular cells. This enzyme is responsible for converting aminoacetone to methylglyoxal, a substance connected with vascular damage. The blood concentration

of methylglyoxal is increased in diabetes, with a strong correlation between levels and degree of diabetic complications (113).

High blood glucose may also directly cause increased proliferation of vascular smooth muscle cells through the induction of NAD(P)H oxidase and subsequent production of superoxide (58).

Smoking - The American Heart Association estimates that 150,000 deaths per year from cardiovascular disease are related to smoking (2). Smoking initiates, aggravates, and/or accelerates lesion formation (114). Several molecular mechanisms have been proposed behind this phenomenon. Nicotine can stimulate DNA synthesis in arterial smooth muscle cells *in vitro* (115), as well as dedifferentiation of vSMCs (116). Carbon monoxide (CO) from smoke may directly damage endothelial cells or increase cell permeability. Benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon that is a component of cigarette smoke, can cause lesion formation in animal models. Both BaP and CO cause oxidative injury in vessel tissues (117-119).

Experimental Models

Diet

Diet-induced atherosclerosis as an experimental model was born after the study of Ignatowski in 1908 showed intimal thickening in rabbits fed a diet of animal tissues including meat, eggs, and milk (reviewed in 120). Five years later, other investigators

demonstrated that lipids were primarily responsible, feeding rabbits with cholesterol dissolved in vegetable oil (reviewed in 121).

Studies have repeatedly demonstrated that diet-induced hypercholesterolemia is sufficient to induce atherosclerotic lesions similar to those seen in humans. This phenomenon is not observed only in rabbits, but also in mice, hamsters, guinea pigs, pigeons, quail, chickens, pigs, dogs, cats, and non-human primates (122). However, several recent reviews of the experimental evidence suggest that the doses required to create atherosclerotic lesions are so high that no useful extrapolation could be made to humans (105, 106).

ApoE Knockouts

Hypercholesterolemia can also be induced genetically, by knocking out critical genes involved in normal uptake of LDL particles. In particular, apolipoprotein E knockout mice have become popular standards for atherosclerosis studies, as they develop atherosclerosis even on normal mouse diets (123). The loss of apoE results in the generation of fewer HDL particles, generating hypercholesterolemia as a result of the loss of HDL scavenging cholesterol from peripheral tissues (124).

Mechanical Injury

Balloon angioplasty is a widely used method in animal models for producing lesions that mimic atherosclerosis. This method mimics restenosis, a common problem

after balloon embolectomy. Many patients experienced accelerated regrowth of lesions after treatment by this method (125, 126).

Hypertension

Hypertension is an important risk factor for development of atherosclerosis. Thus, many models have focused on generating atherosclerosis by inducing hypertension. One early study generated hypertension by suspending animals by their hind limbs (reviewed in 122). Fortunately, modern studies use more humane methods, including infusions of AngII and surgical kidney removal as procedures to generate artificially hypertensive animals. Also, several genetically engineered strains of hypertensive lab animals are commercially available.

Allylamine

Several studies in this laboratory as well as others have demonstrated the viability of a model of chemical oxidation *in vivo* which produces atherosclerotic plaques in laboratory animals. Allylamine ($\text{CH}_2=\text{CHCH}_2\text{NH}_2$), a simple monoaliphatic amine, is an industrial reagent used in the production of vulcanized rubbers, pharmaceuticals, and polymers. When allylamine is administered by gavage to Sprague-Dawley rats, the animals develop aortic lesions reminiscent of those seen in human atherosclerosis (Fig. 4). Allylamine is converted to acrolein and hydrogen peroxide in the vessel wall by semicarbazide-sensitive amine oxidase (SSAO).

Early experiments by Boor and Nelson demonstrated “vigorous” acrolein production in aortic homogenates isolated from rats and humans, a levels six times higher than other tissues (127). Conversion of allylamine to acrolein could be inhibited by semicarbazide.

Semicarbazide-sensitive amine oxidase (SSAO), also referred to as benzylamine oxidase (BAO) is localized extensively in cardiovascular tissues. Levels of this enzyme are increased in the plasma of diabetic patients, as well as those with congestive heart failure (128). This copper-containing enzyme is responsible for the degradation of amino

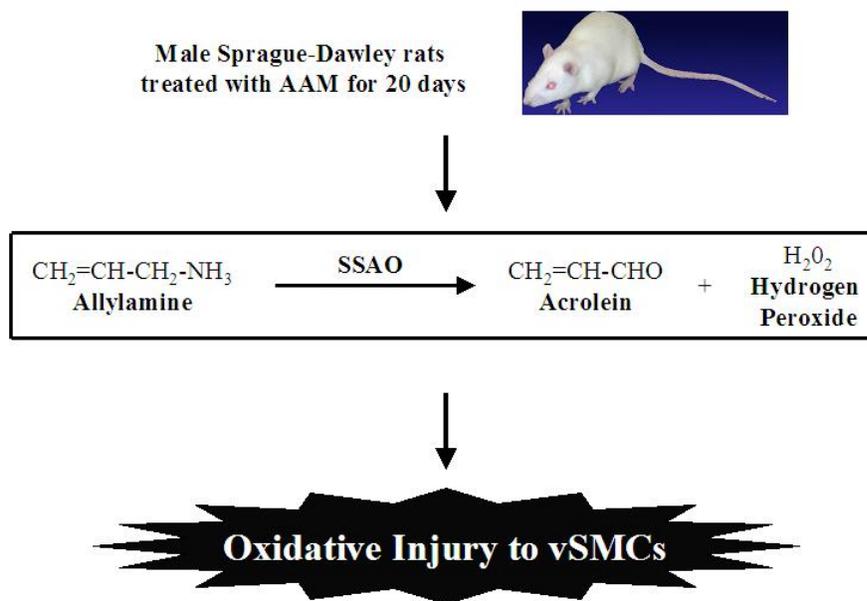


Fig. 4: A schematic representation of the allylamine model. Male Sprague-Dawley rats are treated for 20 consecutive days with allylamine. Allylamine accumulates in vascular tissues, where it is converted to acrolein and hydrogen peroxide by semicarbazide-sensitive amine oxidase (SSAO). These two oxidative by-products cause oxidative stress in the tissue.

acid by-products. Some of its potential substrates include dopamine, histamine, methylamine, and tryptamine (129). It may be responsible for inactivating toxic amines that enter the blood through digestion or inhalation (112). Studies suggest that SSAO is localized in the plasma membrane, possibly facing outward to metabolize amines in the extracellular space (129). The work of Yu and Zuo (130) suggests that circulating SSAO acts on methylamine to create formaldehyde, which injures endothelium and thus contributes to atherosclerosis. Other reports suggest a role for SSAO in the maturation of collagen or elastin (131), much like its relative lysyl oxidase (LyO) (132, 133).

In 1990, Boor, Hysmith, and Sanduja identified SSAO as the enzyme responsible for the conversion of allylamine to acrolein (134). Enzymatic activity responsible for acrolein formation after allylamine exposure resides in the microsomal and mitochondrial fraction of aortic homogenates (135). Other by-products of this reaction include hydrogen peroxide, and ammonia.

Acrolein is a highly reactive aldehyde that can bind to and disrupt the functions of proteins and nucleic acids. The anticancer drug cyclophosphamide is metabolized into acrolein *in vivo* (136, 137). Also, neutrophils produce acrolein at sites of inflammation by enzymatic conversion of threonine by myeloperoxidase (138). Microarray analysis suggested that acrolein enhances expression of stress response genes, including glutathione-S-transferases (GSTs) and γ -glutamyl cysteine synthetases (γ -GCS) (139). Acrolein can conjugate with macromolecules by Michael-type addition (140), and can also promote lipid peroxidation (Fig. 5) (141).

Hydrogen peroxide in the intracellular compartment causes oxidative stress by depleting the pool of available glutathione. Also, through the Haber-Weiss reaction, hydrogen peroxide and superoxide can interact to form destructive hydroxyl radicals ($O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$) (Fig. 6) (139). Not only can these reactive oxygen species damage intra- and extracellular macromolecules, they participate in a variety of physiological signaling pathways, including growth factor-mediated signaling, MAPK activation, and calcium signaling (142).

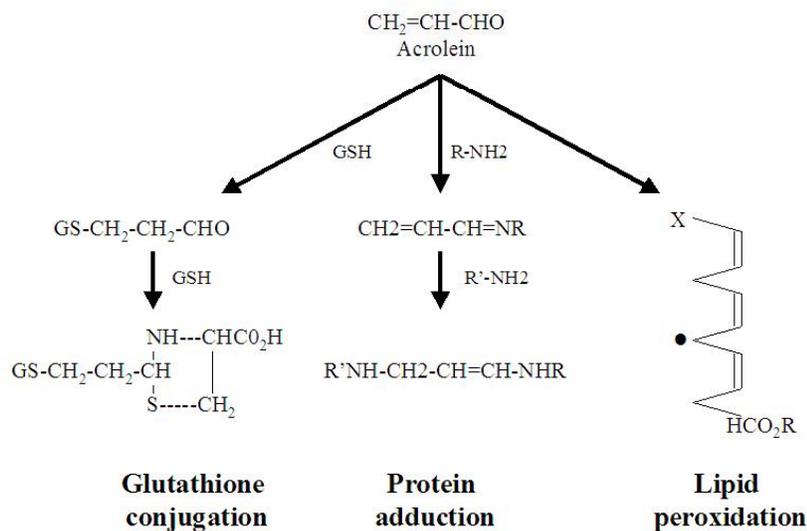


Fig. 5: **Metabolism and toxicity of acrolein.** This by-product of allylamine metabolism causes depletion of glutathione through conjugation, dysfunction of proteins through adduction, and lipid peroxidation.

Modulation of Vascular Smooth Muscle Cell Phenotypes by Allylamine

In Vivo

Previous experiments have demonstrated that chronic oxidative injury by allylamine can induce atherosclerotic lesions in Sprague-Dawley rats. (143). Most of these lesions occur in the descending aorta close to the heart (144). Further experimentation suggested that a result of allylamine treatment was the generation of oxidative stress and lipid peroxidation in the vessel wall (145).

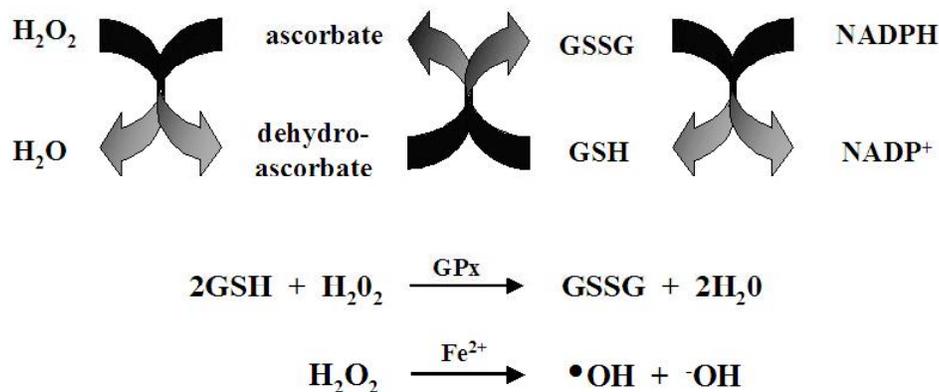


Fig. 6: **Metabolism and toxicity of hydrogen peroxide.** This by-product of allylamine metabolism causes oxidative stress by depleting stores of glutathione and ascorbic acid. Also, hydrogen peroxide can be metabolized to hydroxyl radicals via the Fenton reaction.

In Vitro

When vascular smooth muscle cells are isolated from control and allylamine-treated animals (hereafter referred to as control and allylamine cells), the allylamine cells display marked morphologic variations from control cells (146). As with atherosclerotic vSMCs, the injured cells appeared more rounded and fibroblast-like in comparison to the spindle-shaped control cells. Though the expression of α -actin did not appear to be altered, the organization of the contractile apparatus was markedly reduced (147). Allylamine cells also proliferate much more rapidly than control counterparts (146). The altered phenotype that arises as a result of allylamine injury is sustained over multiple rounds of serial passage.

As matrix plays an important role in atherosclerotic disease, ECM deposition and signaling were the focus of subsequent studies in the Ramos laboratory. The proliferative advantage of allylamine cells was manifest only when cells were seeded on glass, not plastic, suggesting the importance of matrix deposition in the altered allylamine phenotype. Further experimentation revealed that a matrix secreted by allylamine cells increased thymidine incorporation of control cells threefold (147). Also, conditioned media from allylamine cells enhances DNA synthesis in control cells more than 2-fold. Integrin mediated signaling is critical to these enhancements, as thymidine incorporation in allylamine cells increases upon treatment with GRGDS peptides. Some of the alterations in matrix signaling could rely on alterations in expression of integrin subunits (148).

The influence of matrix and RGD-containing peptides is demonstrated by osteopontin. Similar to atherosclerotic vSMCs, allylamine cells overexpress osteopontin, and a 36 kDa fragment thereof. Several studies indicate that osteopontin binding to α_v integrin receptors is critical to the proliferative advantage of allylamine cells (148, 149). Other matrix interactions also influence proliferation in this system. Collagen I, a quiescent influence on vSMCs, inhibits hyperproliferation of allylamine cells, but the proliferative advantage is maintained on fibronectin, laminin or plastic.

The alterations in matrix signaling are reflected in changes in several signaling pathways within the cell. These pathways included ras/raf/MAPK, PI3kinase, and PKC. Allylamine cells exhibit enhanced c-Ha-ras mRNA levels relative to controls (150). The activity of PI3kinase is higher in allylamine cells (151). PKC activity in allylamine-injured vessels, and cells isolated from injured vessels, is enhanced compared to controls (152). Inhibition of PKC by sphingosine and PI3kinase by staurosporine caused a reduction in allylamine cell proliferation, suggesting a pivotal role for these two signaling cascades in maintenance of the altered phenotype.

NF- κ B DNA binding activity is enhanced in allylamine cells compared to controls (148). This finding is consistent with increased activity in several pathways upstream of I κ B phosphorylation by IKK (ie ras/raf/MAPK, PI3K and PKC). Treatment with PDTC preferentially inhibited proliferation of allylamine cells, suggesting a vital role for NF- κ B activity in regulation of the oxidant-induced atherogenic vSMC phenotype.

Perturbations in upstream signaling cascades and NF- κ B DNA binding activity suggested a critical role for this transcription factor in phenotypic maintenance in this oxidant injury model of atherosclerosis. As the promoter region of osteopontin contains a binding domain for NF- κ B, we hypothesized that NF- κ B plays a role in transcriptional regulation of this cytokine. Thus, the following aims were addressed:

- 1) Elucidation of modulated NF- κ B activity and its contribution to atherogenic vSMC phenotypes.

Assessment of NF- κ B activity was performed across a variety of growth conditions. Experiments examined both I κ B degradation and IKK activation, as well as the phosphorylation status of p65/Rel A. The steady-state levels of the Rel proteins themselves were measured both in the cytosolic and nuclear compartments. The importance of matrix signaling in regard to NF- κ B was also assessed. Ultimately, the contribution of NF- κ B activity to the atherogenic phenotype was studied by examining binding profiles across a variety of promoter targets, and also by measuring RNA levels of NF- κ B-regulated genes.

- 2) Analysis of the osteopontin promoter sequence and identification of critical regulatory regions in atherogenic phenotypes.

Using luciferase vectors, osteopontin promoter activity was compared between control and allylamine cells. Sequential deletion and site-directed mutagenesis were used to identify regions of the promoter critical to differential gene regulation.

MATERIALS AND METHODS

Materials

Allylamine (99% pure) was purchased from Aldrich (Milwaukee, WI). Medium 199, trypsin, and antibiotic/antimycotic solution were purchased from Gibco (Grand Island, NY). [³²P]-ATP was purchased from NEN Radiochemicals (Grand Island, NY). NF- κ B consensus oligonucleotide and T4 polynucleotide kinase were purchased from Promega (Madison, WI). Antibodies for Rel proteins, I κ B α , I κ B β , and IKK were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The antibody for phosphoserine was obtained from Zymed Laboratories Inc. (San Francisco, CA). Protein A-sepharose beads were obtained from Amersham Biosciences (Piscataway, NJ). Matrix coated plates (BioCoat) were purchased from Becton Dickinson (Franklin Lakes, NJ). Nitrocellulose membranes were purchased from BioRad (Hercules, CA). Supersignal Dura chemiluminescent substrate was purchased from Pierce (Rockford, IL). Synthetic oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). SYBR Green was obtained from Applied Biosystems (Foster City, CA). Plasmid and RNA purification kits, and DNase-treatment kits were obtained from Qiagen (Valencia, CA). QuikChange Site-Directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA). Fugene 6 and Reverse Transcriptase kits were obtained from Roche (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Aortic Isolation and vSMC Culture

Male Sprague-Dawley rats were gavaged with allylamine (70 mg/kg) or tap water daily for 20 days as previously described (146). Aortas were isolated, and either homogenized for RNA isolation or set aside for primary culture. vSMCs were isolated by successive enzymic digestion of isolated aortas as previously described (153). Subcultures were prepared by trypsinization of subconfluent cultures and maintained in Medium 199 supplemented with 10% FBS and 2 mM L-glutamine in 5% CO₂:95% air at 37°C. Cultures between passage levels 19-25 were used for all experiments. G₀ synchronization was accomplished by incubation of cell cultures for 72 h in Medium 199 containing 0.1% FBS as previously described (148).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared using standard methodologies, quick frozen in liquid nitrogen, and stored at -80° C. For EMSA, 5 µg of nuclear protein was incubated with [³²P]-end-labeled NF-κB consensus oligonucleotide for 1 h. End-labeling of oligonucleotides was performed with T4 polynucleotide kinase and γ[³²P] ATP. Binding reactions were performed in 0.25× HEGDK (25 mM HEPES pH 7.6, 1.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 M KCl) buffer supplemented with 1 mM DTT, 20 µg BSA, and 50 ng poly-dIdC. After addition of loading dyes, reactions were electrophoresed on 7% non-denaturing polyacrylamide gels at 25 mA in 0.5× TBE (0.045 M Tris, 0.045 M Boric acid, 0.001 M EDTA). Gels were dried and exposed to Kodak X-OMAT film with an intensifying screen for autoradiography at -80°C for 48 h.

SDS-PAGE and Western Blotting

Subconfluent cultures were rinsed with PBS and scraped. The pellet was resuspended with RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 10% sodium deoxycholate, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin) and stored at -20°C. Three-10 µg of protein were diluted in sample buffer, boiled for 5 min, and loaded into a 7% denaturing polyacrylamide gel. Electrophoresis proceeded at 18-22 mA until tracking dyes reached the bottom. The gels were equilibrated in transfer buffer for 5 min, and then transferred to nitrocellulose membranes for 9 h at 20V at 4°C. Membranes were blocked in 5% milk in TBS (0.1 M Tris pH 7.5, 0.15 M NaCl) with 0.1% Tween (TTBS) for 18 h, then probed overnight with a 1:1000 dilution of rabbit primary antibody. After 6 × 5 min washes in TTBS, membranes were incubated for 4 h with a 1:25000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP). Following another series of washes, membranes were incubated for 5 min in a 1:2 dilution of chemiluminescent substrate, and then visualized for 30 sec to 2 min on Kodak X-OMAT film.

Immunoprecipitation

Twenty-five µg of protein was incubated overnight at 4°C with 1 µg of antibody against IKK. Fifty µl of a 50% slurry in TE (10 mmol/L Tris, 1 mmol/L EDTA pH 8.0) of Protein A sepharose was added to each immunoprecipitation reaction, and agitated for 4 h at room temperature. The sepharose beads were pelleted, and washed three times

with TBS. Pellets were resuspended in Laemmli sample buffer, and boiled for 5 min. After sepharose beads were pelleted, 10 μ l of supernatant was loaded for SDS-PAGE.

Synthetic NF- κ B Oligonucleotides

Following examination of published sequences for mouse c-Ha-ras promoter, rat osteopontin promoter, and rat matrix metalloproteinase-9, oligonucleotides corresponding to NF- κ B binding domains in these promoters were synthesized. The Genbank accession numbers and sequences used for each are described in Table I. Underlined sequences represent NF- κ B binding motifs.

TABLE I

NF- κ B binding sequences used in this study

DNA sequences used for EMSA are shown. Synthetic complementary oligonucleotides were synthesized, annealed, and used for assessment of NF- κ B binding.

| Gene promoter | Sequence | |
|---------------------------|--|--|
| Mouse H-ras (AF268311) | 5' GGG GGT <u>GGG GTC CTC CCT</u> TTT 3' 3' CCC CCA <u>CCC CAG GAG GGA</u> AAA 5' | |
| Rat OPN (AF017274) | 5' GAT TTG <u>TGG AAT TTC CCT</u> GCA 3' 3' CTA AAC <u>ACC TTA AAG GGA</u> CGT 5' | |
| Rat MMP-9 (AJ428366) | 5' GGG GTT <u>AGG GGG TTC CCC</u> GTG 3' 3' CCC CAA <u>TCC CCC AAG GGG</u> CAC 5' | |

Real Time RT-PCR

A number of genes were selected for examination by Real Time RT-PCR, on the basis of importance to the phenotype and/or putative regulation by NF- κ B. These genes included MMP-2, MMP-9, cyclin D1, and osteopontin. mRNA levels for p65 and p50/p105 were also assessed. β -actin was included as a control. Primers were designed using the Primer Express program by Applied Biosystems (Table II).

TABLE II

Real-Time RT-PCR primers used in this study

Primers designed using Applied Biosystems' Primer Express program for genes of interest are listed.

| Gene | Orientation | Sequence |
|-----------------------------------|-------------|-------------------------------|
| Rat MMP-2 (NM_031054) | Forward | 5' CCCATGAAGCCTTGTTTACCA 3' |
| | Reverse | 5' TGGAAGCGGAACGGAAACT 3' |
| Rat MMP-9 (NM_031055) | Forward | 5' GAGGATCCGCAGTCCAAGAA 3' |
| | Reverse | 5' GCACCGTCTGGCCTGTGTA 3' |
| Rat Cyclin D1 (NM_171992) | Forward | 5' TCTGCGAGCCATGCTTAAGA 3' |
| | Reverse | 5' CCTCTGCACGCACTTGAAGTAA 3' |
| Rat Osteopontin (AB001382) | Forward | 5' TGAGACTGGCAGTGGTTTGC 3' |
| | Reverse | 5' CCACTTTCACCGGGAGACA 3' |
| Rat p65 (XM_219514) | Forward | 5' GAAGAGTCCTTTCAATGGACCAA 3' |
| | Reverse | 5' GGGAAAGGCACAGCAATACGT 3' |
| Rat p50/p105 (L26267) | Forward | 5' CCAACGCCCTCTTCGACTAC 3' |
| | Reverse | 5' CGGTGAGATGGCGCTGTAC 3' |
| Rat β -actin (NM_031144) | Forward | 5' TCTGTGTGGATTGGTGGCTCTA 3' |
| | Reverse | 5' CTGCTTGCTGATCCACATCTG 3' |

Briefly, 1 μg of DNase-treated RNA was incubated with reverse transcriptase, a mix of deoxynucleotides, and oligo dT to a total volume of 12 μL , and allowed to incubate overnight at 14°C. 2 μL of the RT reaction was incubated with SYBR Green Master Mix from Applied Biosystems, forward and reverse primers, and water to a final volume of 25 μL . Real Time PCR was performed in a Biorad iCycler, and assessed using the ΔC_T method (154), using β -actin as a control. Quality of primers was assessed by melt curve to assure that only one PCR product was generated.

Generation of Osteopontin Promoter Constructs

The full-length promoter of rat osteopontin was obtained from Dr. Amy Ridall of the University of Texas Health Science Center in Houston, TX. 2094 base pairs of the promoter was ligated into pGL2-basic using the NheI and XhoI restrictions sites in the multicloning site. This construct was dubbed 2094 rOPNpr. Sequential deletions were generated by Dr. Amy Ridall, which began at 1157 (1157 rOPNpr), 672 (672 rOPNpr), and 127 (127 rOPNpr).

Computerized examination of 2094 rOPNpr by MatInspector v2.2 (available at www.generegulation.de) revealed the presence of a number of transcription factor binding sites, including vitamin D response element (VDRE), AP-1, NF- κ B, and USF1. Site-directed mutagenesis of these sites was performed using Stratagene's QuikChange kit. Mutagenic oligos were designed according to Stratagene's guidelines, and incubated with 2094 rOPNpr as a template. High fidelity PCR followed, and then digestion of the

original template with DpnI endonuclease, which preferentially cleaves methylated DNA. Several clones were sequenced for verification of mutation of the targeted sites. A summary of these mutations is presented below in Table III. The transcription factor binding site is underlined. Mutated bases are in lower case.

TABLE III

Site-directed mutagenesis of osteopontin promoter constructs used in this study

Transcription factor binding sites identified by computerized examination were mutated using Stratagene's QuikChange Site-Directed Mutagenesis kit. These mutants are listed below. Binding sites are underlined, and mutated bases are denoted by lowercase.

| Binding site | | Sequence | | | |
|--------------|----------|----------|---------------------------------|----|--|
| VDRE | Original | 5' | CCTGGAAGGGTCATATGGTTCAGCTCCGAGG | 3' | |
| | Mutated | 5' | CCTGGAATcGTtcTATGGaTCtGCTCCGAGG | 3' | |
| AP-1 | Original | 5' | TCGTGTTGAGTCATTCCTGTGGGC | 3' | |
| | Mutated | 5' | TCGTGTTacaTCATTCCTGTGGGC | 3' | |
| NF-κB | Original | 5' | AGGATTTGTGGAATTTCCCTGCACAGC | 3' | |
| | Mutated | 5' | AGGATTTGaaGAATaaCaCTGCACAGC | 3' | |
| USF1 | Original | 5' | AAACAAACTCATGTGGGTGTCGTGCTT | 3' | |
| | Mutated | 5' | AAACAAACTCAaaTGGGTGTCGTGCTT | 3' | |

Control and allylamine cells were seeded at 4×10^4 cells per well in a 12-well plate. The cultures were allowed to recover for 8 h and then co-transfected with a luciferase construct and a β -gal-expressing plasmid at a ratio of 1.5 μ g luciferase vector to 1.0 μ g β -gal. These plasmids were mixed at a ratio of 6 μ l to 1 μ g DNA in serum-free

medium 199. This mixture was incubated with the control and allylamine cultures for 36 h. The wells were then rinsed with PBS, and harvested using the lysis buffer included in Tropix's Galacto-Light Reporter Assay Kit (Foster City, CA). These lysates were frozen and then thawed.

Twenty-five μL of the reporter lysates was pipetted into two 96-well plates. One set of lysates was injected with 80 μL of a luciferin working solution to assess the luminescence generated. Luminescence was assessed using BMG's Fluostar OPTIMA plate reader. Another set of lysates was mixed with 70 μL of Tropix's β -gal reagent, allowed to incubate for 1 and then injected with 100 μL of the accelerator solution immediately prior to measurement in the OPTIMA plate reader.

Statistics

Real Time RT-PCR data was analyzed using the comparative ΔC_T method as previously described (154). All other statistical relationships were examined by ANOVA and Fisher's least significant difference, unless otherwise noted. Statistical significance was assigned at the $P < 0.05$ level.

RESULTS

Dysregulation of NF- κ B Activity

Repeated cycles of oxidative chemical injury induce proliferative (i.e. atherogenic) vSMC phenotypes characterized by enhanced secretion and deposition of matrix proteins and modulation of integrin-coupled NF- κ B signaling (148). Because nuclear translocation of NF- κ B is dependent upon degradation of I κ B proteins (86), experiments were first conducted to evaluate steady state protein levels of I κ B α and I κ B β across a full synchronization and cell cycle transition regimen in control and allylamine cells. vSMCs were seeded on a plastic substrate and synchronized at G₀ by mitogen restriction over a 72-h period as described (148), and released into growth by addition of 10% serum. The relative abundance of I κ Bs was not affected by growth factor deprivation since comparable protein levels were detected in synchronized cultures, as well as 0.5 hours after mitogen addition. Degradation of I κ B α was observed in activated cells within 1 hr of serum stimulation (Fig. 7), a response that peaked by 2 h and began to subside by 3 h. Modest reductions in I κ B α were observed in control vSMCs, confirming that early activation of NF- κ B by serum mitogens is a key feature of the atherogenic phenotype. No degradation of I κ B β was observed in either cell type at any of the time points examined.

The degradation of I κ B proteins is preceded by phosphorylation by the I κ B-kinases, IKK α and IKK β (155). Analysis of immunoprecipitated IKK in cells seeded on plastic revealed a higher level of serine phosphorylation in allylamine vSMCs,

irrespective of growth status (Fig. 8). Two immunoreactive bands were detected, indicating recognition of both IKK α and IKK β isoforms. Isoform specific analysis revealed that phosphorylation of these kinases was increased in atherogenic vSMCs, and that phosphorylation was dependent upon the degree of mitogenic stimulation.

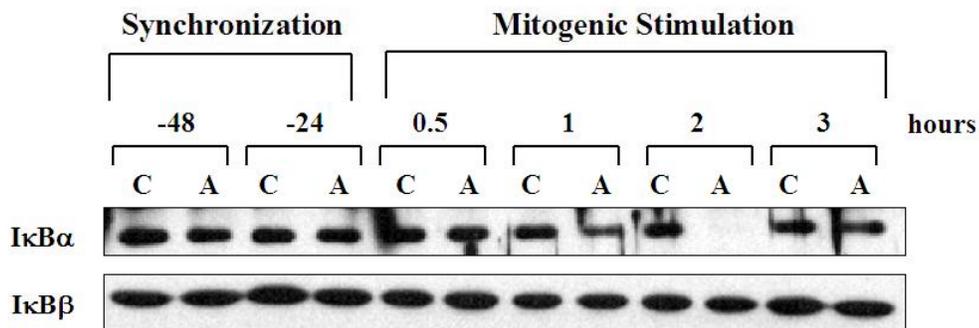


Fig. 7: IκB α and IκB β protein levels in control and allylamine vSMC cultures over a timecourse of synchronization and mitogenic stimulation. Cells were seeded at equivalent densities and serum restricted for 72 h to synchronize in G₀, and then released into growth by addition of 10% fetal bovine serum. Crude protein extracts were harvested at various times after addition of complete medium. Protein extracts were electrophoresed, electroblotted onto nitrocellulose, and probed for each IκB. Similar results were observed in 3 separate experiments. C= control, A = allylamine.

Next, experiments were conducted to examine a time course of NF-κB binding activity to a consensus sequence in quiescent vSMC cultures following mitogenic stimulation. Cells were synchronized as described (150), and stimulated into growth with 10% serum for up to 5 h. The profile of NF-κB binding activity in allylamine

vSMCs was different from control counterparts, with 3 distinct complexes detected at different times (Fig. 8). Constitutive NF- κ B binding activity was enhanced in atherogenic vSMCs relative to controls. This finding is consistent with observed increases NF- κ B activation within the medial wall of aortas isolated from allylamine-treated animals (Partridge et al, unpublished data, 2003). Mitogenic stimulation induced protein binding as early as 0.5 hr in both cell types, but the predominance of individual complexes over time was phenotype-specific (Fig. 9). These findings suggest that different species of NF- κ B transcription factor may be active in vSMCs expressing atherogenic phenotypes. Pursuant to these findings, nuclear proteins were incubated with antibodies to each of the Rel proteins before addition of end-labeled NF- κ B sequences in order to identify proteins present within specific complexes. None of these experiments revealed the composition of the DNA binding complexes observed. These results may be explained by the antibodies used, or as a consequence of the nature of the complexes assembled on the DNA sequence.

Collagen I restricts the proliferative advantage of atherogenic vSMCs, while seeding on plastic, fibronectin, and laminin promotes enhanced mitogenic activity (148). To examine the influence of matrix on profiles of NF- κ B binding activity, vSMCs were seeded on different matrices and processed for nuclear protein binding analysis. The matrix influenced the relative abundance of individual complexes detected (Fig. 10)

NF- κ B binding activity was higher after mitogenic release in allylamine vSMCs compared to control cells on plastic, fibronectin, or laminin, but not collagen I (compare lanes 1 and 7, and lanes 4 and 10). Complexes labeled C1 and C2 were prominent on all

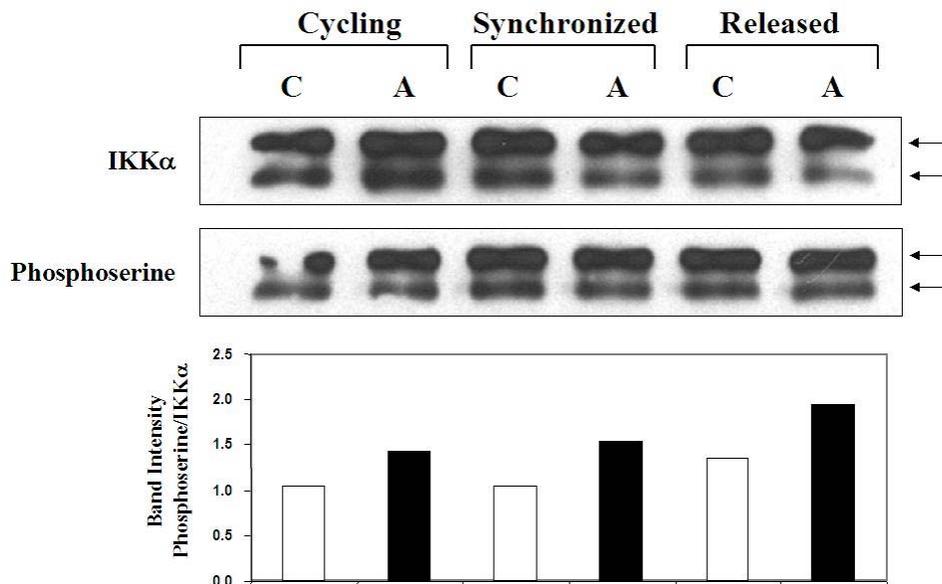


Fig. 8: Total serine phosphorylation of immunoprecipitated IKK α and IKK β in control and allylamine vSMC cultures. Immunoprecipitated IKK α / β was electrophoresed and transferred onto two nitrocellulose membranes and blocked overnight in 5% milk. One membrane was probed for IKK α / β , and the other for phosphoserine, followed by incubation with a horseradish peroxidase (HRP)-labeled secondary. Membranes were then incubated with chemiluminescent substrate and visualized using the Kodak Image Station. Densitometry was performed using Kodak 1D Image Software. C (\square) = control, A (\blacksquare)= allylamine.

matrices, but the intensity of C3 was matrix-specific (compare lanes 1 and 4 for fibronectin versus laminin). On permissive substrates, increased NF- κ B binding activity in allylamine cells was evident after mitogenic release. Binding of C2 and C3 was stronger in allylamine cells than controls seeded on plastic, while enhancement of activity was evident for all 3 complexes in cells seeded on fibronectin and laminin.

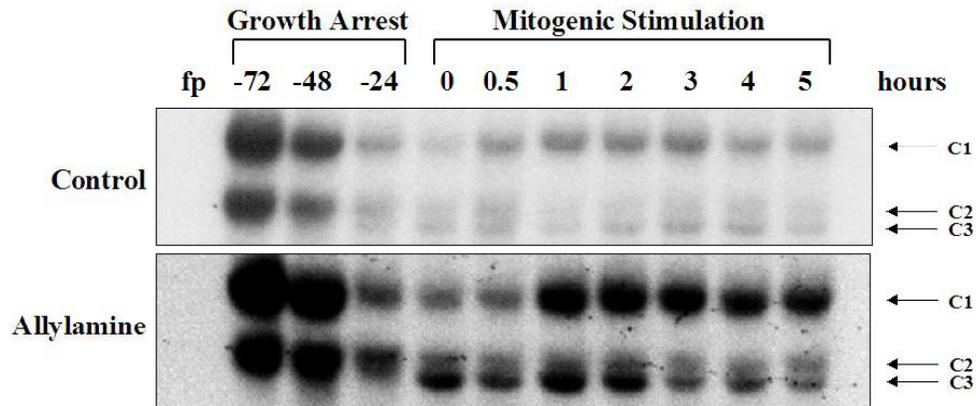


Fig. 9: NF- κ B DNA binding activity in control and allylamine cells over a timecourse of synchronization and mitogenic stimulation. Cells were seeded at equivalent densities on plastic tissue culture dishes. Nuclear extracts were collected at multiple timepoints as noted. EMSAs were performed using end-labeled NF- κ B consensus oligonucleotides as a probe. Arrows denote major NF- κ B binding complexes detected. Similar results were seen in 2 independent experiments. C# represents individual complexes.

Though the intensity of all complexes was higher in growth-arrested allylamine cells seeded on collagen I, this enhancement was abolished upon addition of serum mitogens, suggesting that the response of allylamine cells seeded on a collagen I matrix to mitogenic stimulation is deficient. Competition with excess unlabeled oligonucleotide (lanes 2, 5, 8 and 11) and non-specific oligonucleotide probe (lanes 3, 6, 9, and 12) demonstrated that all complexes were specific to the NF- κ B sequence. The enhancement of NF- κ B binding activity in allylamine cells was most evident on fibronectin (compare lanes 4 and 10), consistent with the notion that fibronectin is a highly permissive substrate for activated vSMCs. Thus, matrix-dependent effects in NF-

κ B binding activity correlate with patterns of proliferative activity in control and allylamine vSMCs (148).

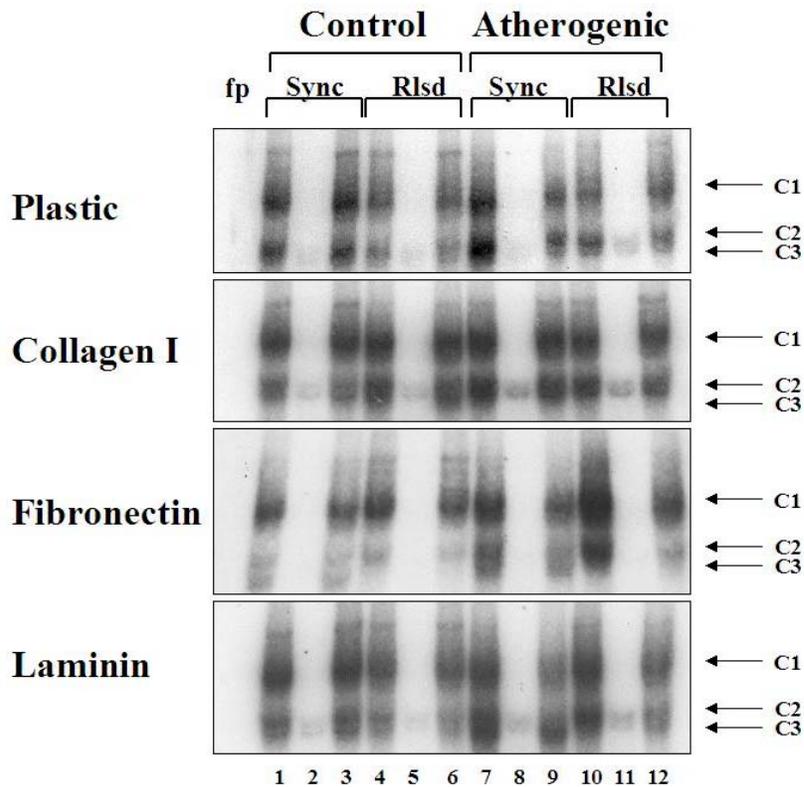


Fig. 10: NF- κ B DNA binding profiles in control and allylamine vSMCs seeded on plastic, collagen I, fibronectin, and laminin. Nuclear extracts were collected after 72 h of synchronization (Sync), or following 2 h of mitogenic stimulation with 10% FBS (Rlsd). EMSAs were performed using end-labeled NF- κ B consensus oligonucleotide as a probe without competitor (lanes 2, 5, 8, and 11), 100-fold excess unlabeled NF- κ B oligo as a specific competitor (lanes 3, 6, 9, and 12), or 50 ng poly dIdC as a non-specific competitor (lanes 4, 7, 10, and 13). End-labeled probe without nuclear protein was run as a control (fp). Arrows denote major NF- κ B binding complexes observed.

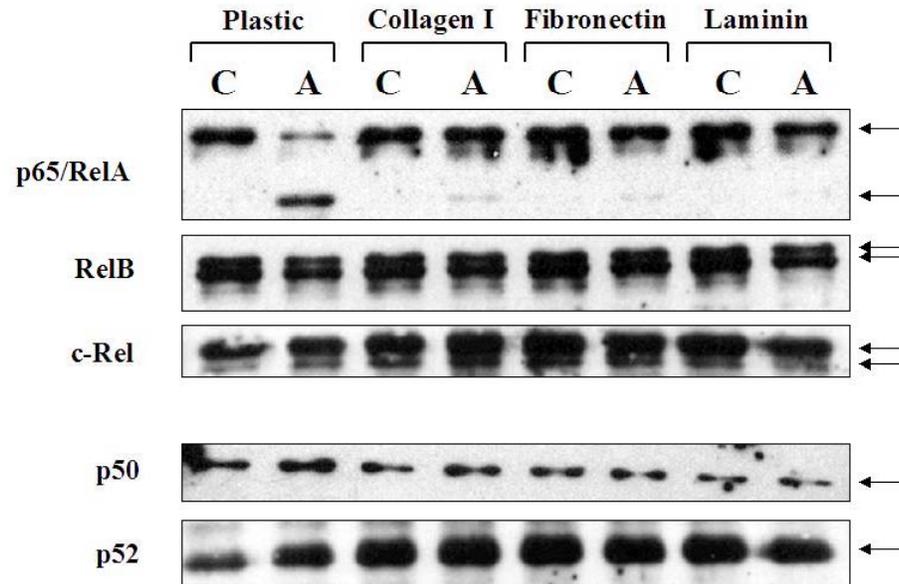


Fig. 11: Expression of Rel proteins in control and allylamine cells seeded on plastic, collagen I, fibronectin, and laminin. Protein extracts from cells at 80% confluence were electrophoresed in a 7% denaturing polyacrylamide gel, transferred onto nitrocellulose and blocked overnight in 5% milk in TBS with 0.1% Tween. Membranes were probed overnight, followed by incubation with horseradish peroxidase-labeled secondary antibody for 4 h, and then incubated with 1:2 chemiluminescent substrate :TTBs for 5 min, followed by visualization through autoradiography. Similar experiments were seen in 2 experiments. C = control, A = allylamine.

Aberrant NF- κ B activity in allylamine cells may be accounted for by differences in Rel protein expression. To evaluate this possibility, control and oxidant-activated vSMCs were seeded on plastic, collagen I, fibronectin, and laminin and total cellular protein extracted and analyzed by SDS-PAGE and Western immunoblotting (Fig. 11). The abundance of RelA/p65 was influenced by matrix and cellular phenotype, with reductions in band intensity observed in allylamine cells seeded on plastic, fibronectin or laminin, but not collagen I. A second immunoreactive band at approximately 45 kDa

was detected exclusively in allylamine vSMCs on all matrices, with signal intensity being most prominent on plastic. The levels of RelB protein were slightly lower in cells of the allylamine phenotype seeded on all substrates, and, as with p65, this reduction was most pronounced on plastic. c-Rel and p52 expression increased in allylamine cells seeded on plastic or collagen I, but decreased on fibronectin or laminin. In contrast, p50 expression was elevated in activated cells seeded on plastic or collagen I, but remained unchanged when cells were seeded on fibronectin or laminin. Analysis of p65 and p105 (the precursor of p50) by Real Time RT-PCR suggests that any change in protein levels in allylamine cells is not a function of increased transcription, either in intact aortas (Figs. 12 and 13), or in cultured cells (Fig. 14).

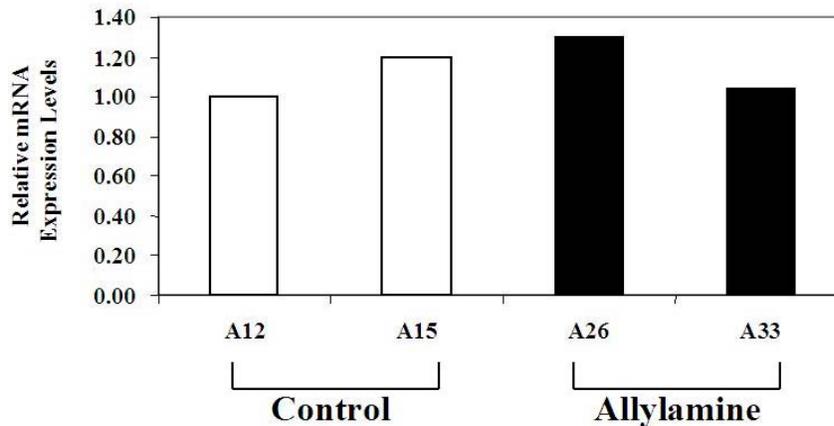


Fig. 12: **RelA/p65 mRNA expression in control- and allylamine-treated aortas.** mRNA was isolated from the aortae of treated animals, and processed for Real Time RT-PCR for quantitation of p65 mRNA levels. Animals A12 and A15 were treated with water (control), and A26 and A33 were treated with allylamine. □ = control, ■ = allylamine.

Next, Rel protein levels were examined in nuclear extracts to determine if changes in cytoplasmic levels are conveyed to the nucleus. Nuclear expression of RelA/p65, c-Rel, and p50 was not influenced by mitogenic conditions or cellular phenotype on a plastic substrate (Fig. 15). In contrast, nuclear RelB levels were decreased, while nuclear levels of p52 were increased in allylamine vSMCs irrespective of mitogenic stimulation. The 45 kDa immunoreactive band detected in RelA/p65 immunoblots was not detected in the nuclear compartment under any of the conditions examined. Thus, differences in total and nuclear Rel protein levels across cellular phenotype under different mitogenic conditions suggest that activation of a different subset of NF- κ B dimers may predominate in allylamine cells.

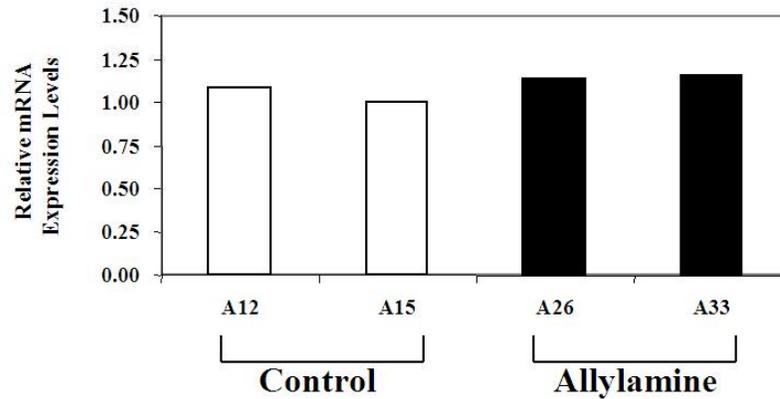


Fig. 13: **p50 (p105) mRNA expression in control- and allylamine-treated aortas.** mRNA was isolated from the aortae of treated animals, and processed for Real Time RT-PCR for quantitation of p50 mRNA levels. Animals A12 and A15 were treated with ater (control), and A26 and A33 were treated with allylamine. . □ = control, ■ = allylamine.

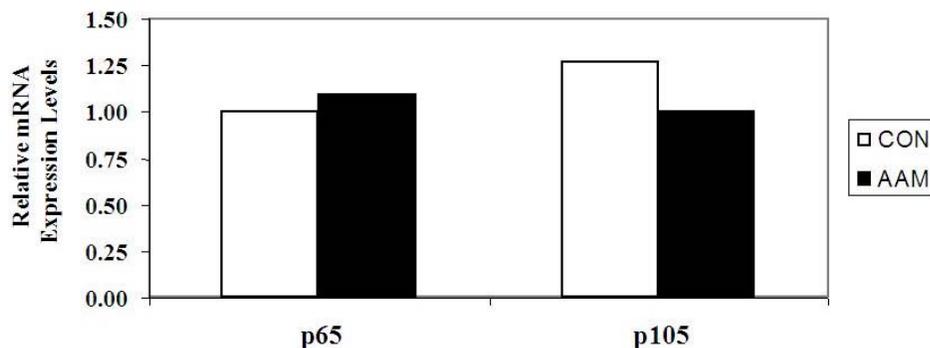


Fig. 14: **Expression levels of p65 and p50 (p105) in control and allylamine cells.** mRNA was isolated from subconfluent cultures of control and allylamine cultures, and processed for Real Time RT-PCR. □ = control, ■ = allylamine.

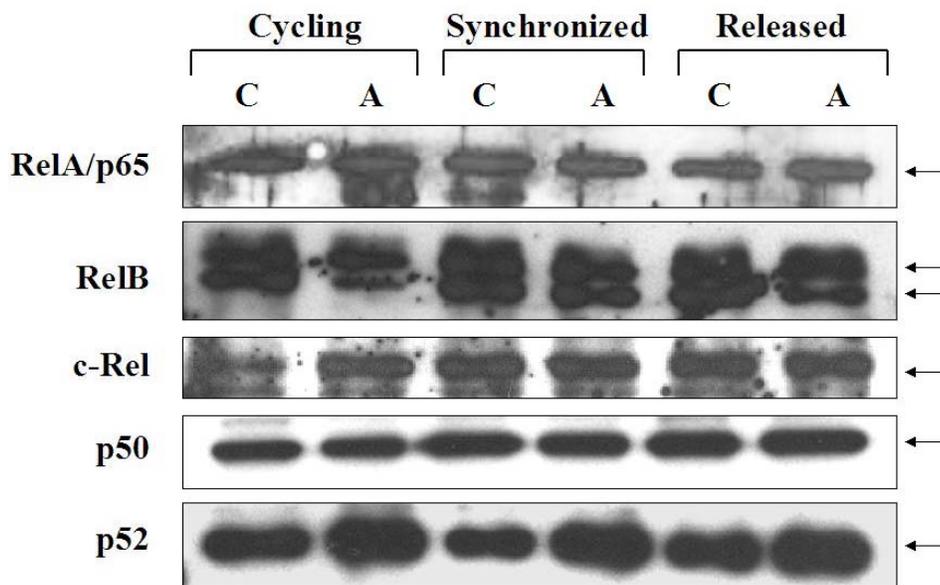


Fig. 15: **Expression of Rel protein in nuclear extracts from control and allylamine cells under various growth stimulatory conditions.** Cells were seeded on plastic culture dishes and allowed to reach 80% confluence. Protein extracts were electrophoresed, transferred onto nitrocellulose, and blocked overnight in 5% milk in TBS with 0.1% Tween. The membrane was probed for each protein, followed by incubation with a horseradish peroxidase (HRP) labeled secondary antibody for 1.5 hours. Membranes were incubated with 1:2 chemiluminescent substrate:TTBS for 5 minutes and visualized through autoradiography. C = control, A = allylamine.

Examination of nuclear Rel protein levels also suggested high constitutive nuclear localization of NF- κ B constituents, which may indicate differential activation pathways in adherent vSMCs, as opposed to immune cells in which prototypical NF- κ B pathways have been described.

Phenotype-specific changes in the prevalence of Rel proteins may translate into variable composition of NF- κ B dimers. Such alterations may influence the profile of transcriptional regulation, as different dimers preferentially recognize different target sequences (79). To test this concept, NF- κ B binding elements from four different promoters were used to evaluate binding activity in nuclear extracts from control and d

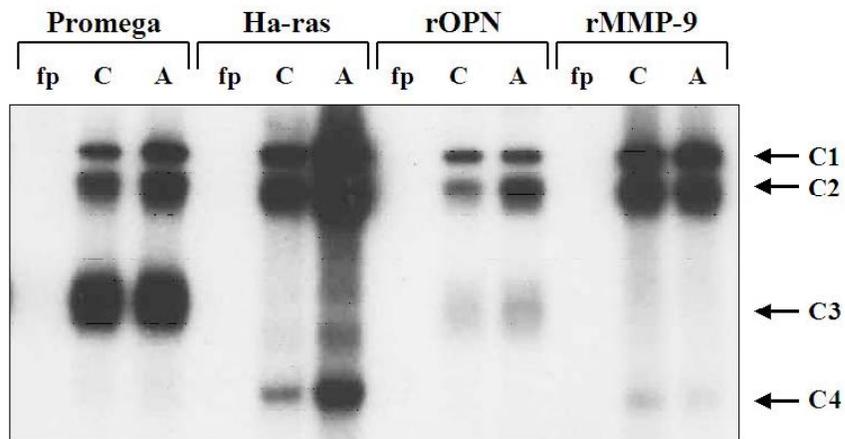


Fig. 16: DNA binding activity to a panel of NF- κ B binding sequences in control and allylamine vSMCs. Nuclear extracts were subjected to EMSA against NF- κ B binding domains from several promoters (Ha-ras, rOPN, and rMMP-9), as well as a consensus oligo (Promega). Gels were dried, and exposed to film for 48 h at -80° C with intensifying screen. Arrows denote NF- κ B binding complexes identified. C = control, A = allylamine.

be identified, two of which were conserved across all of the NF- κ B binding sequences allylamine vSMCs seeded on plastic. Up to four major binding protein complexes could be examined (Fig. 16). With the exception of proteins bound to the NF- κ B sequence from the rat matrix metalloproteinase-9 promoter, band intensities were higher in nuclear extracts from oxidant-activated vSMCs compared to controls, indicating a higher degree of NF- κ B activation. The intensity of the two faster-mobility complexes (C3 and C4)

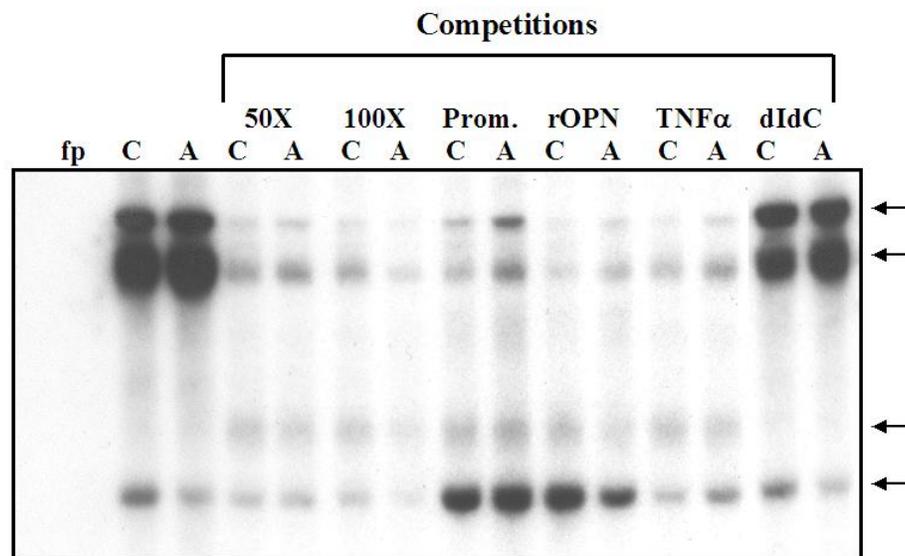


Fig. 17: Cross-competition analysis of DNA binding of nuclear proteins from control and allylamine cells to the c-Ha-ras NF- κ B element. Nuclear proteins from randomly cycling cultures of control and allylamine vSMCs were incubated with the endlabeled c-Ha-ras NF- κ B element. Competitions were performed with 50X and 100X excess unlabeled c-Ha-ras NF- κ B oligonucleotide, 50X consensus NF- κ B oligonucleotide (Promega), 50X rat osteopontin NF- κ B oligonucleotide (rOPN), 50X tumor necrosis factor alpha oligonucleotide (TNF- α), and 50 ng nonspecific DNA oligonucleotide (dIdC). Arrows denote major NF- κ B DNA binding complexes observed. C = control, A = allylamine.

was sequence-specific, with strong binding observed to the Promega consensus and c-Ha-ras sequences. To further examine sequence-specificity of NF- κ B binding within this context, cross-competition analysis was performed to examine the composition of complexes that bound to the c-Ha-ras promoter. Incubation of unlabeled NF- κ B sequences for TNF- α , osteopontin, and a consensus sequence competed away binding to the c-Ha-ras element (Fig. 17). However, varying degrees of competition were observed, suggesting that some complexes, especially the fastest migrating, preferentially bind to the NF- κ B element within the c-Ha-ras promoter.

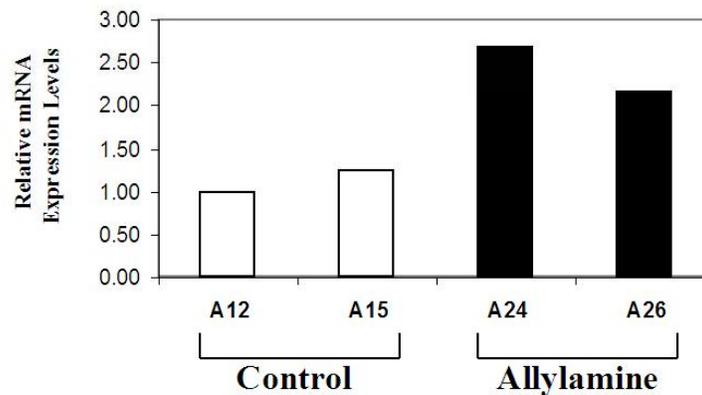


Fig. 18: **Osteopontin mRNA expression in control- and allylamine-treated aortas.** mRNA was isolated from the aortae of treated animals, and processed for Real Time RT-PCR for quantitation of osteopontin mRNA levels. Animals A12 and A15 were treated with water (control), and A24 and A26 were treated with allylamine. □ = control, ■ = allylamine.

As mentioned previously, NF- κ B is a driving influence on the expression of a wide variety of genes (78). Pursuant to observations of promoter-specific enhancements of NF- κ B binding activity in allylamine cells, examinations of RNA levels of several of the targets were performed using Real Time RT-PCR. Increases in ras RNA levels have been described previously in our laboratory (150). Real Time RT-PCR demonstrates increased osteopontin message both *in vivo* (Fig. 18) and *in vitro* (Fig. 19). This observation is consistent with microarray data derived from aortic RNA samples from control and allylamine-treated animals (Partridge et al, unpublished data 2002). However, no such increase in MMP-9 message is observed (Fig. 20). This finding is of particular interest considering the lack of enhancement of NF- κ B binding activity

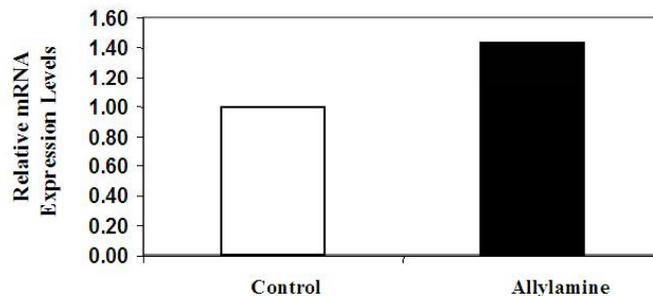


Fig. 19: **Expression levels of osteopontin in control and allylamine cells.** mRNA was isolated from subconfluent cultures of control and allylamine vSMCs, and processed for Real Time RT-PCR. □ = control, ■ = allylamine.

observed against the MMP-9 DNA sequences. However, marked enhancements in MMP-2 RNA levels (Fig. 21), a gene regulated by NF- κ B activity, were observed (78). Thus, the signature of NF- κ B DNA binding activity is consistent with RNA message

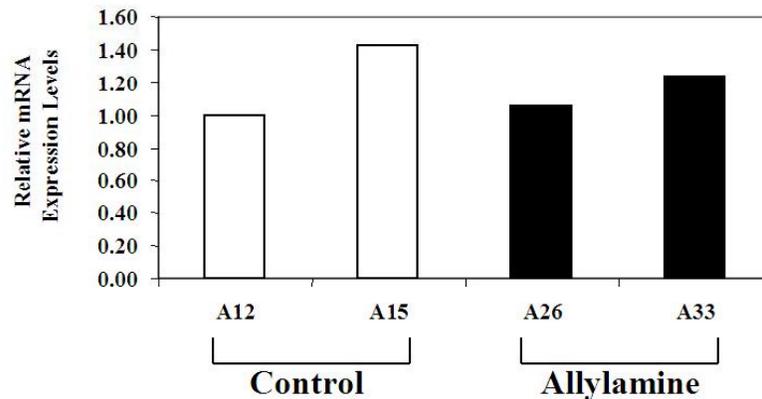


Fig. 20: MMP-9 mRNA expression in control- and allylamine-treated aortas. mRNA was isolated from the aortae of treated animals, and processed for Real Time RT-PCR for quantitation of MMP-9 mRNA levels. Animals A12 and A15 were treated with water (control), and A26 and A33 were treated with allylamine. □ = control, ■ = allylamine.

levels, suggesting that promoter-specific differences in NF- κ B binding to cis-acting elements play a role in differential gene expression in control vs. allylamine cells.

Experiments with PDTC suggest a vital role for NF- κ B in the proliferative advantage of allylamine cells compared to controls. One NF- κ B target that could contribute to enhanced proliferative capacity is cyclin D1, which is responsible for the transition from

G₁ phase to S phase. (98, 156). Examination of cyclin D1 RNA levels in randomly cycling cells revealed a slight increase in message *in vitro* (Fig. 22). No difference

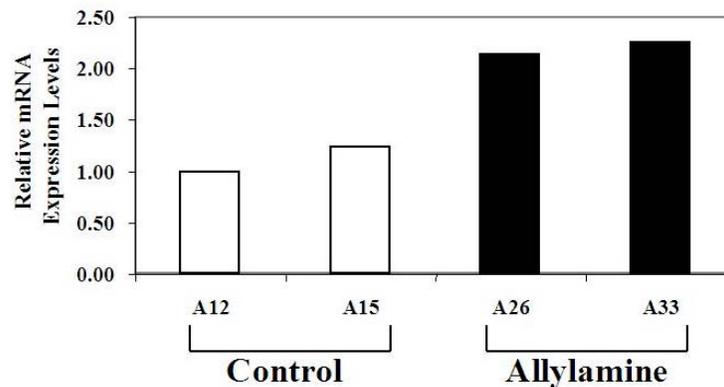


Fig. 21: **MMP-2 mRNA expression in control- and allylamine-treated aortas.** mRNA was isolated from the aortae of treated animals, and processed for Real Time RT-PCR for quantitation of MMP-2 mRNA levels. Animals A12 and A15 were treated with water (control), and A26 and A33 were treated with allylamine. □ = control, ■ = allylamine.

was observed *in vivo* (Fig. 23). However, increased cyclin D1 protein levels were observed in allylamine cells compared to controls (Jones et al, unpublished observations 2003). Thus, the pathway of NF- κ B-mediated dysregulation of proliferation in cultured cells may converge through increased cyclin D1 expression.

Osteopontin Promoter Analysis

Enhanced secretion of osteopontin is a hallmark of the allylamine phenotype. Increased osteopontin RNA levels were observed in from treated vessels by Real Time

RT-PCR, confirming microarray data previously generated in our laboratory (Partridge et al, unpublished data). To evaluate molecular mechanisms of osteopontin regulation, luciferase constructs containing the osteopontin promoter were generated and transfected into control and allylamine cells.

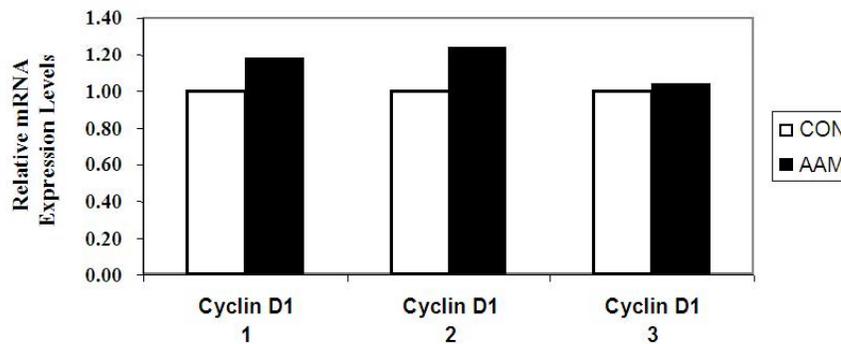


Fig. 22: Expression levels of cyclin D1 mRNA in control and allylamine cells. mRNA was isolated from subconfluent cultures of control and allylamine vSMCs, and processed for Real Time RT-PCR. □ = control, ■ = allylamine. Each set represents an independent experiment.

2094 base pairs of the rat osteopontin promoter (rOPNpr) (AF017274) were spliced into pGL2-basic, a luciferase vector. Deletions were generated of this full-length construct, beginning at 1157, 672, and 129 relative to the beginning of the parent sequence to assess the importance of distinct regions. These deletions are summarized in Fig. 24. The results presented in Fig. 25 show that when these promoter constructs were transfected into control and allylamine cells, the activity of the full-length promoter

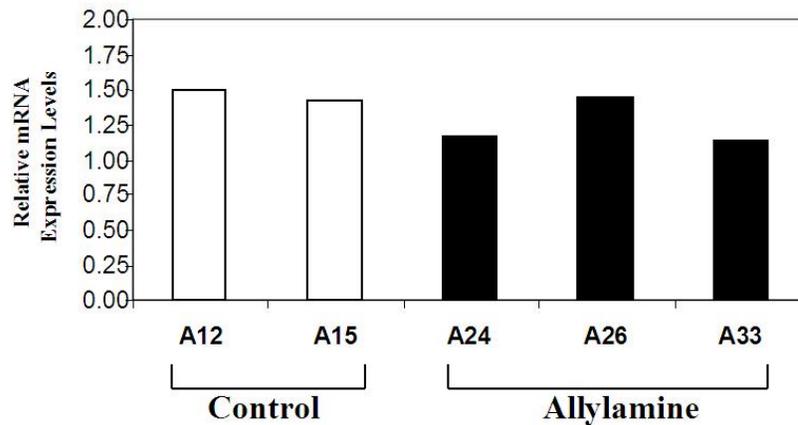


Fig. 23: Cyclin D1 mRNA expression in control- and allylamine-treated aortas. mRNA was isolated from the aortae of treated animals, and processed for Real Time RT-PCR for quantitation of cyclin D1 mRNA levels. Animals A12 and A15 were treated with water (control), and A24, A26, and A33 were treated with allylamine. □ = control, ■ = allylamine.

was lower in allylamine cells compared to controls. Progressive deletions caused an increase in promoter activity in the control cells, suggesting the presence of inhibitory elements between 2094 and 1157, and between 1157 and 672. An increase in activity was also observed in allylamine cells when bases from 1157 to 672 were removed, suggesting that inhibitory elements therein are affected in both phenotypes. The minimal promoter, 127, displayed very low levels of activity. Examination of the sequences between 2094 and 1157 revealed several cis-acting elements, including a vitamin D response element (VDRE), AP-1 binding site, NF- κ B binding site, and an upstream stimulatory factor (USF) binding site.

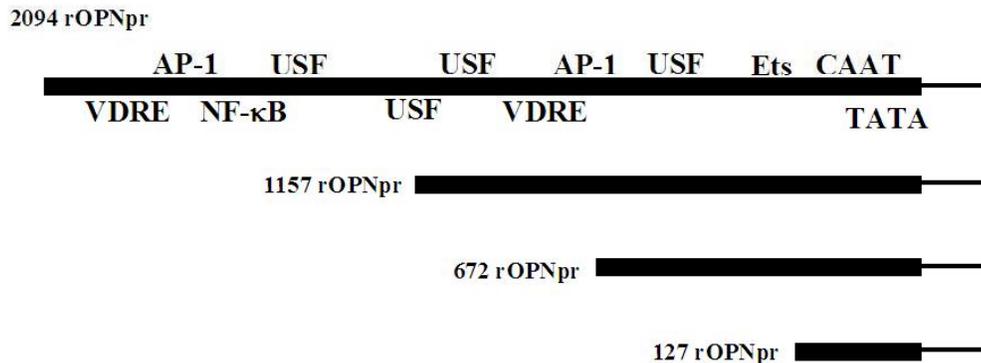


Fig. 24: Schematic representation of the rat osteopontin promoter, cis-acting elements within the full-length promoter, and several deletions thereof.

Several cis-acting elements were identified in the osteopontin promoter using MatInspector v2.2. Therefore subsequent experiments were conducted to examine the relative contributions of specific sites by mutational analysis. Of interest were the vitamin D response element, AP-1 binding domain, NF-κB binding domain, and a USF binding site. Site-directed mutagenesis was performed using the QuikChange Site Directed Mutagenesis kit, with appropriate mutagenic oligos. These mutations are detailed in Fig. 26. Disruption of any of these elements reduced luciferase activity in control cells (Fig. 27), suggesting cooperation between these elements or possibly low-

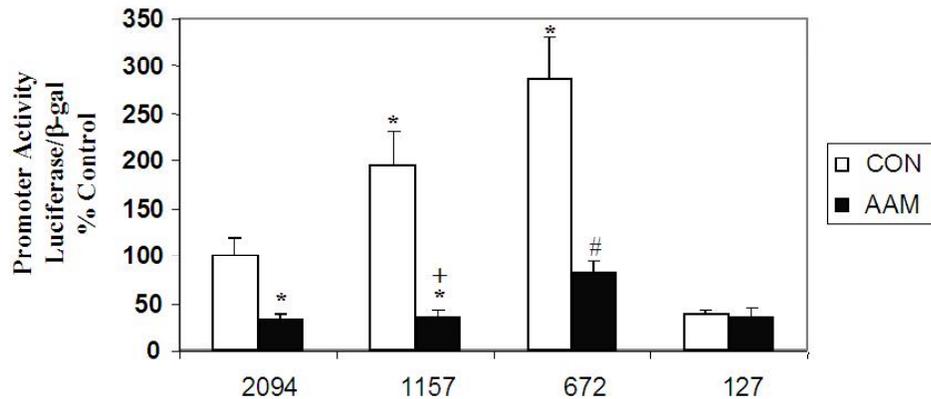


Fig. 25: **Osteopontin promoter activity in control and allylamine cells.** Control and allylamine cells were seeded at equivalent densities and allowed to recover for 8 hours. Cultures were then transfected with the rat osteopontin promoter in pGL2-basic, a luciferase vector, or a deletion thereof (1157 rOPNpr, 672 rOPNpr, 329 rOPNpr, and 129 rOPNpr). Also included as a control was β -galactosidase, to allow normalization of luciferase signal. \square = control, \blacksquare = allylamine. * indicates significant difference from 2094 rOPNpr in control, + indicates significant difference from 1157 rOPNpr in control, # indicates significant difference from 657 rOPNpr in control ($p < 0.05$).

level constitutive activity that can overcome inhibitory influences on promoter activity.

Interestingly, mutation of either the NF- κ B or USF1 site returned the transcriptional

activity in allylamine cells return to full-length control levels, suggesting that

osteopontin promoter activity is under negative control by NF- κ B in cooperation with

USF activity.

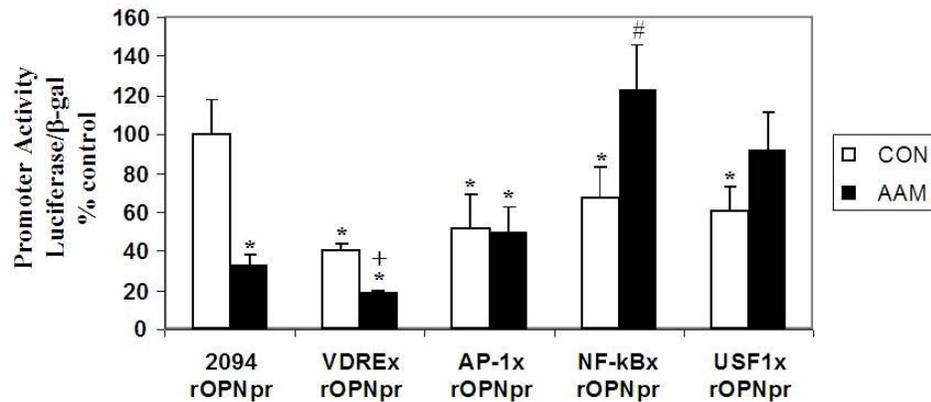


Fig. 27: **Osteopontin promoter activity in control and allylamine cells after site-directed mutagenesis.** Control and allylamine cells were seeded at equivalent densities and allowed to recover for 8 h. Cultures were then transfected with the rat osteopontin promoter (rOPNpr) in a luciferase vector, or a vector in which a cis-acting element has been mutated through site-directed mutagenesis. These mutants are denoted VDREx rOPNpr, AP-1x rOPNpr, NF-κBx rOPNpr, USF1x rOPNpr, USF2x rOPNpr, and USF3x rOPNpr. □ = control, ■ = allylamine. * indicates significant difference from 2094 rOPNpr in control, + indicates significant difference from VDREx rOPNpr in control, # indicates significant difference from NF-κBx rOPNpr in control ($p < 0.05$).

interactions with chromatin remodeling proteins including CBP/p300, and histone acetyltransferases (96). Thus, phosphorylation was examined by Western immunoblotting of immunoprecipitated p65 for phosphoserine. Serine phosphorylation of p65 was noticeably reduced in allylamine cells, suggesting that p65 is less effective at transcriptional activation in allylamine cells than control cells (Fig. 28). This finding is relevant in light of studies suggesting that unphosphorylated p65 associates with histone deacetylases (HDAC1 and HDAC2) which cooperate to repress the expression of NF-κB-regulated genes (97).

Next, the importance of matrix on osteopontin promoter activity was studied by

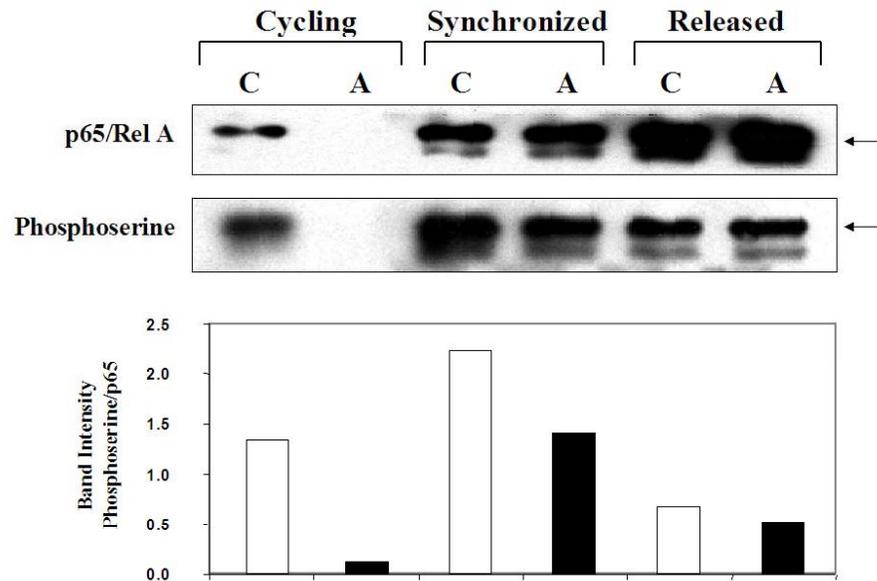


Fig. 28: **Serine phosphorylation of immunoprecipitated p65/RelA in control and allylamine vSMCs.** Immunoprecipitated p65/RelA was electrophoresed on separate gels, and electroblotted onto nitrocellulose membranes. The membranes were blocked overnight in 5% milk in TBS with 0.1% Tween, and then probed for 1.5 h for p65/RelA or phosphoserine. The membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) for 1.5 h, and then with chemiluminescent substrate for 5 min. The membrane was visualized with the Kodak Image Station 4400, and densitometry performed using the Kodak 1D Image software. C (□) = control, A (■) = allylamine.

seeding control and allylamine cells on collagen I, fibronectin, and laminin. These cells were allowed to recover, before transfection with full-length osteopontin promoter in a luciferase construct. Interestingly, the osteopontin promoter activity was 400% of control in allylamine cells seeded on fibronectin (Fig. 29, panel A), suggesting that these cells are primed for osteopontin expression when seeded on a permissive substrate.

The importance of matrix on NF- κ B regulation of osteopontin activity was also assessed by comparing the full length promoter to mutants seeded on these same substrates (Fig. 29, panel B). Deletion of the NF- κ B binding site increased promoter activity in allylamine cells seeded on collagen I. This observation is consistent with the ability of collagen to serve as a repressive matrix for allylamine cells (148), and suggests that NF- κ B plays a role in collagen I-mediated repression of osteopontin expression.

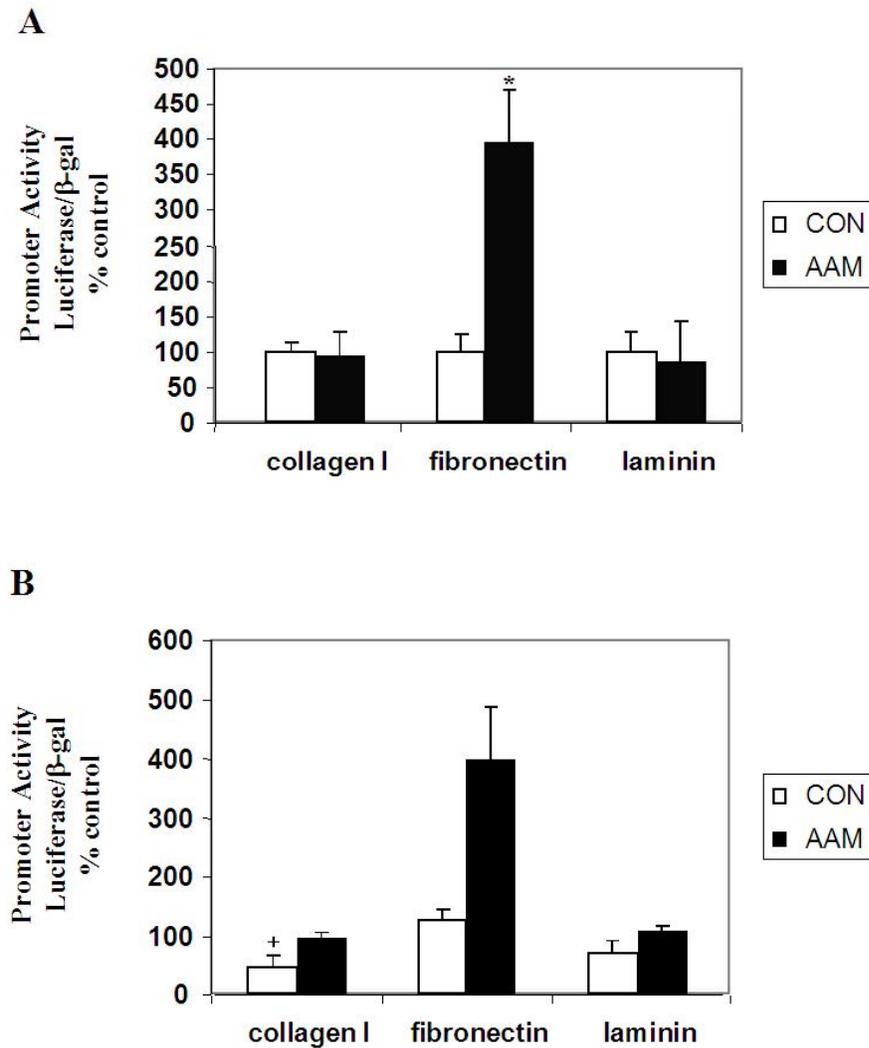


Fig. 29: Activity of osteopontin promoter-luciferase constructs in control and allylamine cells seeded on different matrices. Control and allylamine cells were seeded at equivalent densities on collagen I, fibronectin, and laminin, and allowed to recover for 8 h. Cultures were then transfected with the rat osteopontin promoter (2094 rOPNpr) in a luciferase vector (panel A), and a full length construct in which the NF- κ B site was mutated (NF- κ Bx rOPNpr) (panel B). These mutants are denoted VDREx rOPNpr, AP-1x rOPNpr, NF- κ Bx rOPNpr, USF1x rOPNpr, USF2x rOPNpr, and USF3x rOPNpr. \square = control, \blacksquare = allylamine. * indicates significantly different from control, + indicates significantly different from 2094 rOPNpr in same cell type on same seeding matrix ($p < 0.05$).

DISCUSSION AND SUMMARY

Regulation of NF- κ B Activity

Recent work in this laboratory described increased NF- κ B binding activity in allylamine vSMCs (148). This perturbation is critical for the maintenance of activated phenotypes, as treatment with PDTC specifically inhibited proliferation of activated vSMCs but not controls. These findings are consistent with emerging evidence implicating NF- κ B dysregulation in the pathogenesis of atherosclerosis (158, 159). Giachelli's group have reported that NF- κ B is activated in atherosclerotic vSMCs, but not uninjured vessels (160), and NF- κ B has been implicated in the chronic inflammatory response leading to hyperproliferation of vSMCs during atherogenesis (161). Similar increases in NF- κ B activation have been observed during the course of the atherogenic response in oxidant-injured aortic tissue (Partridge et al, unpublished data, 2003).

The present studies detail the molecular basis for dysregulation of NF- κ B activity in oxidant-activated vSMC phenotypes. We observed enhanced degradation of I κ B α in allylamine cells, indicative of increased nuclear translocation of NF- κ B. This finding suggests a basis for increased proliferation of activated vSMCs, as recent studies using purified I κ B α (162) or antisense p65 (163) have independently demonstrated a vital role for NF- κ B in vSMC proliferation. Not surprisingly, phosphorylation of both IKK α and β was increased in allylamine vSMCs compared to controls. These findings are consistent with previous data suggesting that induction of atherogenic phenotypes by

oxidative injury involves disruption of PI3K, PKC, and ras signaling, all upstream activators of IKK in vSMCs (90, 91, 94).

Processes downstream of IKK activity and I κ B α degradation, such as NF- κ B nuclear localization and DNA binding activity, were also perturbed in activated cells compared to controls. These data are indicative of disruption of NF- κ B activation in atherogenic vSMC phenotypes, in accord with the hyperproliferative state of these cells (100). However, the ability of allylamine vSMCs to proliferate is dependent on extracellular matrix-related signaling, since collagen I, but not plastic, fibronectin, or laminin inhibit their proliferative advantage (148).

Disruption and remodeling of the vessel wall matrix is a pivotal process in atherogenesis. Expression of activated vSMC phenotypes requires disruption of the basal lamina by macrophage metalloproteinases (66). As the process of phenotypic conversion progresses, activated vSMCs produce larger amounts of collagen I that change the relative composition of the extracellular matrix (164). Monomeric collagen I becomes a large component of the matrix (28), as does fibronectin, a protein which promotes dedifferentiation of vSMCs within the lesion (36). Previous data have indicated that collagen I restricts the ability of oxidant-activated cells to proliferate more rapidly than controls, while cells seeded on plastic, fibronectin or laminin display the allylamine phenotype (148). Matrix-dependent increases in NF- κ B binding activity are consistent with this pattern, suggesting a link between matrix signaling and NF- κ B in the regulation of proliferative activity of allylamine vSMCs. Mature collagen I may elicit a reduction in proliferative activity through increased expression of cdk2 inhibitors, as

demonstrated in arterial SMCs (32) . Repression of NF- κ B activity by overexpression of I κ B in HeLa cells is associated with a similar response (165). We have recently shown that a collagen I matrix modulates p21 and cyclin D1 expression in allylamine vSMCs (Jones, Wilson, and Ramos, unpublished observations, 2003), key targets of NF- κ B regulation (98, 99, 166). The underlying mechanisms that translate the interactions between integrins and extracellular matrix proteins to transcriptional responses remain to be fully elucidated. It is clear that in healthy vessels, matrix interactions serve to protect against hyperproliferation of vSMCs. However, in injured vessels, as the composition of the matrix changes, so do the responses of cells within the vessel wall, especially those involving integrin-coupled NF- κ B activation and function.

The appearance of multiple complexes of varying mobility as a function of time and substrate prompted examination of the levels of NF- κ B constituent Rel proteins. Few studies have examined mechanisms that modulate the expression of these proteins. An Ets-family transcription factor was implicated in c-rel expression (167), and the expression of p50/p105 is probably regulated by NF- κ B itself (168). Several matrix-dependent alterations in total cellular Rel protein expression were noted in allylamine vSMCs compared to control counterparts. Changes in Rel protein levels were also noted in nuclear extracts. On the basis of simple stoichiometry, it is likely that altered expression of Rel proteins leads to differences in the prevalence of NF- κ B dimers present at any given point in time (Fig. 30). Because each dimer has a subset of DNA domains to which binding is most favorable (79), a shift in relative Rel protein expression levels could result in differential modulation of gene expression. Such a shift

in dimer prevalence has been described during B-cell differentiation, leading to altered transcriptional activation of the immunoglobulin κ light chain (169). Further, the importance of dimer identity to the effect of NF- κ B binding has been the focus of several recent studies (170, 171). The pattern and intensity of NF- κ B binding activity in

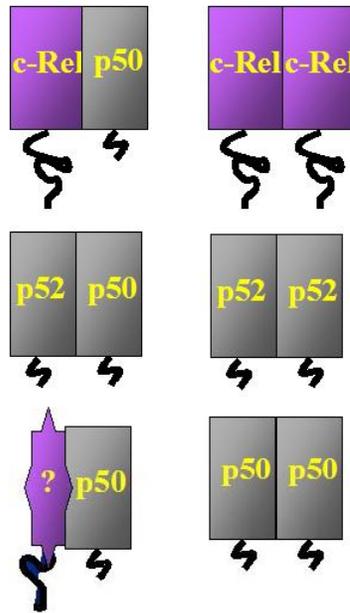


Fig. 30: **Representation of NF- κ B dimers favored in allylamine cells.** Based on relative expression levels, the assembly of these dimers is favored stoichiometrically.

control versus allylamine vSMCs varied among the four different promoter sequences examined, suggesting that a shift in NF- κ B dimer prevalence plays a role in the regulation of allylamine vSMC phenotypes. As p50 and p52 protein levels are increased

in allylamine cells, hetero- and homodimers of these two proteins may be more prevalent. These dimers would act more often as transcriptional repressors.

Further, high levels of constitutive NF- κ B nuclear residence in both control and allylamine cells was noted, though these increases did not translate into increased DNA binding activity. These observations suggest that NF- κ B can be regulated at the level of binding to DNA, consistent with observations that cytosolic modifications of Rel proteins and subsequent changes in DNA binding can affect the ability of NF- κ B to regulate gene expression (172, 173).

Examination of NF- κ B-regulated target genes revealed a pattern consistent with these observations. Osteopontin mRNA levels are increased in response to allylamine injury both *in vivo* and *in vitro*, consistent with several studies which demonstrate increases in osteopontin in atherosclerosis (40, 174). However, no such enhancement was noted for MMP-9 mRNA levels. This finding is in contrast to studies that suggest that NF- κ B is responsible for increases in MMP-9 expression (175), in response to inflammatory cytokines IL-1 α in vSMCs. This difference in response may be attributed to different signaling pathways activated in these separate systems. In any case, patterns of NF- κ B binding to promoter sequences correlate with increased mRNA expression in this oxidant-injury model of atherosclerosis.

As experiments with PDTC have demonstrated, NF- κ B activity is critical to enhanced proliferative capacity in allylamine cells. One NF- κ B target that could account for this phenomenon is cyclin D1 (98, 156). Though only slight increases in mRNA levels were observed *in vitro*, cyclin D1 protein was increased in allylamine cells

(Jones Wilson and Ramos, in preparation 2003). Given that matrix-dependent patterns of NF- κ B enhancement coincide with the proliferative advantage of oxidant-activated vSMCs, and inhibitors of NF- κ B abolish the proliferative potential of allylamine vSMCs (148), we conclude that NF- κ B participates in matrix-dependent regulation of the allylamine vSMC phenotype following oxidative injury.

Osteopontin Promoter Activity in Control and Allylamine Cells

Several observations both in the allylamine model and other models suggest that osteopontin is a critical modulator of atherosclerosis (148, 176, 177). Increased production of osteopontin after injury has been described in balloon angioplasty models of atherosclerosis (40, 174), as well as in this oxidant injury model of atherosclerosis (149).

The findings of the present study demonstrate that alterations in mRNA level in allylamine cells are not mediated at the transcriptional level. This conclusion is based upon the finding that promoter activity in allylamine cells was consistently lower than in control cells irrespective of the length of the promoter, or the deletion of putative activator/repressor sequences. Progressive deletion analysis uncovered strong repressor sequences within the promoter in vascular smooth muscle cells irrespective of growth phenotype. The downstream elements responsible for these patterns of activity remain to be established.

Osteopontin is an acute response gene involved in survival and proliferation (39). As such, immediate activation of the gene in response to stress likely involves

transcriptional activation (178). In contrast, the allylamine phenotype results from long-term adaptation to repeated cycles of oxidative injury and expression of new steady states involving differential regulation of stress response genes. Thus, a lack of transcriptional enhancement of the osteopontin gene in allylamine cells seeded on non-permissive substrates is to be expected. Because the allylamine phenotype is highly dependent on matrix production and deposition, transcriptional enhancement of atherogenic genes follows similar patterns.

Surprisingly, few studies have focused on transcription factors responsible for increases in osteopontin production in atherosclerosis. Several studies have suggested the importance of upstream stimulatory factors (USF1) in injury-induced osteopontin expression (179), and in response to diabetic hyperglycemia (180). AP-1 has also been implicated (180), perhaps as a result of increased ERK1/ERK2 activity (177). Computer assisted analysis of the rat osteopontin promoter revealed binding sites for both of these elements, as well as for the vitamin D receptor (VDRE) and NF- κ B. Analysis of promoters in which these sites were mutated yielded a complex picture which suggests a role for NF- κ B in repression of promoter activity in allylamine cells seeded on non-permissive substrates. Also, data generated after disruption of the USF1 site suggests a degree of cooperation between this factor and NF- κ B, as mutation of either site returns promoter activity in allylamine cells back to control levels. The ability of NF- κ B to repress gene transcription as a function of p50 and p52 hetero- and homodimers has been the subject of several recent studies (83, 181), as has the potential for USF1 binding to repress the transcription of CYP1A1 (182) and plasminogen activator inhibitor-1 (183).

Indeed, a study of NF- κ B binding sites suggests a high affinity of repressive NF- κ B dimers for the osteopontin promoter sequence (81). Cooperativity between these two transcription factors in binding to nucleosome cores has also been recently described (184). Thus, cooperative binding of NF- κ B and USF1 to the promoter of osteopontin may help to effect repression of activity in allylamine cells. However, in control vSMC populations, it is apparent that all of the examined sites are responsible for transcriptional increases to varying degrees, suggesting that recruitment of NF- κ B complexes in control and allylamine cells is altered. This interpretation is consistent with differential patterns of Rel protein expression between these two cell types.

Differential post-translational modifications of Rel proteins may also participate in altered DNA binding activity and/or transactivation potential (96, 157). This phosphorylation event has been attributed to several different kinases, including casein kinase II (185, 186), p38 MAPK (187), IKK α (188), PKA (96), PKC (189), and PI3K/Akt (95, 190). In our system, immunoprecipitated p65 is less phosphorylated in allylamine cells than in control cells, suggesting that NF- κ B dimers which contain this Rel protein would be less likely to transactivate, due to their inability to engage chromatin remodeling machinery (96) and association with histone deacetylases which would inhibit accessibility to DNA binding sites (97).

Signaling through integrin receptors bound to fibronectin increases osteopontin promoter activity in allylamine cells, while no such enhancement is observed in populations seeded on collagen I or laminin. The ability of fibronectin to induce dedifferentiation of vSMCs has been well-documented (191-193), as well as its ability to

induce expression of osteopontin in cultured vSMCs (194). Regulation of osteopontin expression in cells seeded on fibronectin clearly does not involve NF- κ B to a large degree, though this pattern is subject to substrate/matrix regulation on plastic. In that regard, fibronectin may induce a different profile of transcriptional regulators that influence the activity of this promoter. It should be noted, however, that repression of activity on collagen I may be partially accomplished through the NF- κ B element. This interpretation is consistent with a recent study showing that collagen I activates NF- κ B-regulated transcriptional programs (195).

Summary

Dysregulated NF- κ B plays a role in a number of diseases, including atherosclerosis, arthritis, cancer, diabetes, AIDS, and stroke (196). In most of these disease states, inflammation is a key component in pathologic progression. In atherosclerosis, activated NF- κ B has been described *in vivo* (160), and as a critical modulator of vSMC proliferation and phenotypic conversion *in vitro* (161). In the context of atherogenesis, dysregulated NF- κ B may exacerbate the condition by upregulating adhesion molecules VCAM-1 and ICAM-1 (78), inflammatory cytokines including TNF- α , IL-6 (197) and IL-1 β , matrix metalloproteinases 1, 3, and 9 (198) (175), protective factors such as inhibitors of apoptosis (IAPs), Bcl-XL, and c-Myc (199), and cell cycle molecules such as cyclin D1 (98, 99, 156) and cyclin-dependent kinase inhibitor p21^{Waf/Cip1} (166). No specific NF- κ B target has yet been identified that encompasses its role in the atherosclerotic disease process. Most likely, the importance

of NF- κ B in the atherosclerotic response lies in the activation of a subset of genes, and repression of others, as suggested by Tian and coworkers (200). One possibility in this

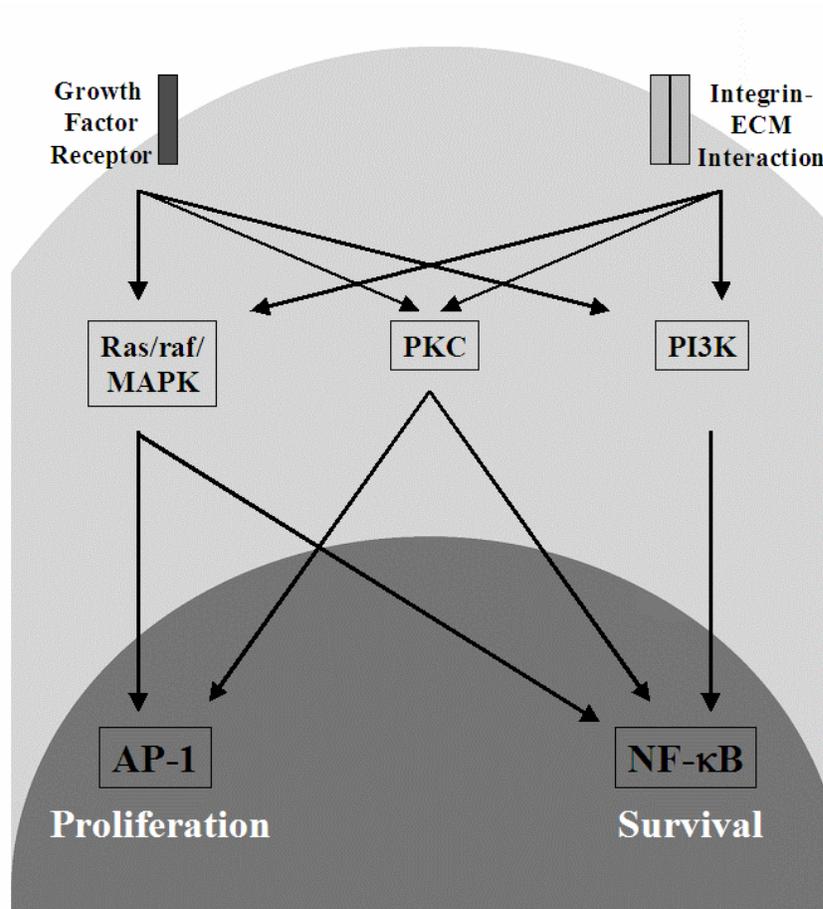


Fig. 31: **Signaling pathways involved in regulation of the atherosclerotic phenotype.** Altered MAPK, PKC and PI3K mediated signaling leads to enhanced activation of AP-1 and NF- κ B. AP-1 may enhance the production of genes important to vSMC proliferation, while the action of NF- κ B protects the cell from apoptosis.

model is that NF- κ B functions to repress apoptosis in atherogenic vSMC phenotypes (198, 199), while AP-1, the activity of which is also increased in allylamine cells (147), mediates enhanced proliferative capacity (Fig. 31) (76). Indeed, the apparent degradation of RelA/p65 could be a result of abortive apoptosis cascades (201-204) as seen in Alzheimer's disease, a related condition (205). Our previously described increases in MAPK activity (Jones, Wilson, and Ramos, submitted 2003) and AP-1 binding activity (148) are consistent with this model of regulation. The interplay between these and other transcription factors in regulation of the atherosclerotic vSMC phenotype will doubtless continue to be the focus of studies in the future.

As noted above, osteopontin is expressed in atherosclerotic lesions *in vivo* (40), and in response to injury *in vitro* (177). Recent studies that employed transgenic mouse models of osteopontin overexpression indicate that this cytokine is necessary but not sufficient to induce neointimal thickening in response to injury (176, 206). Further, osteopontin transgenic mice develop lesions more quickly in response to high cholesterol (207). A recent study by Matsui in which male and female osteopontin knockout mice were compared suggests a gender-related difference in the role of osteopontin in atherosclerosis (208). The work of Parrish and Ramos suggest that perhaps cleavage of osteopontin, which generates a biologically active 36 kDa fragment, is a key facet of its role in atherosclerosis (149).

The molecular basis of osteopontin overexpression in atherosclerotic plaques remains elusive. In other contexts, ras (209), ERK1/ERK2 (177), PKC (210), and transcription factors USF1 (179) and AP-1 (180) have been implicated in osteopontin

expression. In our model of oxidant-induced atherosclerosis, examination of promoter activity suggests a matrix-dependent NF- κ B suppressive influence on osteopontin expression, perhaps in cooperation with USF1. In contrast, the VDRE and AP-1 elements act as enhancers of expression in uninjured cell populations. Thus, as increased osteopontin mRNA levels have been described both in the present studies and in several previous reports (149, 211), the possibility of regulated mRNA stability must be considered. Also, studies that examine mutation of multiple elements within the osteopontin promoter would shed more light on the repressive and stimulatory influences at play in this system.

In the present studies, the details of dysregulation of NF- κ B pathways have been investigated. Evidence has been presented that altered signaling, activation, and composition of NF- κ B dimers leads to differential DNA binding and regulatory activity. These changes lead to repression of osteopontin promoter activity and stimulation of other target genes. Thus, altered prevalence of NF- κ B dimers results in altered profiles of NF- κ B-dependent gene regulation that play a role in maintenance of atherosclerotic vSMC phenotypes induced by oxidant injury.

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