IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF NOVEL GENOMIC TARGETS IN OXIDANT-INDUCED VASCULAR INJURY

A Dissertation

by

CHARLES RANDAL PARTRIDGE

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 2005

Major Subject: Toxicology

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Approved by:

Co-Chairs of Committee,	Kenneth Ramos
	Stephen Safe
Committee Members,	Emily Wilson
	Thomas Spencer
	Robert Burghardt
Head of Department,	Robert Burghardt

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ABSTRACT

Identification and Molecular Characterization of Novel Genomic Targets in Oxidant-Induced Vascular Injury. (December 2005)

Charles Randal Partridge, B.S., Midwestern State University

Co-Chairs of Advisory Committee: Dr. Kenneth S. Ramos
Dr. Stephen H. Safe

Gene expression was examined in vascular smooth muscle cells to study the complex interaction between oxidative injury and the pathogenesis of vascular disease. Extensive vascular remodeling coupled to increased production of 8-epi-PGF2α, nuclear localization of NFκB, and alterations in glutathione homeostasis were identified as major responses of the vascular wall to oxidative stress. Transcriptional profiling studies, supported by immunohistochemistry and in situ hybridization measurements, identified genes involved in adhesion and extracellular matrix deposition (α_1 integrin, collagen), cytoskeletal rearrangements (α-smooth muscle actin, α-tropomyosin), and signal transduction (NFκB, osteopontin, and LINE) as targets of oxidant injury. In the case of osteopontin (OPN), elevation of OPN levels in vSMCs was shown to be mediated by redox-regulated transcriptional mechanisms. A 200bp region located in the 5′ UTR of the osteopontin promoter was found to be responsive to oxidative stress. This regulatory region contained two distinct cis acting elements involved in promoter inducibility. These elements were tentatively identified as NFKB and TIEG-1 binding sites and shown to be highly responsive to hydrogen peroxide and chemical antioxidants.

Collectively these studies answer central questions regarding the mechanisms underlying the vascular response to oxidative stress and the involvement of OPN in diseases of the vascular wall.

DEDICATION

To Manda, Marley and My Parents

For All of Their Support

ACKNOWLEDGEMENTS

The past few years would not have been possible without the help and support of my friends and family. It has been a long and strange journey that has taken us across the country and through many hardships. I would especially like to thank Dr. Kenneth Ramos for his patience, guidance and friendship throughout the years. He gave second chances when he said their were none, his time when there was none to spare and his shoulder when support was needed. I would also like to thank the members of my committee: Drs. Stephen Safe, Robert Burghardt, Thomas Spencer, Emily Wilson and Reza Forough who agreed to substitute at the last minute. I am also greatful for the support from the Department of Toxicology, especially Kim Daniels without whom it would have been a lot more diffiecult to get things done from Kentucky. The people who made my studies possible are far too numerous to mention, but I would like to thank each and every one of them for their support. You know who you are.

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CHAPTER I

INTRODUCTION*

Atherosclerosis and Diseases of the Vascular Wall

Atherosclerosis is the leading cause of death and disability in the United States and many other industrialized nations. However, most forms of atherosclerosis are preventable or even reversible if appropriate intervention is applied. The purpose of this study is to understand the underlying mechanisms leading to the initiation and progression of this pathological condition. One of the critical processes in the pathology of atherosclerosis is the uncontrolled production of reactive oxygen species (ROS). ROS production within the vascular wall can lead to oxidative stress and compromised structural and functional integrity. To study the cellular and molecular bases of this complex interaction, this laboratory has adopted a model of oxidative injury using allylamine, a vascular poison that compromises redox homeostasis leading to activation or repression of redox-regulated genes, peroxidative injury, and cell death.

This dissertation follows the style and format of the Journal of Molecular and Cellular Cardiology.

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Cardiovascular System

The structure and function of the cardiovascular system has been extensivelyreviewed [1]. Briefly, the heart and blood vessels form a circuit for the transport of oxygen and nutrients to tissues throughout the body and the removal of waste products of cellular metabolism. The heart pumps blood through a vascular network that includes arteries, veins, arterioles, capillaries, and post-capillary venules. Oxygen and nutrient exchange at the level of the tissue is regulated by changes of microvascular resistance in response to metabolic demands. Blood returns to the heart through capacitance vessels of the venous compartment.

Cardiac Morphology

The walls of the heart consist of three distinct layers that include the epicardium, myocardium and endocardium. The epicardium is the external layer that originates from visceral connective tissue. The myocardium consists exclusively of muscle cells, while the endocardium consists of a thin sheet of endothelial cells that extends from the coronary vessels to line the chambers and valves of the heart. Cardiac muscle consists of nodal tissue, Purkinje tissue, and muscle [2]. Nodal tissue exhibits a high degree of automaticity – that is the capacity to depolarize spontaneously. Purkinje cells are highly specialized for conduction of electrical impulses, while ordinary muscle cells contract in response to electrical and pharmacological stimulation [3].

Contractility

The sarcolemma and the mitochondrial and sarcoplasmic reticulum membranes are key membrane systems involved in homeostasis of the heart [4]. Conduction of nerve impulses along the sarcolemma triggers the release of intracellular calcium stores from the sarcoplamic reticulum and the opening of voltage sensitive calcium channels. Spontaneous depolarization of specialized cells within the heart mediates conduction of electrical impulses that propagate electrical signals throughout the muscle and trigger ionic changes tightly coupled to muscle contractions. As in other excitable cells, depolarization involves increased conductance of sodium ions across gated channels to reverse the polarity of the membrane and initiate ionic conductances that increase intracellular calcium. Injury to the myocardium is often associated with ionic disturbances and accumulation of calcium at toxic levels. This can be associated with cardiac hypertrophy, necrosis or apoptosis [5].

Cellular Compartments

The sarcolemma consists of two layers: an inner plasma membrane that regulates ionic permeability and myocardial contractility, and an outer membrane involved in calcium binding. Mitochondria function in energy generation via electron transport and participate in ionic transport and accumulation. The sarcoplamic reticulum is a tubular system that comes in close contact with the sarcolemma and functions in calcium exchange and accumulation. The sarcolemma is the membrane that surrounds each myofiber. It consists of invaginations called T-tubules. When an action potential reaches

the muscle cell and the sarcolemma is depolarized, this depolarization spreads down the t-tubule to the interior of the cell, where it stimulates the release of Ca++ from the sarcoplasmic reticulum. Recent studies have identified cellular targets capable of decoding frequency-encoded intracellular calcium signals, indicating that calcium pulses as opposed to net calcium levels control downstream targets intracellularly [6]. At low frequency, calcium oscillations stimulate calmodulin kinase II followed by kinase deactivation between spikes. During high frequency calcium oscillations the activity increases to maximal levels.

Calcium

Calcium is critical to myocardial contraction. In muscle cells, the regulation of this critical ion involves sarcolemmal pumps and exchangers that mediate energy-dependent sequestration of free calcium [7]. Events that stimulate muscle activity by raising sarcoplasmic calcium begin with neural excitation at neuromuscular junctions. Excitation induces local depolarization of the sarcolemma, which spreads to the associated T tubule system and deep into the interior of the myofiber. T tubule depolarization spreads to the sarcoplasmic reticulum (SR), with the effect of opening voltage-gated calcium channels in the SR membranes. This is followed by massive, rapid movement of cisternal calcium into the sarcoplasm close to nearby myofibrils. The appearance of calcium very close to the Tn-C subunit of troponin results in the production of multiple myosin power strokes, as long as the available calcium concentration remains greater than about 1 to 5 micromolar [8]. The process of

myocardial contraction is associated with calcium influx through the sarcolemma and release of intracellular calcium stores. Calcium then binds troponin to remove the inhibition exerted by the troponin-tropomyosin system on actin and myosin. A reduction of cytosolic calcium is associated with relaxation of the myocardium. Cytosolic calcium concentration is lowered via energy-dependent mechanisms. Dysregulation of these calcium-mediated processes lead to cellular injury and impairment of cardiac function. Elevation of smooth muscle calcium to about 10^{-5}M induces formation of Ca^{2+}Calmodulin (CaCM) complexes which activate thin filaments by binding caldesmon (Cald) and freeing myosin binding sites on thin filaments. CaCM also binds and activates myosin light chain kinase (MLCK). Active MLCK phosphorylates myosin p-light chain activating the actomyosin ATPase activity of myosin headpieces. Epinephrine binding to b-adrenergic receptors raises cAMP, activates cAMP-dependent protein kinase (PKA), which reduces the affinity of MLCK for CaCM and modulates the strength of contractions generated by elevated cytosolic calcium [9].

Arterial Morphology

The blood vessel wall of arteries is made of three distinct layers. The innermost layer is the tunica intima and represents a single layer of endothelial cells resting on a thin basal lamina. The medial layer consists of several sheets of smooth muscle cells dispersed in a matrix of collagen and elastin. The outermost layer is formed by fibroblasts that provide structural support to the vessel, and participate in the regulation

of medial smooth muscle function. Capillaries are endothelial tubes that rest on a thin basal lamina to which pericytes readily attach [10].

Contractility

In contrast to the heart, blood vessels require hormonal or pharmacological stimulation for initiation of contraction. Smooth muscle relies heavily on calcium entering from the extracellular space to raise intracellular calcium levels and support prolonged energy efficient contractions, using approximately 1% of the energy of striated muscle [11]. Because of this contractile efficiency, prolonged contractile states can provide tone to vessels. Reduction of extracellular calcium can lead to SMC proliferation that is similar to that seen in atherogenic phenotypes [12].

Vascular Smooth Muscle Cells

Vascular smooth muscle exhibits multiple phenotypes that can be distinguished by their relative expression of smooth muscle-specific genes and their degree of proliferative activity. In their most differentiated state, vascular smooth muscle cells are characterized by low mitotic rates, predominance of smooth muscle-specific proteins, a well-defined myofilament network, and contractility in response to physiologic stimulation [13]. Vascular smooth muscle cells can also exhibit less differentiated phenotypes characterized by enhanced proliferative, migratory, and synthetic capabilities and loss of smooth muscle-specific properties. Less differentiated and apoptotic phenotypes make up the majority of the smooth muscle cells localized within

atherosclerotic lesions in both experimental and human disease [14]. As such, the transition of vascular smooth muscle cells from a quiescent phenotype to a succession of less differentiated proliferative phenotypes is the hallmark of atherosclerosis onset and progression [15]. These alterations in phenotype span the entire spectrum of cellular behaviors from quiescence to migration, apoptosis, differentiation, and proliferation. Subpopulations of vascular smooth muscle cells within the normal vessel that differ in their responsiveness to stimuli have also been described [16].

Extracellular Matrix

The extracellular matrix is another key component of the myocardial and vascular environment. This matrix provides a structural, chemical, and mechanical substrate essential in cardiovascular development, growth, and responses to pathophysiological signals. Metalloproteinases present in the myocardium degrade all the matrix components of the heart and as such, are the driving force behind myocardial matrix remodeling. Matrix metalloproteinases (MMPs) represent a family of extracellular and membrane-bound proteases involved in maintaining extracellular matrix (ECM) integrity and modulating interactions between cells during development and tissue remodeling. MMPs have been implicated in the pathogenesis of numerous diseases. There are 24 matrix metalloproteinases (MMPs) known in the human genome. They may be secreted or present on the cell-surface as membrane-bound molecules. They bind and cleave a variety of substrates, in a zinc-dependent fashion. [17].

Integrins

Transmembrane integrin receptors provide a dynamic interaction of environmental cues and intracellular events. Integrins orchestrate multiple functions in the intact organism including organogenesis, regulation of gene expression, cell proliferation, differentiation, migration, and death [18]. Cell shape and mechanical stimulation (stretch) can regulate growth and differentiation in both myocardial and vascular smooth muscle cells. The shape of cells is altered by cytoskeletal reorganization mediated by the extracellular matrix, suggesting that altered extracellular matrix-cytoskeletal dependent events may induce spatial changes that modify biological functions (see figure 1) [19]. Regulation of growth and differentiation by mechanical forces also act at the level of the extracellular matrix-cytoskeleton. Increasing evidence indicates that the integrin family of cell adhesion receptors is involved in transduction of biochemical signals from the extracellular matrix to the cell interior in order to modulate cell growth and differentiation. The myocardial extracellular matrix transmits mechanical forces generated by cardiomyocytes to cardiac cavities [20]. Integrins are members of a family of cell surface receptors that are comprised of α and β subunits. There exists at least 16 α and 8 β subunits that are capable of combining into at least 20 different receptors [21]. The α and β subunits are composed of a large extracellular domain, a region that spans the cell membrane and a short domain that is contained in the cytoplasm [22]. The binding of ligand to the receptor and the clustering of the ligand bound receptors are critical for the activation of intracellular integrin mediated responses [23]. Divalent cations are also critical for integrin stability. The binding of the cations

regulates the specificity and affinity of the receptor for ligands [24, 25]. Integrin binding and clustering initiates a signaling cascade which is capable of transmitting extracellular signals to the inside of the cell and can affect gene expression [26]. Integrin binding provides a way to link extracellular matrix proteins on the extracellular side of the plasma membrane to cytoskeletal proteins and actin filaments in the cytoplasm. Ligand binding and the resulting clustering occur at sites called focal adhesions [27]. Focal adhesions initiate the nucleation of a large number of cystoskeletal proteins. These proteins play an important role in cellular adhesion and migration. The proteins localized in focal adhesions include, α actin, talin, vinculin, and tensin [28]. Integrin binding and the resulting complex of proteins regulates cell morphology, adhesion and motility. The complex of proteins associated with the focal adhesions can also serve as scaffolding for various other proteins that mediate integrin signaling [21]. Clustering of integrins also elicits as enhancement in tyrosine phosphorylation termed focal adhesion kinase (FAK) [29]. FAK plays a critical role in integrin mediated transduction of signals. FAK is targeted to focal adhesions via a focal adhesion targeting sequence (FAT) [30]. Several proteins localized to focal contacts also contain SH2 and SH3 domains [31]. PI-3 kinase and PLC are two other proteins that are activated in response to integrin binding [32]. Grb2 and SOS also associate with focal adhesions following integrin binding [33].

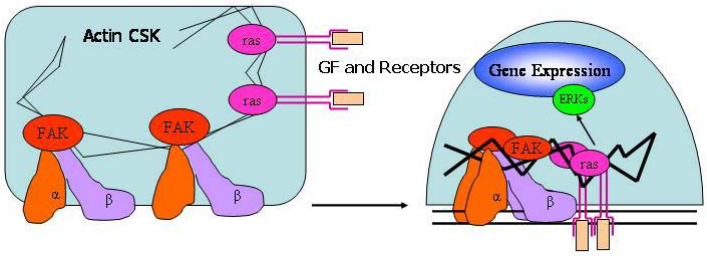


Figure 1. Coupling of the ecm/integrin and growth factor RTK pathways.

Upon integrin and growth factor bindings there is a reorganization of the actin cytoskeleton. This reorganization that occurs with the integrin clustering provides as spatial and biochemical coupling if the integrin and growth factor pathways. This coupling of pathways leads to activation of MAPK and can ultimately affect gene expression

Remodeling

Network rearrangement and enlargement is an essential component of vascular remodeling at various pathological stages. In hypertrophic cardiomyopathy, the primary changes occur within cardiomyocytes that are subjected to either necrosis or mutation of genes that code for contractile proteins. A distortion of the mechanical link between the contractile apparatus and the collagen matrix may disturb force transmission in both directions and lead to decreased developed pressure and increased systolic volume. Thus, extracellular matrix alterations may be the primary factor in the pathogenesis of many cardiomyopathies [34].

Response to Injury

In both heart and blood vessels, injury is associated with recruitment of inflammatory cells. These cells play a very important role in determining outcomes of injury to the cells of the cardiovascular system. Although cardiovascular cells are not prolific cells in the adult differentiated phenotype, a common response to injury involves activation of genes involved in the regulation of growth and differentiation. Cardiac myocytes are terminally differentiated cells that undergo hypertrophy in response to injury. This is often seen in smokers and those living in polluted cities [35]. Likewise, during the early phases of atherosclerosis, chemoattractants and molecules generated by the endothelium, smooth muscle, and monocytes such as monocyte chemotactic protein 1, osteopontin, and modified low-density lipoprotein, attract monocytes and T cells into the artery. Monocyte-derived macrophages and specific subtypes of T lymphocytes are found at every stage of the disease [36]. Fluid pressure and physical forces acting on endothelial cells cause fluid shear stress, which effects endothelial cell morphology and facilitate movement into the vessel [37]. The balance between pro-inflammatory and anti-inflammatory cytokines may be decisive for the progression of the atherosclerotic lesion. Class I major histocompatibility complex deficient mice demonstrated a three-fold increase in lesion area [38]. Vascular smooth muscle cells in the media of arteries, as well as lesions are surrounded by different types of matrix, largely type I and III fibrillar collagen in healthy smooth muscle cells and proteoglycan intermixed with scattered collagen fibrils in atherosclerotic lesions. Other matrix types, such as fibronectin and heparan sulfate, may play a role in cell-matrix regulation of the

expression of chemokines by macrophages [36]. Oxidized LDL or, LDL cholesterol that has been bombarded by free radicals, has been attributed a key role in the development of atherosclerosis. Previous studies have demonstrated increased plasma levels of oxidized LDL in patients with established vascular disease [39]. Free radicals and oxidized low-density lipoprotein are cytotoxic for macrophages, smooth muscle cells and endothelium, whose death contributes to the central necrotic core as the lesions advance. Studies of human and animal models of atherosclerosis suggest that apoptosis plays an important role in formation of the necrotic core [40]. Thus, the balance of cell death and proliferation is an important aspect of atherosclerosis. Both proliferation and apoptosis are enhanced in undifferentiated vascular smooth muscle cell phenotypes [41]. Transforming growth factor-β_1 and other growth factors and cytokines stimulate proliferation and apoptosis. [42]. The over-accumulation of lipid is toxic to cells and leads to necrosis and complex lesion formation [43].

Vascular-specific Injury

In serving circulatory functions, cells of the heart and vasculature are repeatedly exposed to blood-borne toxicants and their metabolic byproducts. Toxic moieties compromise cardiovascular function by interference with specialized cellular functions. Because cardiotoxic insult interferes with pumping of blood through the vasculature, blood flow to major organs can be compromised leading to end-organ dysfunction [44]. Acute myocardial injury may induce disruption of electrical activity and/or contractility, degradation of extracellular matrix and myocellular death. Likewise, acute vascular

toxicity involves cellular death accompanied by destruction of extracellular matrix components. Angiotoxicity may also cause alterations of arterial pressure due to changes in contractility and blood flow. Cardiovascular injury is often associated with recruitment of inflammatory cells and changes in the growth of cardiovascular cells [45]. Chemically-induced injury to cells of the heart or vascular system may also involve excessive accumulation of toxic chemicals within the tissue and/or cell-specific bioactivation of protoxicants.

Atherosclerosis

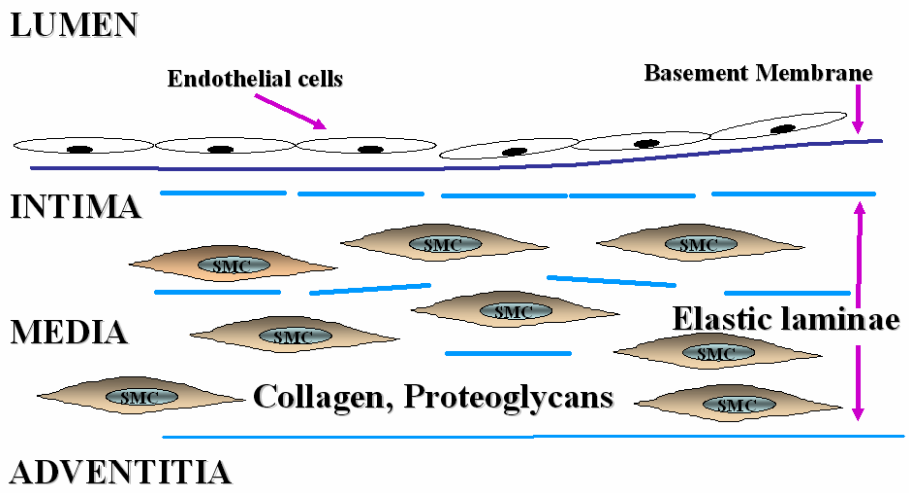
Heart disease is the number one leading cause of death in the United States. Cardiovascular disease is a primary or secondary cause in 60% of deaths in the United States, with 75% of these deaths resulting directly from atherosclerosis [46]. Atherosclerosis is a progressive disease of the arterial wall that is characterized by the migration of vascular smooth muscle cells (vSMCs) from the media to the intima, where they proliferate and lead to plaque formation in large to medium sized arteries. This proliferation if left unchecked, can lead to compromise of lumen diameter and the eventual formation of thrombi and the complications that follow. The differences between normal and atherosclerotic vascular morphology can be seen in figure 2. Unfortunately atherosclerosis produces no symptoms until the damage to the arteries is at a severe level, consequentially it takes ten to twenty years for symptoms to become evident. This makes it extremely difficult to diagnose this disease in its early stages. As

of yet, identification of cells predisposed to proliferation has been an elusive target for this disease.

Monoclonal Hypothesis

In order for the atherosclerotic plaque to progress, which is essentially a benign neoplastic tumor, there has to be a "switch" of vascular smooth muscle cells (vSMCs) from a contractile phenotype to a proliferative phenotype [47]. It is this initial "switch" that is the first step in a series of biochemical malfunctions that is extremely important in atherosclerosis. Indeed it is in the proliferation of a cellular population that is at the core of the diseases. If one starts to look at atherogenesis as being neoplastic in origin, then these biochemical malfunctions become more apparent. It was Earl Benditt who first hypothesized that atheromous plaques may indeed be neoplastic [48]. This observation had huge implications in the field of cardiovascular disease research because it was in direct conflict with what was the conventional wisdom of the time, which was that an atherosclerotic lesion was a proliferative response to toxic products that build up in the wall of an artery over time [49]. Without a doubt, this controversy continued for many years and has led to volumes of research being produced both for and against this divisive hypothesis. It was only in 1998 that Schwartz proved Benditt correct by a new PCR based method on micro-dissected tissue from an atherosclerotic lesion to show that indeed the vSMCs present in lesions were monoclonal in origin [49]. Regardless of the origin of this monoclonal expansion, atherosclerotic plaques undergo a cellular

A.



B.

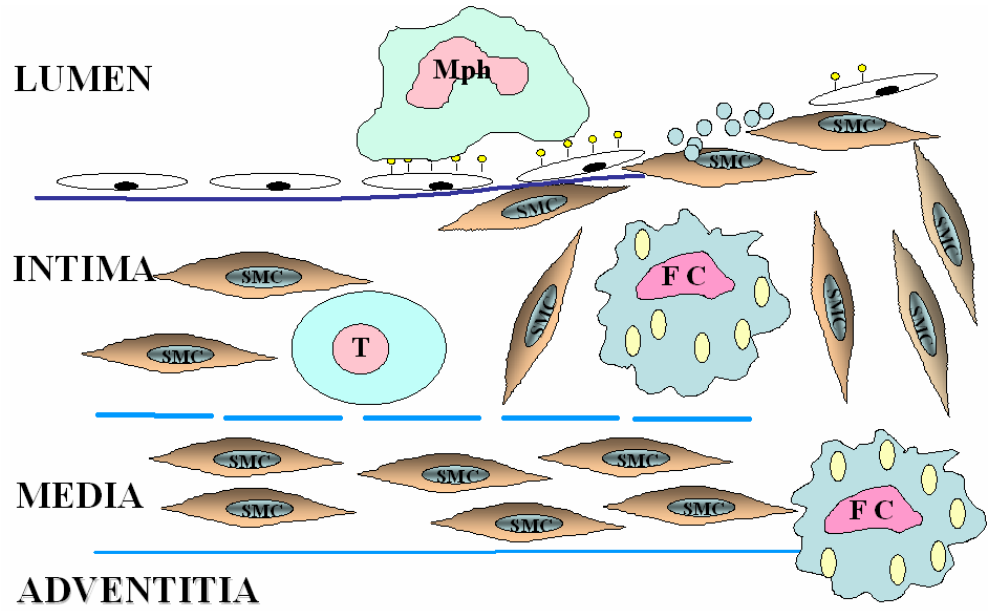


Figure 2. Normal and atherosclerotic vascular morphology.
A) Morphology and composition in the normal vasculature, with an intact endothelial layer and smooth muscle cells (SMC) contained mainly in the medial layer. In the atherosclerotic vessel (B) smooth muscle has migrated and proliferated into the intimal space, macrophages have attached to the endothelium and migrated into in intima. Once there, the macrophages become foam cells (FC) that deposit a lipid core upon death. These events can lead to a thrombus formation and vSMCs proliferation that will compromise the vessel lumen.

proliferation stage that is associated with progression of the disease. This "phenotypic switching" is at the heart this disease.

Carcinogen-Induced Atherosclerotic Vascular Disease

A logical extension of the clonal theory of atherogenesis is that known chemical mutagens and carcinogens cause vSMC mutations and atherosclerotic lesions. While the search for mutational targets has proven elusive, chemical mutagens have consistently been shown by different laboratories to induce atherosclerosis in experimental animals [50]. The body of knowledge implicating chemical carcinogens as etiologic factors in atherosclerosis coincided with Benditt's original report. Perhaps most relevant were the studies of Roy Albert and colleagues examining the atherogenicity of environmental chemical carcinogens [51]. Indeed, exposure of chickens to benzo(a)pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) present as a byproduct of the incomplete combustion of organic chemicals, initiates and/or accelerates atherosclerosis without altering serum cholesterol [52,53]. A similar response can be elicited by 7, 12-dimethyl-benz [a, h] anthracene or 3-methylcholanthrene. The ability of these carcinogens to cause atherosclerotic lesions has been associated with cytochrome P450 mediated conversion of the parent hydrocarbon to reactive intermediates that adduct cellular macromolecules. Aortic homogenates of chickens possess a cytochrome P-450-dependent monooxygenase activity that bioactivates BaP to mutagens that bind covalently to DNA in vitro [54]. BaP hydroxylase activity is also expressed within the aortic wall in humans, monkeys and rabbits [55, 56]. Human fetal aortic smooth muscle

cells metabolize BaP and 7, 12-dimethylbenz[a]anthracene to phenols and 12-hydroxymethyl-7-methylbenz[a]anthracene,7-hydroxymethyl-12-methylbenz[a] anthracene, 7, 12-dihydroxymethyl benz[a]anthracene, and trans-8, 9-dihydrodiol-7, 12-dimethylbenz[a]anthracene, respectively.

The majority of the activity responsible for the biotransformation of BaP is associated with the smooth muscle layers of the aorta, but activity is also localized in the aortic endothelium. Interestingly, aortic aryl hydrocarbon hydroxylase activity correlates with the degree of susceptibility to atherosclerosis in avian species. Other carcinogens implicated in animal or human atherosclerosis include 2, 4- and 2, 6-dinitrotoluenes, nitrosamines, arsenic and dioxin [50]. Because the inherent genotoxic potential among this diverse group of carcinogens is highly variable, clearly complex mechanisms are at play in the initiation and progression of atherosclerosis by environmental carcinogens. Regardless, the evidence suggests that atherosclerotic plaques and tumors both require disruption of mechanisms involved in control of cellular proliferation and differentiation. Thus, "phenotypic switching" is at the heart of both diseases.

Risk Factors

Stroke, angina or heart attacks are just a few of the cardiovascular conditions that are preceded by, or accompanied by, atherosclerosis. Risk factors for these diseases parallel those for atherosclerosis. While there are risk factors that can not be modified

such as, genetics, familial history or age, there are many factors that can be modified to lower the occurrence or decrease the severity of atherosclerosis.

Smoking

The association between cigarette smoking and atherosclerosis is well established. Smoking is one of the most important and most easily modifiable risk factors for this disease. Epidemiological studies have shown that smoking increases the incidence of atherosclerosis in both men and women and even in those exposed to passive secondary smoke [57]. Smoking has been established as increasing the incidence of cardiovascular disease. Conversely, the stopping of smoking will reduce the risk of atherosclerotic disease [58]. The pathophysiology of tobacco-related disease is complex with many confounding factors, the least of which is that cigarette smoke contains approximately 4000 compounds many of which are known atherogens and carcinogens [59]. These toxic compounds cause damage along many different biochemical pathways. This is just one of the many reasons why it is difficult to identify the mechanisms that lead to diseases that are tobacco related. However, if one identifies and dissects out key compounds and metabolic by-products of cigarette smoking, then the process of identifying the mechanisms responsible becomes an easier task. Another problem with identifying the mechanisms of cigarette smoke induced disease is that in addition to the compounds themselves being pathogenic, the metabolic activation of the compounds present in tobacco can be highly pathogenic as well. Sometimes these byproducts can even be more toxic that the parent compound found in smoke.

Experimental studies have shown that exposure to cigarette smoke induces the mutagenicity of heterocyclic amines found in smoke. This increased mutagenicity occurs by metabolic activation of the compounds via the cytochrome p450 family of enzymes. Smoking has been shown to specifically induce cyp1a2, cyp2a6, and cyp2a8 [60, 61].

Sex Hormones

Estrogens and androgens have both been linked to the development of atherosclerosis. Based on early epidemiological studies hormone replacement therapy (HRT) was recommended for all post-menopausal women [62]. This recommendation was based mainly on the positive biological effects that HRT seemed to have on cardiovascular disease. However, recent clinical trials have shown that HRT may not be the best course of action in all cases because estrogen can produce undesirable effects in selective tissues. Selective estrogen receptor modulation as well as tissue specific compounds may allow for clinicians to dissociate the favorable cardiovascular effects of estrogen from the unfavorable effects on breast and endometrial tissue [63]. Testosterone has also been implicated in the development in atherosclerosis [64]. However, like estrogen the causative effects of male hormones on atherogenesis are poorly understood and much more needs to be learned.

Age-Related

A relatively new concept has come about in medicine and biology that goes a long way in defining and describing pathologies that are seen in age-related diseases such an atherosclerosis. This new hypothesis approaches these diseases as being the result of evolutionary pressure and is referred to as the Darwinian-evolutionary concept. According to this concept, man and other animals are designed as a compromise in form and function to guarantee survival until the time of reproduction. These biological compromises that are made to ensure that the organism reaches a reproductive age whereby genetic material can be passed down to the next generation can be detrimental in later stages of life [65]. This compromise is not problematic for the majority of the animals in the world, because without human interference, the lifespan of the average animal has not dramatically increased. However, this is not the case for man. In the past hundred years, man's lifespan has increased more than it has in the past 10,000 years. The compromises and evolutionary pressures that allowed man early on in his existence to be fit and survive to an age of reproduction, comes to be harmful later in life. The genetic traits that allowed survival until reproductive age are now harmful in the aged [66, 67]. One clear example of this in atherosclerosis is immunity to HSP60. HSP60 is highly conserved and is a highly immunogenic constituent of viruses, bacteria and parasites. As a result, humans have developed immune reaction against microbial HSP60. However, this can present problems because humans also have a HSP60 and this immunity to microbial HSP60 can lead to an autoimmune reaction against the hosts own HSP60 later in life [68].

Diet

Given the central role that inflammation has on atherosclerosis, it should come as no surprise that diet can directly affect the pathogenesis of this disease. Diet can have either a positive or negative effect, depending on the types and amounts of foods eaten. The most direct effect that diet can have on the inflammatory process is increasing the levels of antioxidants that are available to the body [69]. Alterations in these levels will enable the body to be better equipped to handle oxidative stress conditions. Many studies have shown that increasing the intake of antioxidants either indirectly through the diet or directly via vitamin supplementation can afford a protective effect against inflammation in atherosclerosis [70]. Diet can also indirectly affect atherosclerosis in many other ways. For example, it is known that the modification of cholesterol levels by dietary habits can, and does, have a beneficial effect on the progression and regression in atherosclerosis [71]. It has also been shown that there exists a strong correlation between dietary fat intake and predisposition for certain types of heart disease. The toxins and mutagens that are also present in foods, either by the way it was processed, environmental contamination or as naturally occurring, can lead atherosclerotic disease [72].

Mechanisms of Vascular Disease

Atherosclerosis is a multifactoral disease. As such, the mechanisms underlying the initiation and progression of atherosclerosis are complex and convoluted at best.

However, there are fundamental mechanisms that are common to all risk factors for atherosclerosis.

Inflammation

If there is one unifying concept that you can get the scientific community to agree on regarding the pathogenesis of atherosclerosis, is that inflammation and the reactions resulting from inflammation are key components in the disease. Numerous pathophysiological observations in animals as well as in humans have led to the theory that chronic inflammation and the inflammatory process in general are key components in atherosclerosis. Many of the key stages and components in atherosclerosis have a response to inflammation or the inflammatory pathway; migration, proliferation, gene expression, apoptosis and cell cycle regulation to name just a few. Inflammation is characterized by macrophage activation, production of cytokines, inflammatory mediators, chemokines, acute phase proteins and mast cell activation [73]. All of these processes work in conjunction to activate and promote the inflammatory process. Many of the inflammatory stimuli can affect nearby cells as well, such as endothelial cells, monocytes macrophages, platelets, and smooth muscle. Inflammation can cause the production of cytokines by lymphocytes, macrophages, epithelial, or mesenchymal cells as well. Chronic inflammation is often accompanied by, or followed by, the increased formation of ROS or even reactive nitrogen species. This increase in oxidative stress can either be localized to the area of inflammation, or it can be systemic, all of which depends on the level of inflammation that is being experienced. Continual inflammation

can result in a localized micro-environment in which there is an increase in the number of macrophages and lymphocytes. This localized increase can lead to the formation of a lesion in the affected areas. The inflammatory reactions that are seen in atherosclerosis are essentially the same inflammatory reactions seen in other inflammatory diseases such as rheumatoid arthritis, cirrhosis, and pulmonary fibrosis. The only difference is that the cellular response in the arteries exhibits specific characteristics. For example, in the chronic inflammatory response in the vessel wall there are three different types of macrophages present, each regulated by different T-cell cytokines. Likewise, other inflammatory reactions share the same general characteristics as atherosclerotic inflammation with some minor changes specific to the cells type and location of where the inflammation is taking place [74, 75]. The initial damage to the blood vessel wall results in an inflammation response. Monocytes enter the artery wall from the bloodstreams. The monocytes differentiate into macrophages, which ingest oxidized cholesterol, slowly turning into large foam cells. Foam cells have a changed appearance resulting from numerous internal cytoplamic vesicles and resulting high lipid content. Microscopically, the lesion now appears as a fatty streak. Foam cells eventually die, and further propagate the inflammatory process.

Infections

Injury due to infections, whether bacterial or viral, can activate the inflammatory cascade and processes that are associated with it, and contribute to the pathology of atherosclerosis. Infectious pathogen burden has been implicated in the atherogenic

process. Recent epidemiological studies have suggested that there may be an association between infection and atherosclerosis [76]. The impact of infectious burden on the progression of atherosclerosis follows the same etiology as that found in cancer. This is that infection leads to inflammation, which in turn leads to oxidative stress, which affects the cellular homeostasis and cells ability to adequately deal with perturbations in homeostasis. It should be noted that the relative risk of developing atherosclerosis seems to be related to the actual number of pathogens that an individual is infected with. When this happens, this can lead to a whole sequence of events that ultimately end up in the initiation of atherosclerosis [77].

Oxidative Stress

In the early to intermediate stages of atherosclerosis there is a synthesis of DNA in lesions. Many exogenous and endogenous factors can damage DNA via cellular stress. One cause of such stress is the production of oxygen radicals. The unregulated or prolonged production of oxidants has been linked to mutations and altered gene expression in many cell types [78]. It is through ROS that much of the damage occurs. ROS include superoxide anion, hydrogen peroxide, and hydroxyl radicals. ROS have been linked to a variety of environmental stressors such as tobacco smoke, environmental pollutants, heat shock and UV irradiation. Oxidative stress can lead to inflammation, cellular proliferation and lesion formation and is thought to be an important part in the initiation stage of atherosclerosis. The consequences of oxidative stress and the pathways that lead to oxidative stress in the vasculature can be seen in

figure 3. Oxidative stress has been linked with DNA instability, hypermethylation, mutations in repair genes, modification of macromolecules and cell cycle deregulation [79]. Such perturbations lead to an imbalance in cellular redox homeostasis, and can initiate the pathogenic cascade of oxidative stress. This pathogenic cascade can lead to the expression of proteins not normally found in the cells. An example of the role of oxidative stress in atherogenesis lies in p38mitogen-activated protein kinase (MAPK). p38 MAPK is involved in cellular migration, growth and apoptosis [80]. It has been shown that oxidative stress can induce p38MAPK which can lead to activation of several pathways that are detrimental in both of these diseases [81].

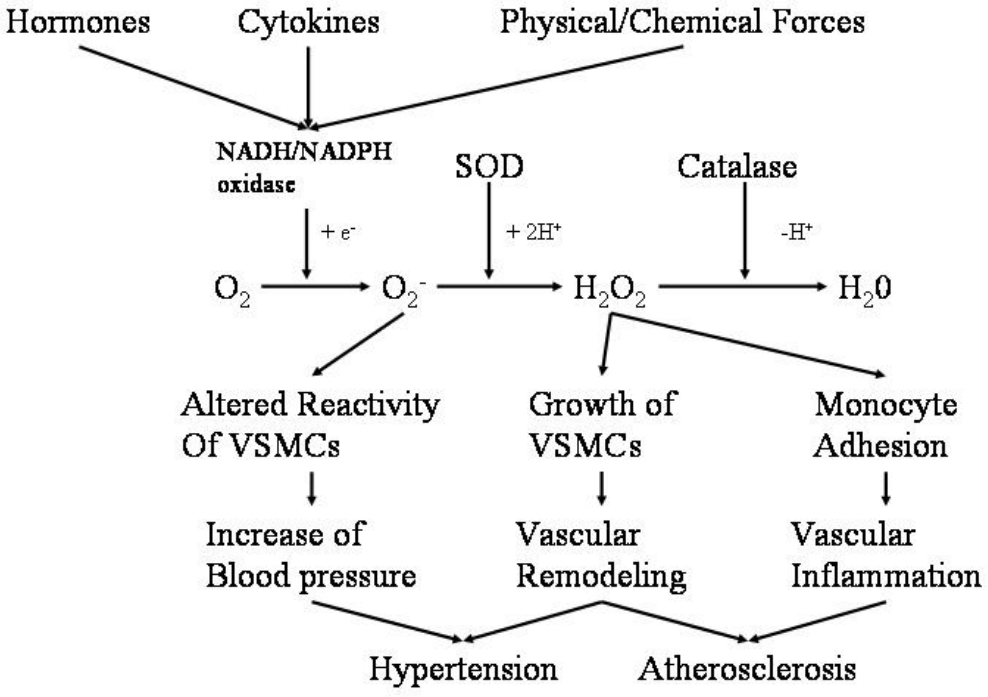


Figure 3. Biological consequences of oxidative stress.

Schematic representation of some of the causes and consequences of oxidative stress in the vasculature.

Genomic Targets in Atherogenesis

The growth and progression of atherosclerotic plaques requires a "phenotypic switch" of vSMCs from contractile to proliferative phenotypes. This initial "switch" is at the core of atherogenesis and therefore, the study of oncogenes and related growth factors has dominated research efforts in the field of atherosclerosis for many years. These studies have proven critical to the elucidation of mechanisms of deregulation of vSMC proliferation in atherosclerotic plaques. Platelet-derived growth factor (PDGF) has been identified as a key mediator of proliferation in atherosclerotic lesions [82]. This protein is the product of the c-sis proto-oncogene, the first found as part of a retrovirus isolated from a monkey sarcoma. Barrett and Benditt in 1987 assayed the expression of PDGF, PDGF-B, and v-sis gene expression in carotid plaques removed by endarterectomy and found an excess expression of PDGF-B message in the lesions compared with that found in normal artery wall [83]. PDGF was originally considered a likely oncogenic target in atherogenesis because its B-chain gene is almost identical to the transforming gene of the simian sarcoma virus [84]. This virus transforms many PDGF-responsive cells in tissue culture, including SMCs, fibroblasts and NIH 3T3 cells, and the phenotype of virus-transformed cells is relatively benign, being intermediate between that of normal cells and that produced by Kirsten mouse sarcoma virus. Many cells transformed in culture by agents other than simian sarcoma virus or obtained from naturally occurring tumors produce a PDGF-like protein. Whether this represents species-specific differences, or whether the population of vSMCs is different remains to be elucidated. Little is known about the mechanism of c-sis activation. Bejcek et al.

suggested that transformation by this gene is due to internal activation of the PDGF receptor before reaching the cell surface [85]. Other studies have implicated the myb gene in atherogenesis [86]. The c-myb and v-myb proteins bind DNA in a sequence-specific manner [82], and trans-activate or trans-repress promoters containing the binding sequence [87]. Antisense studies showed that local delivery of antisense c-myb oligonucleotide suppresses intimal accumulation in a rat carotid SMC injury model [88]. Other reports have implicated fos and jun in atherogenesis [89].

c-Ha-ras is a proto-oncogene that is a critical regulator of proliferative phenotypes and cellular differentiation of vSMCs. Studies established a link between the expression of proliferative vSMC phenotypes and alterations in ras-regulated phospholipid signaling [90]. In subsequent studies, c-Ha-ras was identified as a critical regulator of vSMC growth and differentiation [91].

Exposure of vSMCs to the atherogenic hydrocarbon benzo-a-pyrene, BaP, enhances c-Ha-ras gene expression [92]. This response correlates with binding of BaP to protein(s) that interact with cis-acting regulatory elements in the 5'UTR of the c-Ha-ras gene to increase ras transcription rates [93]. Sequence analysis of the c-Ha-ras promoter identified an Ahr response element (AhRE) at -30 and an antioxidant response element (ARE] at -543. These elements were shown to interact in the regulation of redox-mediated transcriptional activation of the c-Ha-ras gene.

Murine Models of Cardiovascular Disease

In the study of cardiovascular disease, the use of murine models has proven to be invaluable tool. It can safely be said that without the murine model system far less would be known about the etiology and pathology of cardiovascular disease.

In Vivo Models

In vitro methods of molecular analysis play a critical role in the laboratory today. However, discrepancies can arise between the in vitro and in vivo testing methods. In vitro tests do not take into account the potential impact of factors such as plasma protein binding, tissue distribution, formation of active metabolites or hemodynamic effects. During in vitro analysis there exists the real possibility that the testing results will provide false positive or negative results when applied to the whole animal studies. For example, drug induced histamine release was not detected by in vitro experiments in the screening of some antibiotics and was only realized when applied to an in vivo model system [94]. For this reason, tests in experimental animals have to be carried out at some point to confirm in vitro data. The advantages of a whole animal study is that it allows for the study of the integrative actions of the drug in question in a real pathological context [95]. It is only the combination of both in vitro and in vivo testing methods that the pharmacodynamics of the response can be fully appreciated.

LDL Model

During the process of atherogenesis, macrophages and vascular smooth muscle cells interact with low density lipoproteins (LDLs) to form foam cells. Gesquiere et al. (1999) demonstrated that cholesterol metabolism is altered in the presence of free radicals in vascular smooth muscle cells [96]. In this manner, cholesterol and cholesteryl esters accumulate to stimulate formation of foam cells. The lipid model proposes that an elevation in levels of plasma LDL will result in infiltration of LDL into the arterial wall. This infiltration leads to an increase in lipid accumulation in cells of the vascular wall, specifically smooth muscle cells and macrophages. LDL is also responsible for increases in smooth muscle cell hyperplasia and migration into the subintimal and intimal region in response to growth factors. Under these conditions, LDL is modified [39]. This altered LDL is rendered more atherogenic. The modified or oxidized LDL that is found in the vascular wall acts as a chemokine to monocytes. This action promotes their migration into the intima, their early appearance in the fatty streak, and their transformation and retention in the subintimal compartment as macrophages. Also found on the surface of macrophages are scavenger receptors. These receptors facilitate the entry of oxidized LDL into the vSMCs. The entry of modified LDL transfers them into lipid-laden macrophages and foam cells. Oxidized LDL has also been found to be cytotoxic to other cell types, endothelial cells, and may be responsible for their dysfunction or loss from the more advanced lesion [97]. In a porcine model of atherosclerosis, pigs were fed a cholesterol-rich Western diet. Within 2 weeks of inducing hypercholesterolemia in these animals, monocytes become attached to the

surface of the arterial endothelium through the induction of specific receptors. These monocytes then migrated into the subendothelium space, and started to accumulate lipid and started the transformation into foam cells [39]. Proliferating smooth muscle cells also accumulate lipid at an increased rate compared to non-proliferating cells. As the fatty streak and fibrous plaque enlarge and bulge into the lumen, the subendothelium becomes exposed to the blood at sites of endothelial retraction or tear, and platelet aggregates and mural thrombi form. Release of growth factors from the aggregated platelets may increase smooth muscle proliferation in the intima. Alternatively, organization and incorporation of the thrombus into the atherosclerotic plaque may contribute to its growth.

ApoE Model

In the early 90s, the development of apoliprotein E mutant mice provided a genetically modified model that replicates many of the features of the human disease [98]. This model replicates many of the changes observed in human populations linked to heightened susceptibility to atherosclerosis. The progressive series of atherosclerotic lesions developed in these animals is similar to those found in humans [99,100]. A recent report investigated the interaction between hypercholesterolemia and BaP in apoE mutant mice. These experiments showed that BaP accelerates the progression of atherosclerotic plaques in apoE mutant mice via mechanisms that involve a local inflammatory response [101]. Apolipoprotein E is a lipoprotein (a protein connected to a fat). Lipoproteins are responsible for packaging cholesterol and other fats, carrying them

through the bloodstream, and processing them. In particular, apolipoprotein E is a major component of specific lipoproteins called very low-density lipoproteins (VLDL). A key function of VLDLs is to remove excess cholesterol from the blood and carry it to the liver for processing. Maintaining normal levels of cholesterol is essential for the prevention of atherosclerosis. There are at least three slightly different versions (alleles) of the APOE gene. The major alleles are called e2, e3, and e4. The most common allele is e3, which is found in more than half of the population [102]. Apolipoprotein E is also associated with cardiovascular disorders. People who carry at least one copy of the APOE e4 allele are at increased risk for atherosclerosis, which is an accumulation of fatty deposits in the lining of the arteries [103]. People who carry two copies of the APOE e2 allele are at risk for a condition known as hyperlipoproteinemia type III. This condition is characterized by increased levels of cholesterol, triglycerides (fats), and molecules called beta-VLDLs, which carry cholesterol and lipoproteins in the bloodstream [104].

AAM Model

Allylamine (AAM), 3-aminopropene is a precursor used in the manufacturing of several commercial and pharmaceutical by products [105]. AAM has been found to preferentially localize within large to medium sized arteries. AAM is a selective vascular toxicant that causes the induction of vascular lesions that are similar to those found in atherosclerosis [106]. The toxicity and specificity of AAM is mediated by a vascular specific enzyme semicarbazide specific amine oxidase (SSAO). SSAO

catalyzes the conversion of AAM to the reactive metabolites hydrogen peroxide and acrolein [107]. For a schematic representation of the metabolism of AAM see figure 4. The hydrogen peroxide that is generated during the deamination process contributes to cellular injury by causing the formation of oxygen radicals [108].

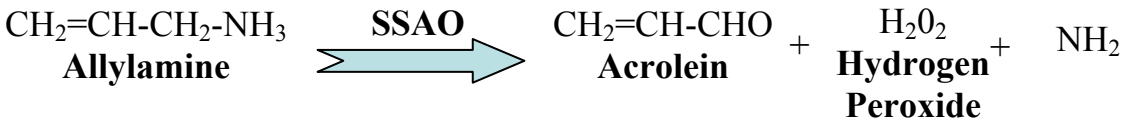


Figure 4. Allylamine metabolism.

Allylamine accumulates in vascular tissues where it is converted to acrolein and hydrogen peroxide by semicarbazide-sensative amine oxidase (SSAO). These two oxidative by products cause oxidative stress in the tissue.

The oxidative injury resulting from the metabolism of AAM can be inhibited by semicarbazide [107]. Recent studies from this lab and others have shown that vSMCs treated with AAM have an enhanced mitogenic responsiveness and this enhancement involves alterations in the regulation of growth by the ECM. Cells plated on tissue culture dishes precoated with ECM components from AAM treated cells are afforded a growth advantage [109]. Cells from AAM treated rats display a rounded morphology, loss of contractile capacity and acquisition of enhanced mitogenic responsiveness [110]. The enhanced proliferative capacity in AAM treated cells involves enhanced

phophalidylinositol metabolism, increase c-Ha-ras proto-oncogene expression and deposition of ECM components [111].

Experiments in the late 60's and early 70's established a model of atherogenesis in which chronic exposure to a cardiovascular-specific poison induces vascular lesions of atherosclerotic morphology. In these experiments, male Sprague-Dawley rats are gavaged with allylamine for up to 20 days to allow preferential accumulation of the amine within the vascular compartment and conversion to acrolein and hydrogen peroxide by a vascular-specific amine oxidase [112]. Elevated levels of this enzyme in vSMCs account for highly specific oxidant injury to the media by acrolein and hydrogen peroxide, and induction of atherogenic vSMC phenotypes [113,114,115,116]. An example of the induction of the atherogenic phenotype by AAM through altered gene expression of osteopontin is shown in figure 5. In vitro analysis of vSMCs isolated from oxidant-treated animals revealed disorganization of the contractile apparatus, increases in endoplasmic reticulum, and emergence of a prominent nucleolus. Oxidant-activated vSMCs acquire a proliferative advantage that allows them to proliferate into the luminal space and contribute to plaque formation in vivo.

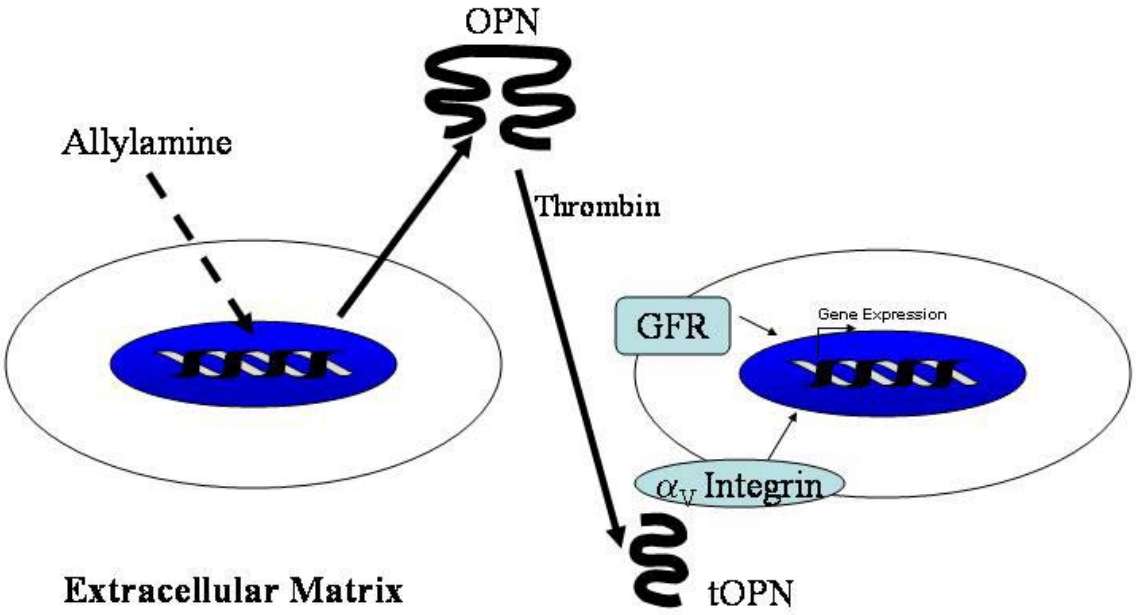


Figure 5. Mechanism of allylamine-induced smooth muscle proliferation.

The vascular specific compound allylamine (AAM) induces an increase in the expression of osteopontin. This increased level of osteopontin, once it is secreted into the extracellular matrix is cleaved via thrombin. This thrombin cleaved fragment, tOPN, then binds in the αV integrin and causes and increase in many genes responsible for phenotype in vSMCs.

In Vitro Models

Primary cultures can be established with relative ease from cell suspensions of cardiac and vascular tissue. Vascular endothelial and smooth muscle cultures can also be established by the explant method in which pieces of tissue are placed in a culture vessel to allow for cellular migration and proliferation in vitro. Neonatal and embryonic cells of cardiac origin proliferate readily under appropriate conditions in vitro [93]. The

ability of adult cardiac myocytes cells to divide is repressed, but not completely lost [117]. Myocardial cell division can be stimulated by stable insertion of the large tumor antigen from the SV40 virus into the myocyte genome [118].

Vascular endothelial and smooth muscle cells derived from large and medium-sized vessels of embryonic, neonatal, or adult animals proliferate readily under appropriate conditions in vitro [119]. As such, cultures can be propagated to prepare cell strains that retain variable degrees of differentiation as a function of cultivation in vitro. Bastaki et al. (1997] completed studies to establish three mouse endothelial cell lines from aorta (MAECs)[120], brain capillaries (MBECs), and heart capillaries (MHECs). These cell lines exhibit endogenous expression of specific markers, as evidenced by angiotensin-converting enzyme, acetylated LDL receptor, constitutive endothelial NO synthase, and vascular cell adhesion molecule-1 and bind Griffonia simplicifolia-I lectin. In other studies, an in vitro model of vascular injury by menadione-induced oxidative stress in bovine heart microvascular endothelial cells was developed [121].

VSMCs in suspension can be separated from non-muscle cells by a differential pour-off technique based on the rate of attachment of cells in suspension to the substratum [122]. Because the percentage of fibroblasts in culture increases logarithmically, if no attempt is made to separate individual cell types, fibroblasts will eventually dominate the cultures. A monoclonal antibody to cell surface adhesion factors has been used to enrich preparations of cultured vSMCs [123]. Addition of

mitotic inhibitors and maintenance of cultures in glutamine-free or serum-free media have also been successfully used to enrich culture purity [124].

Matrix molecules modulate cellular behavior and toxicological responsiveness [125]. Recent work has established a role for ECM interactions in the induction of proliferative vascular smooth muscle cells phenotypes following oxidative chemical injury [126]. Other important considerations related to the use of cultured cell systems includes recognition that the presence of serum modulates antioxidant capabilities in vitro [127], and that cardiovascular cells in culture undergo variable degrees of dedifferentiation [128,129,130].

Of particular interest from a toxicological perspective is that CYP (cytochrome p450) activities are present in cells of the cardiovascular system, and mediate metabolism-dependent toxicities. For instance, vertebrate cardiac endothelial cells of the marine scup express CYP1A1 [131]. Although CYPI family members are often associated with metabolism of exogenous substrates, studies have linked P450s to eicosanoid formation and metabolism. This is significant given the prominent role of these intermediates in regulation of cellular functions [132,133]. The pattern of constitutive and inducible expression of CYPIA1 and CYPIB1 genes in cultured human vascular endothelial cells and smooth muscle cells was recently defined [134]. In view of the role of CYPs in oxidation of exogenous and endogenous substrates, a link between CYP-mediated arachidonic acid metabolism, vascular SMC signaling and oxidant-induced atherogenesis has been proposed.

Because toxicity is often due to interactions with, or disruption of antioxidant defense systems, measurements of the redox status of cells may be particularly useful in the elucidation of mechanisms of toxicity [135]. Ionic homeostasis can also be used as an index of disturbances in the structural and functional integrity of the plasma membrane [136]. Co-culture systems of muscle and non-muscle cells have been used to evaluate critical cell-cell interactions. Co-cultures of vascular endothelial and smooth muscle cells [137,138], or cardiac myocytes and neurons have been employed to reconstruct the complexities of the cellular environment in vivo, while retaining the advantages of cell culture. The complexity of co-culture systems is exemplified by studies which show that endothelial cells modulate the extent of binding, internalization, and degradation of low-density lipoproteins by arterial smooth muscle cells [139], and produce growth factors for both smooth muscle cells and fibroblasts [140].

Particularly relevant are the paracrine influences exerted by inflammatory cells recruited to sites of injury, such as macrophages. Co-culture systems in which cells are seeded individually on separate surfaces and separated by semipermiable membranes have been developed [141,142,143]. Smooth muscle cells are seeded on conventional tissue culture plates, while macrophages are seeded on tissue culture inserts made of a semipermeable membrane of varying pore sizes. Because this co-culture model obviates direct cell-cell contact, individual cell populations can be separated at any time, while allowing soluble factors secreted from one cell type to interact with the other.

In other studies, rat aorta vascular smooth muscle cells and rat heart beating myocytes were challenged with allylamine and beta-aminopropionitrile singly and in

combination [144]. A combination of these agents provides myocardial protection from allylamine injury, but lead to severe aortic injury. These responses are due to increased accumulation of allylamine metabolites in the aorta. He et al. used cultured rat vascular smooth muscle cells to demonstrate that glutathione S-transferase is important in the defense against electrophilic atherogens [145]. This study emphasized the importance of vascular smooth muscle xenobiotic metabolism in the generation of reactive oxygen species, and cellular defense against oxidative stress and ensuing atherogenicity.

In Depth Study of Vascular Responses to Chemically-Induced Vascular Injury

As outlined in the previous sections, oxidative stress induced by inflammatory cells, viruses, bacteria, smoke, or chemicals plays a central role in the onset and progression of atherosclerosis. Therefore, studies were conducted to evaluate critical gene targets in oxidant-induced vascular injury and the mechanisms of osteopontin (*opn*) gene dysregulation. Studies were carried out to characterize the mechanisms by which *opn* contributes to the atherogenic phenotype. Specifically, two hypotheses were tested: 1) Modulation of vascular smooth muscle cells (vSMCs) to the atherogenic phenotype via oxidative stress, in vitro and in vivo, involves activation of osteopontin (OPN). 2) The phenotypic transition and activation by oxidative stress that is seen in vSMCs is due to up-regulation of osteopontin, which is controlled at the transcriptional level by redox-regulated cis-acting elements.

CHAPTER II

NOVEL GENOMIC TARGETS IN OXIDANT-INDUCED VASCULAR INJURY[†]

Synopsis

To study the complex interactions between oxidative injury and the pathogenesis of vascular disease, vascular gene expression was examined in male Sprague Dawley rats given 35 or 70 mg/kg allylamine, a synthetic amine converted to acrolein and hydrogen peroxide within the vascular wall. Vascular lesions and extensive vascular remodeling, coupled to increased production of 8-epi-PGF2α, nuclear localization of NF-κB, and alterations in glutathione homeostasis, were observed in animals treated with allylamine for up to 20 days. Transcriptional profiling, immunohistochemistry, and in situ hybridization showed that genes involved in adhesion and extracellular matrix (α_1 integrin, collagen), cytoskeletal rearrangements (α-smooth muscle actin, α-tropomyosin), and signal transduction (NFκB, osteopontin, and LINE) were altered by oxidant treatment. To evaluate mechanisms of gene dysregulation, cultured aortic smooth muscle cells were challenged with allylamine or its metabolites and processed for molecular analysis. These agents increased formation of reactive oxygen species and elicited changes in gene expression similar to those observed in vivo. Oxidative stress and changes in gene expression were inhibited by N-acetylcysteine, a precursor of

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glutathione. These results indicate that genes along the extracellular matrix-integrin-cytoskeletal axis, in addition to LINE, are molecular targets in oxidant-induced vascular injury.

Introduction

The development of atherosclerosis involves damage to endothelial cells and vascular smooth muscle cells (vSMCs), influx of inflammatory cells to sites of injury, release of inflammatory mediators, uncontrolled proliferation of vSMCs, and accumulation of lipids and matrix proteins. The uncontrolled proliferation of vSMCs is a major factor contributing to lesion progression, and involves reprogramming of phenotypic expression from a quiescent to a mitogen responsive, highly proliferative state [146]. This phenotypic switch has been extensively studied, but little is known about the complex gene-gene interactions that participate in phenotypic control. Of relevance is the finding that vascular oxidative stress caused by endogenous or exogenous reactive oxygen species (ROS) is a major contributor to phenotypic modulation and atherogenesis [147].

Many of the vascular disorders involving phenotypic modulation of vSMCs have roots that can be traced to ROS and its effects. For example, Angiotensin II induced hypertrophy of vSMCs is dependent on hydrogen peroxide produced intracellularly [148]. Likewise, there is a clear cut requirement for hydrogen peroxide in PDGF-induced vSMC proliferation [149]. However, much of the work completed to date examining the impact of oxidative stress on vascular cell proliferation has relied solely

on the use of in vitro models, therefore hampering the study of the complex biological interactions in vivo.

The study of vascular oxidative stress in vivo in this laboratory has been facilitated in experiments where oxidative injury is localized to cells of the cardiovascular system. To this end, a chemical model of vascular injury has been developed where allylamine, a synthetic aliphatic amine converted to acrolein and hydrogen peroxide, is administered to rats for varying times [150]. In this model, repeated cycles of injury promote the development of vSMC lesions and phenotypic modulation of vSMCs. The oxidative injury induced by allylamine is highly specific because metabolism of the parent amine is catalyzed by a vascular-specific amine oxidase expressed predominantly in vSMC [151]. Both acrolein and hydrogen peroxide promote peroxidative injury and activate the cellular stress response in vSMCs [152,153,154]. These metabolites induce phenotypic modulation of vSMCs in vivo, characterized by morphological and ultrastructural alterations [155], as well as changes in mitogenic signaling, extracellular matrix production and integrin expression [156].

In this study we test the hypothesis that that repeated cycles of oxidative injury alter the genomic circuitry of vSMCs. Evidence is presented that the production of reactive intermediates from allylamine leads to changes in gene expression caused by compromised redox homeostasis. As such, the injury induced by oxidative metabolites was fully preventable by N-acetylcysteine, an intracellular precursor of glutathione. Our findings defined genes within the vessel wall affected by oxidative stress and showed

that the adaptive response to oxidative injury involves disruption of growth regulatory genes and extracellular matrix-integrin-cytoskeletal associated genes.

Materials and Methods

Animals

Six week old (175-180 g) male Sprague-Dawley rats were gavaged daily with 35 or 70 mg/kg/day allylamine (99% purity) (Sigma, Milwaukee, WI), or water (1ml/kg/day) for 20 days. All experimental procedures involving animals were in compliance with federal and institutional guidelines.

Antibodies

α_1 Integrin and NF-κB were purchased from Chemicon (Temecula, CA), OPN from Santa Cruz (Santa Cruz, CA), α smooth muscle actin from Sigma (St. Louis, MO) and isoprostane from Assay Designs (Ann Arbor, Michigan).

Immunofluorescence

Vessels were removed, cut into 5 mm sections and placed in OCT embedding media (TissueTek, Torrence, CA), snap frozen in liquid nitrogen, and stored at –80°C. Tissues were sectioned at 8-10 μm, fixed in –20°C methanol for 15 minutes, and processed as described in Molecular Probes ELF-97 Protocol MP 06600 (Molecular Probes, Eugene, OR). Tissues were incubated for 5 minutes with 4', 6-diamidino-2-

phenylindole, dihydrochloride (DAPI). Slides were mounted in ELF-97 immunohistochemical mounting medium from Molecular Probes.

Western Analysis

Thoracic aortas were removed and completely stripped of adventitia. Vessels were placed into liquid N_2 and powdered using a mortar and pestle. Powdered aortas were then placed into T-PER (Pierce, Rockford IL) containing 1X HALT protease inhibitor cocktail (Pierce), and proteins extracted according to a manufacturer's protocol. Protein concentrations were determined using a Biorad protein assay (Hercules, CA). Twenty-five μg of protein/lane were loaded onto NuPage 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA), run at 10V for 1 hr and then transferred onto Invitrolon PVDF (Invitrogen). Gels were stained with Gelcode Blue stain reagent (Pierce) and the membranes stained with Ponceau S to determine loading efficiency. Membranes were blocked overnight using 10% Blotto non-fat dry milk (Santa Cruz) and incubated with primary and secondary antibodies as noted below. Primary antibody concentrations were as follows; α-smooth muscle actin (0.4 μg/ml), osteopontin (0.4 μg/ml), alpha-1 integrin (1 μg/ml), and alpha-tropomyosin (0.67 μg/ml). Blots were developed using the Pierce SuperSignal West Dura substrate. Films were scanned and analyzed using 1D Image Anaysis Software version 3.5 (Kodak, Rochester, NY).

Fluorescence In Situ Hybridization

The probe for the α-tropomyosin target (Tpm1) was a 50 nucleotide antisense oligo: 5′ CAA GAC TCC TTC ATC AAG CCG GAT GTC CCA CCT CTC TGA GCT CTT TTT CG 3′. Sense probe was used as a control for non-specific binding. The oligos were designed according to manufacturer's recommendations, labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR) and purified by PAGE. Pretreatment and hybridization of sections was completed as specified in the Starfish (Fluorescent In Situ Detection kit (Genisphere, Hatfield PA).

Microscopy

Labeled tissues were visualized using a Bio-Rad RTS200MP Confocal Microscope (Bio-Rad, Hercules, CA) equipped with 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) long-pass (LP) and fluorescein-5-isothiocyanate (FITC) filter sets. Sections were excited with the Tsunami Laser set to 790 nm. ELF-substrate excitation and emission wavelengths were 360 and 535 ±18 nm, respectively. UV band-pass (BP) excitation filter 340–380 nm and LP suppression filter (425 nm) were used for the collection of DAPI images. For FISH experiments, the images were captured using a Zeiss Axiovert200 equipped with an Axiocam HRm cooled CCD camera (Zeiss, Thornwood, NY).

Image Analysis

Image analysis was carried out using either Metamorph Image Analysis software version 6.0 (Universal Imaging Systems, Downington PA) for immunofluorescence or KS3000 ver. 3.0 image analysis software (Zeiss, Thornwood,NY) for fluorescence in situ hybridization. In each vessel, autofluorescence originating from elastin, cholesterol and lipids was corrected prior to quantification of antigen levels [157]. The extent of non-specific binding was also determined using secondary antibody only, or non-related IgG, and this intensity was used to normalize the signal of all antibodies examined. Frozen sections (8-10 μm in thickness) of thoracic aorta from at least 3 different animals were examined for each antigen.

Vessel Isolation for Microarray Analysis

Thoracic aortas were harvested from Sprague-Dawley rats treated with 35 or 70 mg/kg allylamine for 20 days. Following removal of the adventitia, isolated vessels were placed into RNAlater (Ambion, Austin, TX), snap frozen in liquid nitrogen, and stored at

-80°C for later use, or immediately homogenized in Trizol (Invitrogen, Carlsbad, CA) for RNA isolation.

RNA Isolation

Frozen aortas were thawed on ice and rinsed with sterile PBS to remove excess tissue storage solution. Tissues were placed in 1ml of TRIZOL Reagent and

homogenized using a polytron homogenizer. RNA was isolated as specified in the manufacturer's protocol except for an additional overnight incubation at –20° C in isopropanol.

Affymetrix GeneChip

Double stranded cDNA synthesis and biotinylated cRNA preparation were conducted as recommended in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Microarray analysis was repeated twice using different sources of RNA.

Data Analysis

After scanning, each image was inspected for major chip defects or abnormalities in hybridization signal as a quality control and analyzed using Affymetrix Microarray Suite Software 5.0 version for absolute and relative analyses. The data was then normalized to the 50th percentile of the median intensity for each chip. All data sets used for microarray analysis are publicly available at the NCBI GEO repository under the following accession numbers GSM9246, GSM9248, GSM9249 and GSE610.

Clustering

K-means clustering was used to infer gene groupings before and after induction of oxidative stress. The K-means clustering algorithm updates the cluster assignment of individual genes after each iteration. Similarity between genes was measured based on a

minimum distance measure known as the Euclidean distance. The process was repeated until the grouping stabilized. In our study, we initialized the algorithm with 5 cluster groups.

Cell Culture

vSMCs were isolated by enzymatic digestion of aortas from adult male Sprague–Dawley rats [158]. Subconfluent cultures were split at a 1:4 ratio twice weekly and maintained in Medium 199 supplemented with 10% FBS and 2 mM glutamine in 5% CO_2:95% air at 37 °C. Cell identity was confirmed by α smooth muscle actin immunofluorescence as described [159].

ROS Detection

DCFDA (Molecular Probes, Eugene OR) was used to measure ROS in naïve vSMCs seeded at $2x10^5$ cells/well in 48 well tissue culture plates (Costar, Corning, NY). Cells were grown to ~80% confluence in M199 without phenol red (Invitrogen, Carlsbad,CA) containing 10% FBS. Media was aspirated and plates washed with sterile PBS. Cells were pretreated for 1hr with M199 media containing 0.5% FBS plus or minus 0.5mM N-acetylcysteine. Cells were then incubated for varying time points with allylamine or its metabolites in media containing 0.5% FBS and 10μM DCFDA. DCFDA fluorescence was determined on a Fusion plate reader (Perkin Elmer, Wellesley, MA) at an excitation of 480nm and emission of 530 nm.

Real Time PCR

The double-stranded DNA-binding dye method was used to measure RNA levels. Primers were chosen using Beacon Designer 2.1 (Premier Biosoft, Palo Alto, CA). Real-time PCR amplification was performed using a Biorad iCycler (Biorad, LaJolla, CA). For each run, 25 μl of 2X SYBR Master mix (Perkin-Elmer Applied Biosystems) and 0.4 μM of forward and reverse primers along with 10 μl each of appropriate transcriptase were mixed. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes, 50 cycles at 95°C for 15 seconds, and 65°C for 1 minutes. All experiments were performed in duplicate. The primer sequences used are as follows; osteopontin Forward (F) 5'AGCCAGCCAAGGACCAACTAC 3',Reverse (R) 5' TGCCAAAC TCAGCCACTTTCAC 3';α-1 integrin F 5'AGAGGCACAATCCAGGACTG3',R5' AGGAGGAGCGAGACATTCAC 3'; alpha-tropomyosin F5' CCTCCCAAGACTC CTTCATC 3', R 5' ACCTCTGTGACAATAAGAAAGC; LINE F5' GTGCGGCT TCCAACATTCC 3', R5' AAGGTGGGCGTGTCTACAG 3', GAPDH F5' TGTTGAAGTCACAGGAGACAACCT 3',R5' TGTTGAAGTCACAGGAG ACAACCT 3'.

Results

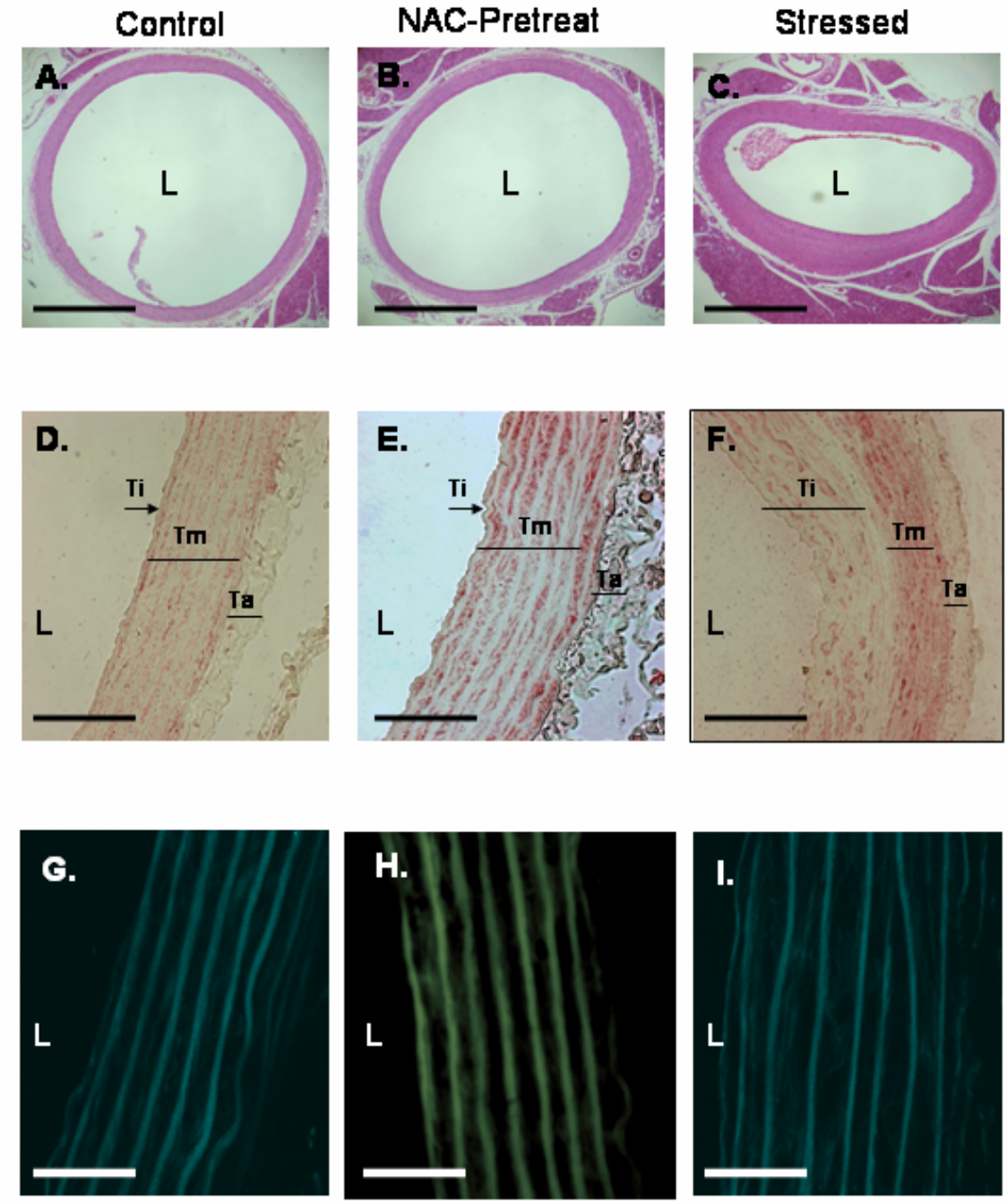
Vascular Injury

Male Sprague-Dawley rats were gavaged daily with 35 or 70 mg/kg/day allylamine or water (1ml/kg) for 20 days to induce vascular injury. At the end of this

dosing regimen, thoracic aortas were removed and processed for morphological analysis. Allylamine treatment induced medial thickening as early as 7 days (Figure 6., compare panels A and C). Pre-treatment with NAC prevented increased thickening in vessels from stressed animals (Figure 6., compare panels B and C). Extensive remodeling and general disorganization of elastin fibers over the course of the treatment period was observed in vessels from allylamine-treated animals (Figure 6., compare panels G and I). To determine if vascular injury involved oxidative stress, frozen sections were probed with an antibody for 8-epi-PGF2α, an isoprostane formed in vivo by a non-enzymatic free radical peroxidation step. Isoprostanes are regarded as mediators of oxidant-induced atherosclerosis [160], and reliable indices of oxidative stress in vivo [161].

Figure 6. Morphological changes in Sprague-Dawley rats subjected to oxidative challenge.

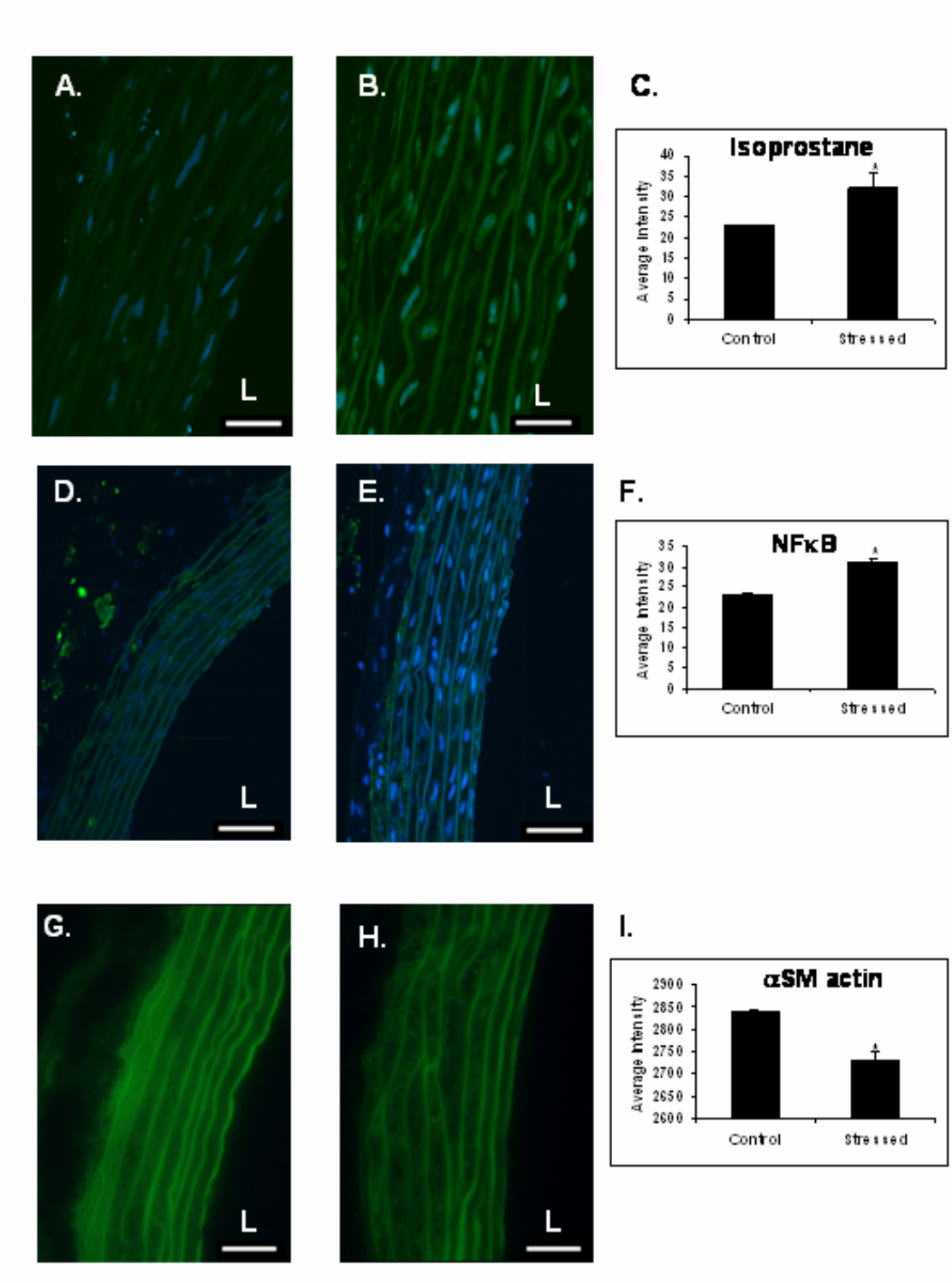
Images are representative of sections from animals that were control treated (panels A,D and G), pre-treated with NAC followed by AAM treatment (panels B,E,and H) or animals challenged with 70 mg/kg allylamine for various times (Panels C,F, and I). Medial thickening of the thoracic aorta is seen as early as 7 days after the start of the dosing regimen. (Panels A, B and C) Bar=250μm. H&E staining of lesion area from control (D) ,NAC pre-treated (E)and stressed animals (F) showing anatomy of aorta and lesion. Bar=50μm. Elastin disorganization and vascular remodeling in animals treated with allylamine for 20 days (Panels G,H and I) Bar= 25μm. These results are representative of vessel sections from 7 animals. Ti=Tunica intima, Tm=Tunica media, Ta=Tunica adventita, L=Lumen.


Control
NAC-Pretreat
Stressed
A.
B.
C.
L
D.
E.
F.
Ti
Tm
Ta
G.
H.
I.

The fluorescence signals for isoprostane in aortas from control and stressed animals are depicted in Figure 7 (panels A and B). Significant increases in 8-epi-PGF2α signal intensity were observed in stressed animals relative to controls, as quantified using Metamorph™ software (Figure **7** C). Induction of oxidative stress was also evidenced by increased nuclear localization of NFκB (Figure 7, panels D-F), as well as alterations in glutathione homeostasis. Of note was the finding that amine treatment for 20 days increased vascular levels of oxidized glutathione 3-fold (0.11 ± 0.011 versus 0.39 ± 0.16 nmol/mg protein in control compared to treated animals, n = 7). Further evidence for a role of oxidative stress in allylamine injury came from studies showing that daily pre-treatment with N-acetylcysteine prevented lesion occurrence in allylamine treated rats (Figure 6, panels B and C or E and F). Next, the expression of α-smooth muscle actin, a marker of smooth muscle differentiation, was evaluated. A marked decrease in α-smooth muscle actin staining was observed in oxidatively stressed animals (Figure 7, panels G-I). These findings collectively indicate that induction of vascular lesions by allylamine involves oxidative stress within the aortic wall and changes in α-smooth muscle actin. Western blot analysis of α-smooth muscle actin was confirmed the results of immunohistochemistry experiments (Figure 9, panel A).

Figure 7. Expression of selected markers within the vascular (aortic) wall of chemically stressed Sprague-Dawley rats.

Panels A-C show isoprostane expression in control and oxidatively stressed animals, respectively. Isoprostane, a marker of lipid-mediated oxidative stress, was increased in AAM-treated animals. NfκB expression in control and stressed vessels is shown in Panels D-F, respectively. NfκB, a transcription factor implicated in the inflammatory response to vascular injury, was significantly elevated in vessels from allylamine-treated animals. Immunofluorescence analysis of alpha smooth muscle actin expression in the thoracic aorta of control and stressed animals is shown in Panels G-I. Notice the marked reduction in alpha smooth muscle actin signal. These results are representative of the average immunofluorescence intensities of vessel sections from treated animals. Quantified signals are shown for all markers examined in panels C, F and I, respectively. * denotes statistically significant differences at $p<0.05$. A-B Bar = 50μm, D, E, G and H Bar = 100μm. L=Lumen. Average immunofluorescence intensities ± SEM of vessel sections from 3 separate animals for each group are shown.


A.
L
B.
L
C.
Isoprostane
Average Intensity
40
35
30
25
20
15
10
5
0
Control
Stressed
D.
L
E.
L
F.
NFκB
Average Intensity
35
30
25
20
15
10
5
0
Control
Stressed
G.
L
H.
L
I.
αSM actin
Average Intensity
2900
2850
2800
2750
2700
2650
2600
Control
Stressed

Genomic Analysis

Aortic RNA was hybridized to the rat genome U34A Affymetrix GeneChip Array. Raw data images were acquired with the Agilent GeneArray Scanner and analyzed using Genespring microarray analysis software package version 4.1.5. Genechip analysis was repeated in duplicate using different sources of RNA to ensure reproducibility. A total of 263 genes were significantly altered by oxidant treatment, 40 of which were present in the allylamine groups at levels two-fold or less than control. K-means clustering was used to infer gene groupings before and after induction of oxidative stress by allylamine. A cluster set of five was selected and after eight iterations the data converged. Table 1 lists the genes by clusters that are involved in the vascular response to oxidative injury. These genes could also be classified into one of three categories based upon their presumptive function: 1) adaptive response; 2) growth regulation; and 3) matrix association. Of interest were OPN, α_1 integrin, LINE and α-tropomyosin, genes that exhibit redox-dependent profiles of regulation in vascular cells.

Table 1. Genes Involved in the Response to Oxidant Induced Vascular Injury

	Genes	35AAM	70AAM
Cluster I	Protocadherin 6	↑	↓
	Solute carrier family 14, member 2	↑	↓
	Development-related protein	↑	↓
	C-terminal binding protein 1	↑	↓
	Regulator of G-protein signaling 19	↑	↓
	proteasome activator rPA28 subunit alpha	↑	↓
	chytochrome P-450	↑	↓
	Ral guanine nucleotide dissociation stimulator	↑	↓
	p67	↑	↓
	Glutamate oxaloacetate transaminase 2	↑	↓
	Arginine vasopressin (Diabetes insipidus)	↑	↓
	Alanine-glyoxylate aminotransferase	↑	↓
	Homeobox A2	↑	↓
	Methylmalonate semialdehyde dehydrogenase	↑	↓
	Ribosomal protein S15	↑	↓
	Cystatin beta	↑	↓
	Ca++/calmodulin-dependent protein kinase II	↑	↓
	Beta-carotene 15, 15'-dioxygenase	↑	↓
	rRNA promoter binding protein	↑	↓
	Amiloride binding protein	↑	↓
	Superoxide dismutase 1, soluble	↑	↓
Cluster II	Synaptogyrin 2	↑	↑
	Cyclin D3	↑	↑
	Annexin V	↑	↑
	protein tyrosine phosphatase epsilon M	↑	↑
	Agrin	↑	↑
	beta-galactoside-alpha 2,6-sialyltransferase	↑	↑
	ATPase, Ca++ transporting, cardiac muscle	↑	↑
	Ras homolog enriched in brain	↑	↑
	DNA polymerase beta	↑	↑
	insulin-like growth factor binding protein	↑	↑
	Dnase1	↑	↑
Cluster III	Nuclear protein E3-3 orf1	↑	↑
	ORF 2	↑	↑
	MRNA for ribosomal protein S9	↑	↑
	Mismatch repair protein	↑	↑
	Procollagen, type I, alpha 1	↑	↑
Cluster IV	Inpp1-inositol polyphosphate-1-phosphatase	↑	↑↑↑
	COXI-cytochrome oxidase subunit I	↑	↑↑↑
	NG,NG dimethylarginine dimethylaminohydrolase	↑	↑↑↑
Cluster V	Embigin	↑↑	↑↑↑↑
	LINE	↑↑	↑↑↑↑
	Tma2-striated-muscle alpha tropomyosin	↑↑	↑↑↑↑
	Alpha 1 Integrin	↑↑	↑↑↑↑
	OPN	↑↑	↑↑↑↑
	Cytoplasmic beta-actin	↑↑	↑↑↑↑
	Glutathione-S-transferase, alpha type (Ya)	↑↑	↑↑↑↑
	Superoxide dismutase 2, mitochondrial	↑↑	↑↑↑↑
	beta-2-gpl	↑↑↑	↑↑↑
	COLIA1-alpha-1 type I collagen	↑↑↑	↑↑↑
	Cytochrome b5	↑↑↑	↑↑↑
	Ribosomal protein S29	↑↑↑	↑↑↑
	Hemoglobin, beta	↑↑↑	↑↑↑

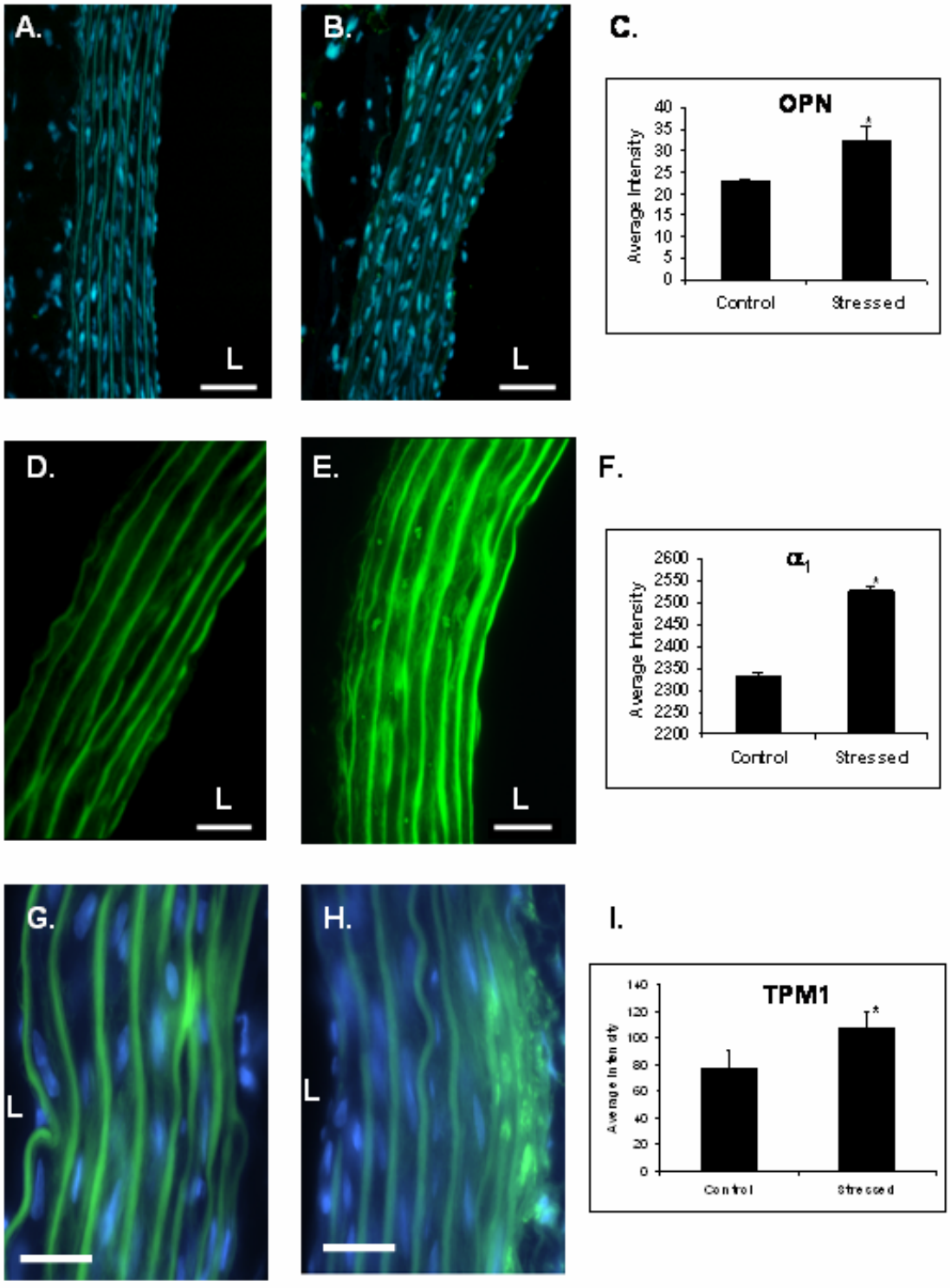
Data in Table 1 was analyzed by K-means clustering; analysis was performed on genes present in the 35 and 70 allylamine (AA) groups. A cluster set of five was selected and after eight iterations the data converged. Similarity was measured by standard correlation. ↑=1.5-3 fold regulation, ↑↑=3-5 fold regulation, ↑↑↑=5-7, ↑↑↑↑=>8 fold differential regulation.

OPN, α_1 Integrin, and Tropomyosin Expression

OPN has come to light in recent years as a cytokine and matrix molecule involved in medial thickening and neointima formation [162]. Figure 8 (panels A-C) and Figure 4 (panel B) shows that, consistent with genomic data, a marked increase in OPN expression at the protein level was observed in oxidatively-stressed animals compared to controls. α_1 integrin mediates collagen and laminin cell-matrix interactions and plays a role in the regulation of cellular proliferation in collagenous matrices [163]. As with OPN, significant increases in α_1 integrin fluorescence intensity were observed in treated animals (Figure 8, panels D-F and Figure 9C). Next, control and stressed vessels were examined for α-tropomyosin expression, an actin-binding structural protein that increases during transition of vSMCs from contractile to proliferative phenotypes [164]. Fluorescence in situ hybridization showed that α-tropomyosin mRNA (Figure 8, panels G-I) and protein (Figure 9D) increased in injured vessels relative to controls. Altogether, these observations support the conclusion that vascular injury by allylamine triggers vessel remodeling and shifts vSMCs within the aortic wall toward atherogenic phenotypes.

Figure 8. Osteopontin, alpha-1 integrin, and α-tropomyosin expression in the thoracic aorta of oxidative stressed Sprague-Dawley rats.

Panels A-B,D-E, and G-H show signals for control and stressed animals, respectively. Panels A-F refers to immunofluorescence data using antibodies against the respective proteins. Panels G-F refers to fluorescence data obtained using a RNA probe against tropomyosin. Average intensities ± SEM of vessel sections from 3 separate animals are shown in graphical form in panels C, F, and G (* = $p<0.05$). For panels A-F the bar = 25μm. For panels G-I the bar = 50 μm.


A.
L
B.
L
C.
OPN
Average Intensity
40
35
30
25
20
15
10
5
0
*
Control
Stressed
D.
L
E.
L
F.
α1
Average Intensity
2600
2550
2500
2450
2400
2350
2300
2250
2200
*
Control
Stressed
G.
L
H.
L
I.
TPM1
Average Intensity
140
120
100
80
60
40
20
0
*
Control
Stressed

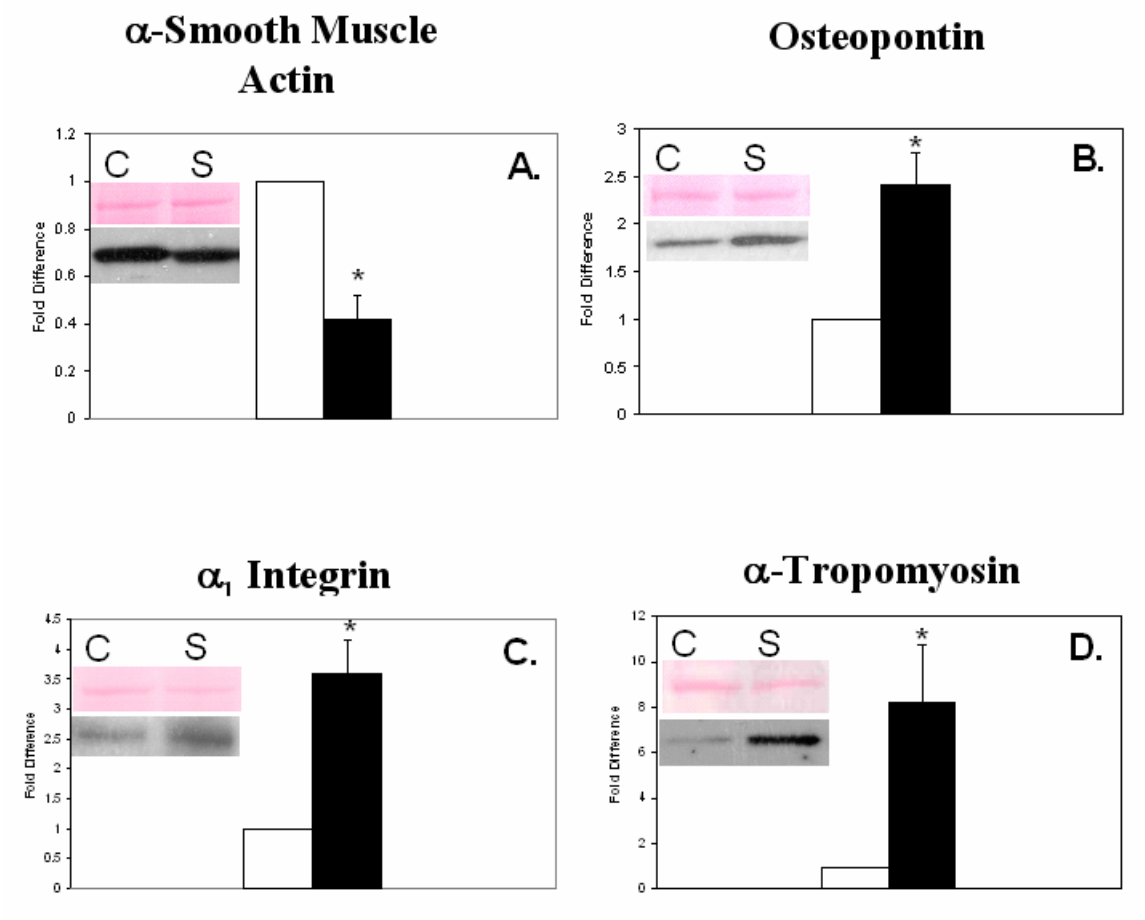


Figure 9. Western blot confirmation of immunofluorescence results.
α-smooth muscle actin, osteopontin, alpha-1 integrin and α-tropomyosin results of immunofluorescence were confirmed via Western blot analysis of total cell lysates of complete thoracic aortas. Data represents average intensities ± SEM for three different animals from two independent experiments normalized to control (white=control, black=stressed, n=3). Top bands are ponceau S staining of PVDF membranes as loading control. Bottom bands are representative of Western results (C=control, S=Stressed).

Mechanisms of Gene Dysregulation

To evaluate mechanisms of gene dysregulation following oxidant-induced vascular injury, naïve cultured vSMCs were treated with allylamine or its metabolites, acrolein and hydrogen peroxide, and processed for measurements of reactive oxygen species (ROS). Treatment of vSMCs with allylamine (10 nM - 10μM) elicited

concentration-dependent increases in ROS production (Figure 10). Gradual reductions in signal intensity were observed as cell viability decreased over time. Allylamine-induced ROS production was inhibited by N-acetylcysteine, a precursor of the antioxidant glutathione in vSMCs [165]. Challenge of vSMCs with the allylamine metabolites, acrolein or hydrogen peroxide, also increased ROS production. However, the stress response differed both in terms of intensity and temporal dependence. Concentration-dependent increases in ROS production in peroxide-treated cells subsided over time, while the response to acrolein was more erratic. As predicted, N-acetylcysteine afforded protection against metabolite injury, except in cells treated with 1 μM hydrogen peroxide (Figure 11). The protective actions of N-acetylcysteine are in keeping with the finding that allylamine and its metabolites compromise glutathione homeostasis and increase vascular levels of oxidized glutathione, and cause oxidative stress. To determine if modulation of gene expression involved oxidative mechanisms, real time PCR for selected genes was completed. The results showed that modulation of OPN, LINE and α-tropomyosin gene expression by allylamine and hydrogen peroxide was fully prevented by pre-treatment with N-acetylcysteine (Figure 12) Consistent measurements of gene expression could not be made for acrolein treated cells; a pattern attributed the erratic nature of the injury response observed.

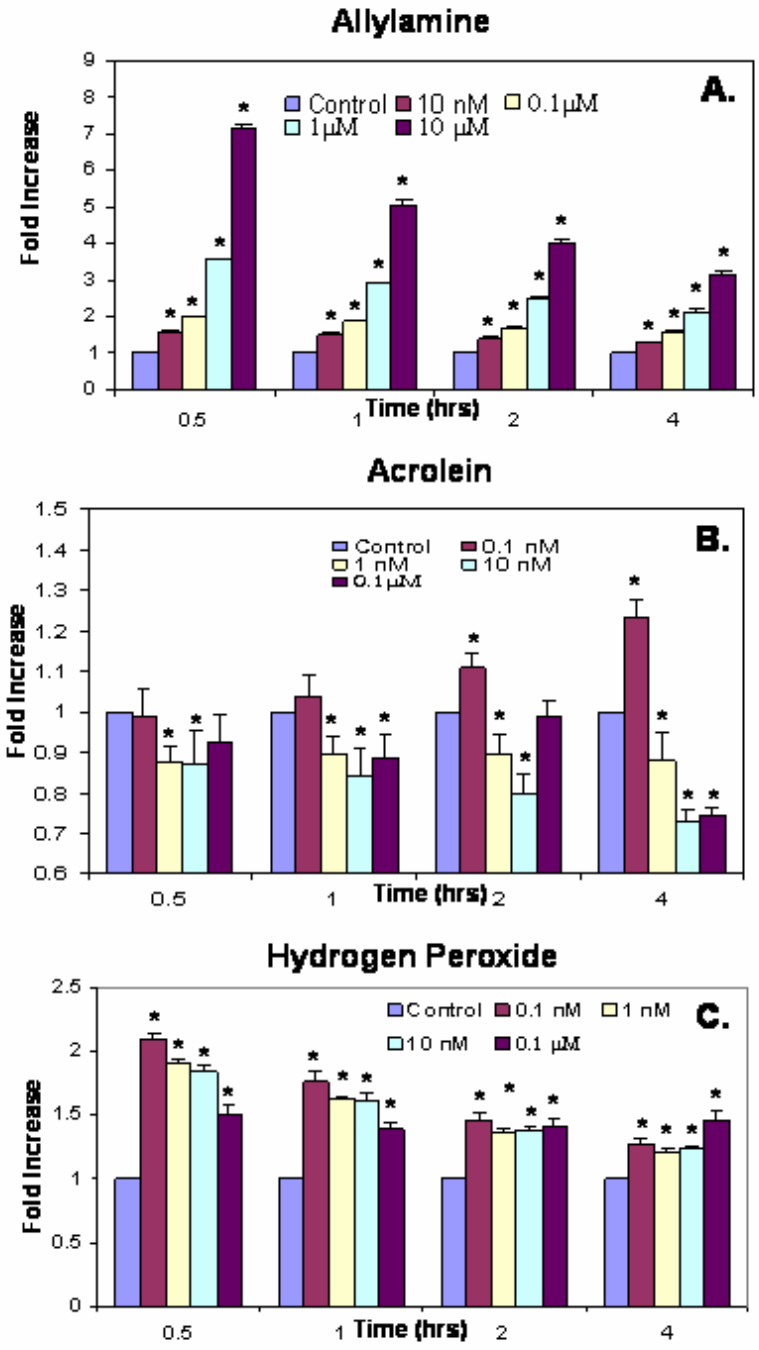


Figure 10. ROS detection in vSMCs treated with AAM or primary metabolites. Average DCFDA fluorescence ± SEM is shown as fold increase over control for varying chemical concentrations over time. (* = $p<0.05$, n=4 for each group).

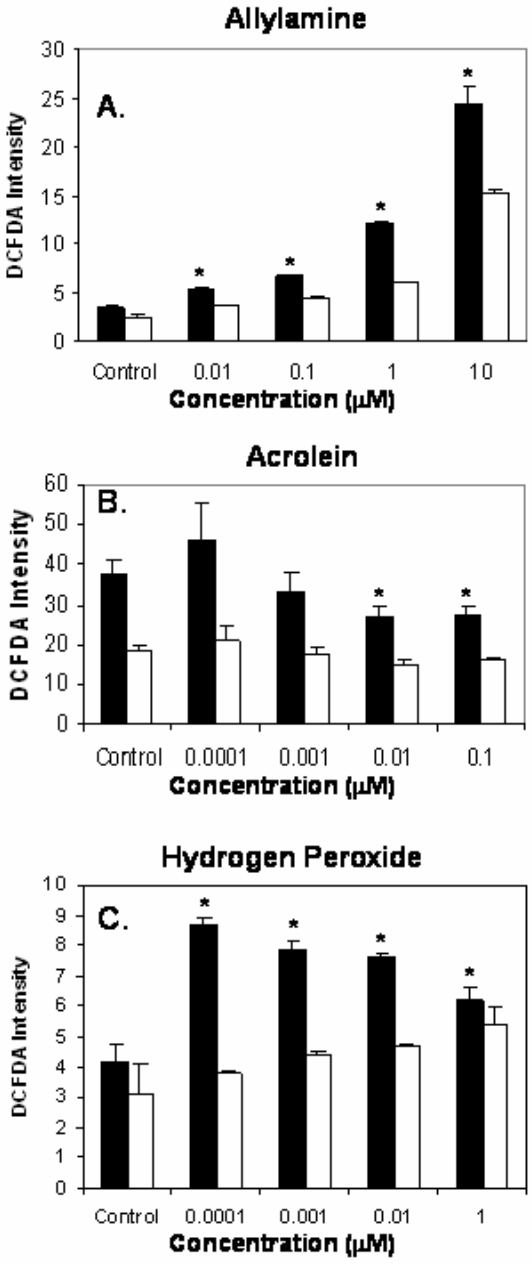


Figure 11. Protective effects of N-acetylcysteine in vSMCs.
ROS detection after 30 minutes of treatment with allylamine or its primary metabolites. Varying oxidant concentrations alone are represented as (solid bar) or following pre-treatment with NAC for 0.5 hours (clear bar). The bar graph on the right shows actual DCFDA intensities ± SEM. (* = $p<0.05$, n=4 for each group).

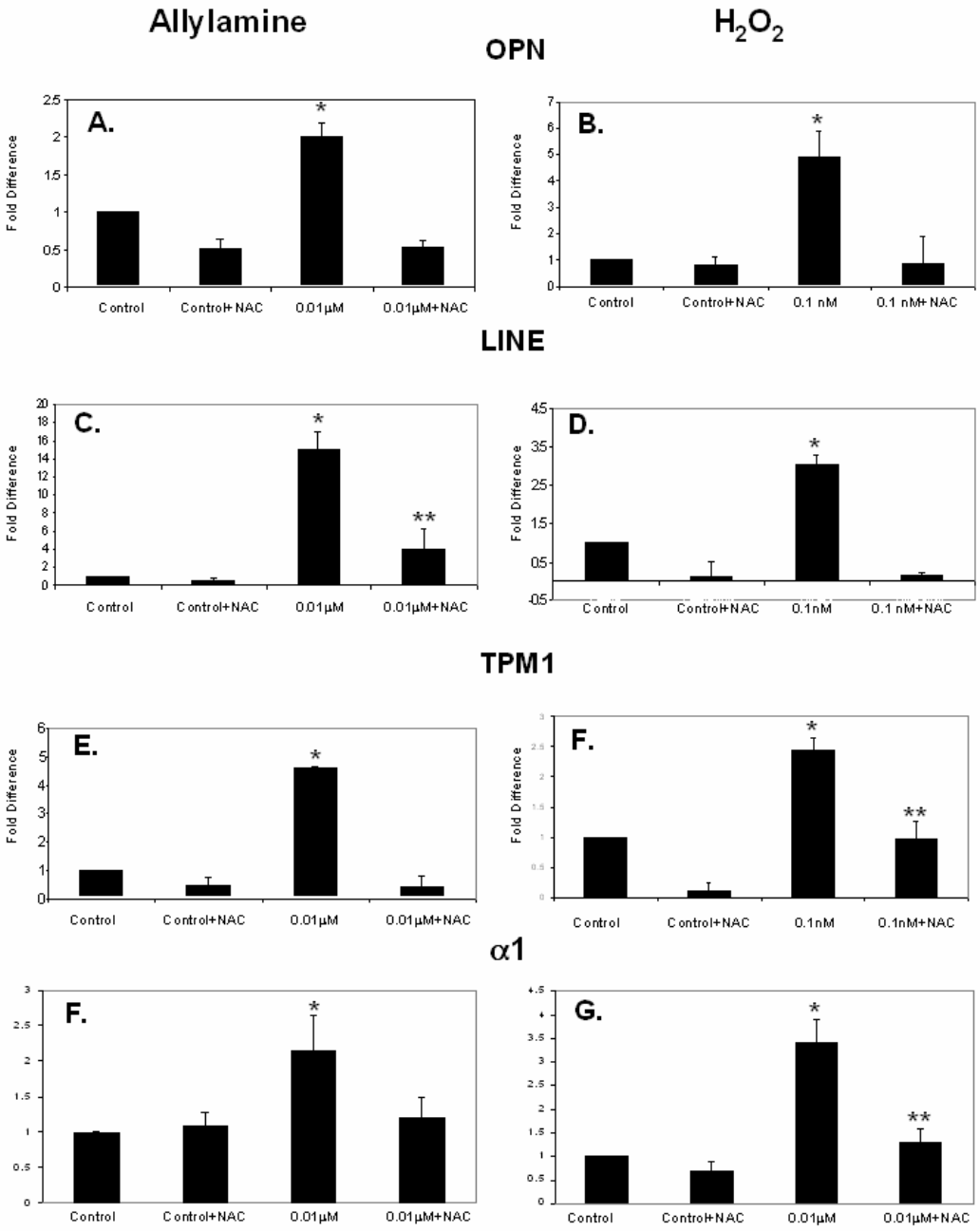


Figure 12. Real time PCR confirmation of genomic changes.
Cultured vSMCs were treated with either AAM or hydrogen peroxide and mRNA extracted for gene analysis. For NAC protection studies, cells were pretreated with NAC for 0.5hr, followed by oxidant treatment and RNA extraction. Data shown as average fold increase ± SEM over control. OPN-osteopontin, L1Rn-LINE, TPM1-α tropomyosin. (* = $p<0.05$ compared to control, **=$p<0.05$ compared to NAC-control, n=4 for each group). n=4 for each group.

Discussion

Oxidative injury is a critical event in the pathways leading to initiation and progression of vascular diseases, such as atherosclerosis and hypertension [166]. While the vascular response to oxidative injury involves altered patterns of gene expression, critical genomic targets and molecular mechanisms of gene dysregulation have remained poorly understood. In this study, experiments were conducted to evaluate the complex nature of such interactions in vessels challenged with allylamine, a pro-oxidant converted within the vessel wall by enzyme-catalyzed oxidative deamination to acrolein and hydrogen peroxide [167]. Acrolein is a reactive aldehyde that covalently modifies macromolecules and induces lipid peroxidation, and is found widely in the environment or formed endogenously via lipid peroxidation [168,169]. Acrolein conjugates with reduced glutathione in vascular cells and initiates peroxidative injury and changes in gene expression [170,171]. Hydrogen peroxide is a direct oxidant that modifies cellular macromolecules and induces oxidative stress [172]. The vascular response to allylamine is similar to that seen with methylamine in diabetes where increased production of formaldehyde and hydrogen peroxide disrupts redox homeostasis within the arterial vasculature and gives rise to protein cross-linking and angiopathy [173,174].

Medial thickening and vascular remodeling were observed in rats treated with allylamine. These changes likely involve reprogramming of redox-regulated genomic targets, including extracellular matrix-integrin-cytoskeletal axis genes. Vascular injury is mediated by oxidative stress since isoprostane levels significantly increased in treated vessels, and these changes correlated with nuclear localization of NF-κB and increases

in oxidized glutathione levels. Isoprostanes are stable peroxidation products of free radical mediated oxidation of arachidonic acid [160,175,176]. Increased levels of isoprostanes have been directly associated with the occurrence of atherosclerotic disease [161,176]. In its latent form, NF-κB exists in the cytoplasm of unstimulated cells as a dimer bound to an inhibitory protein, IκB. Upon activation by oxidative stress, NF-κB dissociates from the inhibitory IκB protein and translocates to the nucleus where it regulates the expression of genes involved in diverse cellular functions, including inflammation and matrix remodeling [177]. In diseases such as hypertension and atherosclerosis, vSMCs can shift from a contractile state where the expression of α-smooth muscle actin is high, to a proliferative state characterized by loss of α-smooth muscle actin expression [178]. Consistent with these correlations, oxidative injury by allylamine down-regulated α-smooth muscle actin expression, and this change was coupled to loss of normal architecture and increased thickness of the vessel wall

Clustering analysis of genomic data indicated that the adaptive response to allylamine injury, a.k.a. "the stress response", involved genes required for redox balance, such as superoxide dismutase, as well as genes involved in the extracellular matrix-integrin-cytoskeletal axis. Such relationships are consistent with existing knowledge, and suggest that gene-gene interactions regulated by oxidative stress may contribute to the progression of oxidative vascular injury by allylamine. Initiation of oxidative stress is likely due to poor maintenance of the redox flux caused by changes in GSH balance [179,180]. Of interest was the finding that many of the genes altered by oxidant injury were genes that exhibit some level of regulation by NFκB, such as OPN and α_1 integrin.

To validate genomic findings, and to define the molecular bases of the oxidant response, OPN and α_1 integrin levels in vivo were probed at the protein level. Considerable fidelity of the genomic response was observed at the protein level, at least for these two targets. OPN is a secreted acidic phosphoprotein involved in ECM/integrin interactions and proliferative control of vSMCs [167]. Elevated levels of OPN have been observed during neointimal formation and atherosclerotic disease [162]. Because the proliferation of vSMCs is a key feature of atherogenesis, OPN is an ideal marker of modified vSMC phenotypes. Integrins on the other hand, are a family of heterodimeric transmembrane glycoproteins composed of α and β subunits that allow cells to identify changes in ECM composition and activate intracellular signaling pathways [181]. ECM remodeling, a critical step in the pathogenesis of atherosclerosis, is highly dependent on the expression of matrix components and their receptors. For instance, α_1 integrin is a collagen/laminin receptor implicated in cellular proliferation, abnormal laminin deposition and inflammatory responses [163]. In inflammation, the α_1 integrin is responsible for monocyte adhesion to damaged tissue. Blockade or deletion of the α_1 integrin inhibits accumulation of matrix proteins, and may be of therapeutic value in the management of inflammatory disorders [182]. Recent reports from several laboratories, including our own, have demonstrated that cellular adhesion molecules provide signaling specificity during the vascular response to injury [150,183].

Tropomyosin is a component of the contractile mechanism of the cell, and is present in both stress fibers and thin filaments. Isoform inter-conversions may be coordinated with changes in the vSMC phenotype [184]. Tissue-specific regulation and

developmental expression of this gene is regulated by alternative splicing of duplicated isotype-specific exons [185]. The expression of an alternate tropomyosin isoform in vSMCs may be partially responsible for a shift from contractile to proliferative phenotypes. In keeping with this observation, evidence was obtained that oxidative injury increases the levels of α-tropomyosin within the vascular wall, as well as in vSMCs in culture. Interestingly, gene expression in vivo was mainly localized to vSMCs in close proximity to the advential side of the vessel wall. This localization is intriguing in light of evidence implicating the adventitia in neointima formation in the rat carotid injury model [186].

The finding that oxidative injury increased the expression of several members of the LINE gene family is also of potential significance. LINEs (Long Interspersed Nuclear Element) encode a reverse transcriptase and perhaps other proteins [187]. Transcriptional activation of the LINEs can increase up to 70-fold after exposure to stressors, such as UV light and ionizing radiation [188]. Recent studies in this laboratory have shown that activation of LINE expression in vSMCs is mediated by redox-regulated transcription factors that interact with the antioxidant response element [189]. As such, it has been hypothesized that activation of retrotransposons following oxidative injury may induce genomic instability and contribute to phenotypic changes following oxidative injury [187,189].

In order to characterize mechanisms responsible for gene dysregulation, the influence of N-acetylcysteine on oxidant-induced oxidative stress and gene expression was examined. Oxidative stress in cells challenged with allylamine or its metabolites

was inhibited by pre-treatment of the cells with N-acetylcysteine. This finding is consistent with previous studies showing that NAC neutralizes the effects on allylamine on α7 integrin expression [150]. Because N-acetylcysteine upregulates reduced glutathione levels and antioxidant defense in vSMCs [165], these findings implicate glutathione depletion and oxidative stress in the gene dysregulation response by allylamine and its metabolites. GSH may participate in the regulation of transcription factor binding to DNA. For instance, in COS2 cells depleted of GSH, or treated with hydrogen peroxide, significant decreases in glucocorticoid receptor-DNA binding have been reported [190]. Thus, decreases in glutathione and oxidative stress may work to effect gene expression in oxidant-injured cells. In this context, we have previously shown that depletion of cellular glutathione with buthionine sulfoximine is associated with redox-dependent transcriptional activation of ras proto-oncogene in vSMCs [165].

Repeated exposure of rats to allylamine modulates vSMCs from quiescent to proliferative phenotypes [191]. This transition is viewed as a hallmark of oxidant-induced atherogenesis, and involves increased production and secretion of extracellular matrix (ECM) components [192]. The results presented here identified the extracellular matrix-integrin- cytoskeletal axis, along with LINEs, as genomic targets of oxidative injury. As such, gene modulation in this model system appears to be mediated by redox-dependent mechanisms involving increased production of ROS and modulation of glutathione homeostasis. Ongoing efforts in this laboratory are focusing on the resolution of discrete networks of biological activity using Boolean and coefficient of determination methodologies [193].

CHAPTER III

COOPERATIVE REDOX REGULATION OF THE OSTEOPONTIN GENE IN VASCULAR SMOOTH MUSCLE CELLS: A ROLE FOR NF-κB AND TIEG-1

Synopsis

Osteopontin has come to light in recent years as a primary cytokine and matrix-associated protein playing an important role in medial thickening and neointima formation. OPN has a broad spectrum of biological activities ranging from regulation of extracellular matrix/integrin interactions to proliferative control of vascular smooth muscle cells (vSMCs). Osteopontin is produced by vSMCs, activated macrophages, leukocytes and activated T-lymphocytes, is present in extracellular fluids and found at sites of inflammation and injury. This laboratory has undertaken the task of dissecting the transcriptional regulatory mechanisms controlling the expression of osteopontin in response to oxidative injury/stress. Experimental data show that the transcriptional activity of the osteopontin promoter is regulated in a matrix dependant manner when activated vSMCs isolated from allylamine treated animals are seeded on a permissive fibronectin matrix. This increased transcriptional activity leads to increases in mRNA and protein levels of OPN, as confirmed by RT-PCR and in situ immunofluorescence in vivo. Here we show by promoter analysis and DNA-protein binding experiments that elevated levels of osteopontin mRNA in vSMCs involve a redox-regulated transcriptional mechanism. Specifically, a 200 bp region located in the 5′(UTR of the

osteopontin promoter was identified that mediates the redox regulatory response. Mutational analysis identified two distinct cis acting elements, NF-κB and TIEG-1, which are responsible for the promoter response to oxidative stress. The redox regulation of these elements was confirmed using two antioxidants, N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PTDC), to inhibit promoter activity. Further, DNA-protein binding assays confirmed the responsiveness of the OPN promoter to oxidative stress and antioxidant treatment and identified RelA(p65) as a protein present in redox regulated DNA-protein complexes. Collectively these studies help answer central questions regarding the mechanisms underlying increased OPN expression in diseases of the vascular wall.

Introduction

Cardiovascular disease is a primary or secondary cause in 60% of deaths in the United States, with 75% of these deaths resulting directly from atherosclerosis. Atherosclerosis is a chronic inflammatory disease associated with thickening of the arterial wall and reduction in vessel lumen size. The atherosclerotic lesion is termed a plaque, and consists of a mixture of immune cells, extracellular matrix, fatty deposits, and proliferative (i.e. atherogenic) vSMCs [59]. The activation of vSMCs to acquire atherogenic phenotypes is a critical event in atherogenesis, and oxidative injury is a critical event in the pathways leading to initiation and progression of this phenotypic transition [146]. Critical genomic targets and molecular mechanisms of gene dysregulation during the course of atherogenesis have remained poorly understood.

Previous studies in this, and other, laboratories have identified osteopontin as a putative molecular target in atherogenesis. Osteopontin (OPN) was first isolated as a protein that plays a key role in bone remodeling. This cytokine is secreted as a 55-80 kDa acidic phosphoprotein with varying degrees of post-translational modification. OPN is regarded as a matricellular protein, proteins that do not serve a primary structural role but rather function as modulators of cell-matrix and cell-cell interactions [194]. As such, OPN participates in the regulation of ECM/integrin interactions and proliferative control of vSMCs [162,195].

OPN has come to light in recent years as a key cytokine and matrix molecule playing an important role in medial thickening and neointima formation. Elevated levels of OPN have been observed during neointimal formation and atherosclerotic disease [196]. Because the proliferation of vSMCs is one of the key features of atherogenesis, OPN is an ideal marker of the modified (i.e. activated) vSMC phenotype. However, it is not yet clear if OPN expression is a cause, or a result, of atherosclerosis. Osteopontin is expressed in atherosclerotic lesions in vivo [197] and in response to injury in vitro [198]. 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. Further, osteopontin transgenic mice develop lesions more quickly in response to high cholesterol diets [197]. The work of Parrish and Ramos suggests that cleavage of osteopontin, which generates a biologically active 36 kDa fragment, is a key aspect in atherosclerosis [198, 199]. To this end we have adopted a model of oxidative

injury to study the molecular mechanisms governing the response of vSMCs to redox stress.

The study of the molecular mechanisms of transcriptional regulation is currently one of the most active areas of research in cell and molecular biology. Research in this field has been fueled by increased awareness of the crucial role of transcription in normal cell physiology and disease. For instance, transcription factors are involved in the regulation of vital cellular functions including cell growth, differentiation, apoptosis, metabolism, and secretion. In addition, alterations in transcription factor activity are increasingly being identified as causes of human diseases, including cancer and atherosclerosis [200]. Consequently, knowledge derived from cis-trans interactions in the regulation of gene expression has profound implications in medicine [201].

The present studies were conducted to evaluate a critical gene targets in oxidant-induced vascular injury (OPN). Studies were carried out in vitro to characterize the mechanisms by which *opn* contributes to the atherogenic phenotype. Specifically, one hypothesis will be tested: That the phenotypic transition and activation by oxidative stress that is seen in vSMCs is due to up-regulation of osteopontin, which is controlled at the transcriptional level by redox-regulated cis-acting elements. Following the identification and experimental confirmation of the functionality of these regions, site-directed mutagenesis was performed. Transient transfection and gel-shift analysis was then used to identify the regions responsive to oxidative injury, along with the proteins binding.

Materials and Methods

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). Medium 199, trypsin, and antibiotic/antimycotic solution were purchased from Gibco (Grand Island, NY). Antibodies for NFKB and p65 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Nitrocellulose membranes were purchased from BioRad (Hercules, CA). Synthetic oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). QuikChange Site-Directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA). Fugene6 and Reverse Transcriptase kits were obtained from Roche (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Nuclear Extracts

Nuclear extracts were prepared as previously described [202]. In brief, cells were washed twice with ice-cold HEGD (25mM HEPES-Cl, pH 7.6, 1mM dithiothreitol (DTT), 1.5mM EDTA, 10% glycerol, 0.5mM PMSF) and scraped from plates using 1mL HEGD. Cells were then transferred to a Dounce homogenizer and lysed with 20 strokes. Nuclei were pelleted at 16 x g, 2 minutes, 4°C in a variable speed microfuge, and the supernatant discarded. Nuclei were resuspended in 50 ml HEGDK (HEGD plus 0.5 M KCl) and incubated on ice for 2-3 hours to extract nuclear proteins. Nuclear ghosts were removed by centrifugation at 16 x g, 10 minutes, 4°C, in microfuge, and supernatant was

quick frozen in liquid nitrogen and stored at -80°C. Protein concentration was determined by the method of Bradford.

Construction of Luciferase Deletion Plasmids

Genomic DNA was prepared from the rat vSMCs using Wizard Genomic DNA Purification Kit (Promega). Oligonucleotides, FOR and REV, were designed on the basis of genomic DNA sequence of 5-flanking region of the OPN gene, to amplify a portion of DNA starting -2200 bp upstream of the identified transcription start site (+1). In addition to the template (genomic DNA) and primers FOR and REV, the 50 μl reaction mixture contained 0.2 mM dNTPs, Pfu DNA polymerase buffer and 5units of Pfu DNA polymerase (Promega), and was subjected to 35 cycles of amplification (60 seconds at 94 °C, 60 seconds at 55 °C, and 120 seconds at 72 °C). The PCR product was recovered from the agarose gel and used as template in PCR reaction. In this PCR amplification we used the primers FOR-Kpn and REV-Kpn tailed with a Kpn I restriction endonuclease recognition sequence (ggggtacc). The 50 μl reaction mixture contained the template, obtained as described above, primers For-Kpn and REV-Kpn, 0.2 mM dNTPs, PfuDNA polymerase buffer and 5 units of Pfu DNA polymerase (Promega). The reaction was subjected to 30 cycles of amplification (60 seconds at 94 °C, 60 seconds at 54 °C and 120 seconds at 72 °C). The PCR product was loaded on 1.5% agarose gel, recovered from the gel, purified and ligated into pGL3-basic reporter vector Kpn I digested, and dephosphorylated; the resulting plasmid was inserted in the pGL3-basic reporter vector and the correct orientation were verified by DNA sequencing. A set of OPN promoter

reporter constructs containing 5′ deletion was prepared by PCR using specific 5′ primers (Table 1 for details), and a common 3′ primer (REV-Kpn). PCR amplification products were loaded on 2% agarose gel, recovered from the gel, purified and ligated into pGL3-Basic reporter vector Kpn I digested. The resulting plasmids were analyzed by DNA sequencing to ensure the fidelity of amplification and the correct orientation.

Site-Directed Mutagenesis

Mutagenesis experiments were carried out using the QuickChange Mutagenesis kit according to the manufactures recommendations (Stratagene, La Jolla CA). Mutagenic oligonucleotides were designed according to the manufactures guidelines, and incubated with 2094bp rat OPN promoter as template. High fidelity PCR followed by the digestion of the original template with DpnI endonuclease. Clones were sequenced for verification of mutation of the targeted sites.

Transient Transfections and Luciferase Assays

rvSMCs cells were plated in 24-well plates 2 days before transfection. The day of transfection, cells were washed twice with PBS solution and replaced with serum-free M199 media (not containing penicillin-streptomycin). Cells were transfected using Fugene6 (Invitrogen) with 1 μg of the respective construct. rvSMCs were cotransfected with 200 ng of pGL3-renilla (Promega) (a plasmid containing the SV40 promoter upstream the renilla gene) to normalize for transfection efficiencies. After 4 hours of incubation at 37°C, the transfection solution was withdrawn and replaced with the

complete medium described above, and cultivated for an additional period of 48 hours at 37°C. Transfection were performed in duplicate, and repeated at least three times. Measurement of luciferase activity was performed 48 hours after transfection using the Luciferase Assay Kit (Promega) according to the manufacturer's protocol. Briefly, cells were washed with PBS solution, 100 µl of Passive Lysis Buffer (Promega) was added to each well and collected after 10 minutes at room temperature on orbital shaker. The lysates were centrifuged at 12000 x g for 2 minutes to remove cell debris. Luciferase activity was determined using 10 µl aliquots of supernatants in a Turner Biosystems 20/20 Luminometer. Each lysate was measured twice and activities were normalized for renilla activity in each extract to correct for transfection efficiency. Reporter gene expression was expressed as relative light units and the luciferase activity of each construct compared with that of the promoter less pGL3 Basic vector.

EMSA

Nuclear extracts from vSMCs were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Approximately 2 x 10^6 cells were trypsinized, collected and washed once in 5ml of PBS. The cell pellet was then processed as described by the manufacturer. The protein concentration of the nuclear extract was determined using the bicinchonininc acid assay (BCA; Pierce), and BSA as standard. The nuclear proteins were kept at -80°C and used in band shift assays. The sequences of the oligonucleotides used are reported in Figure 13. To prepare the double-stranded oligonucleotides, single-stranded forward and reverse oligonucleotides were annealed by

heating to 95°C and cooling slowly to room temperature in TE Buffer (10mM Tris, 1 mM EDTA). The 20 μl of binding solutions contain 3 μg of nuclear extract with 20 fmol of 5′ end-biotinylated DNA target in the presence or absence of competitor, 2.5% (v/v) glycerol, 5 mM MgCl2, 50 ng/l poly (dI-dC), 0.05% (v/v) NP-40, and the binding buffer provided with the kit. Binding reactions were incubated at room temperature for 20 minutes 5 μl of 5x Loading Buffer was then added to each 20 μl binding reaction. The complex was separated on a 6% nondenaturing polyacrylamide gel in 0.5x Tris-Borate Buffer (TBE) for 1.5 hour at 100 V at room temperature. Complexes were then transferred to a nylon membrane using an electrophoretic transfer unit (Bio-Rad) according the manufacturers instructions, in 0.5x TBE cooled to 4°C, for 1 hour at 380 mA. DNA was cross-linked to the membrane with UV-light cross-linker instrument. Complexes were detected according to Panomics EMSA Kit instructions. 20 ml of blocking buffer were added to the membrane, and this was incubated at room temperature for 15 minutes. This buffer was replaced with blocking buffer added with Stabilized Streptavidin-Horseradish Peroxidase Conjugate, and the membrane was incubated for 15 minutes at room temperature. The blot was washed five times for 15 minutes each in 20 ml of 1x Wash solution and incubated in 30 ml of Substrate Equilibration Buffer for 5 minutes. The membrane was placed in Substrate Working Solution for 5 minutes and then placed in a film cassette and exposed to X-ray film. For antibody supershift assay, 4 μg of NF-κB antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction mixture prior to the addition of the probes and incubated at room temperature for 30 minutes. Protein-DNA complexes were

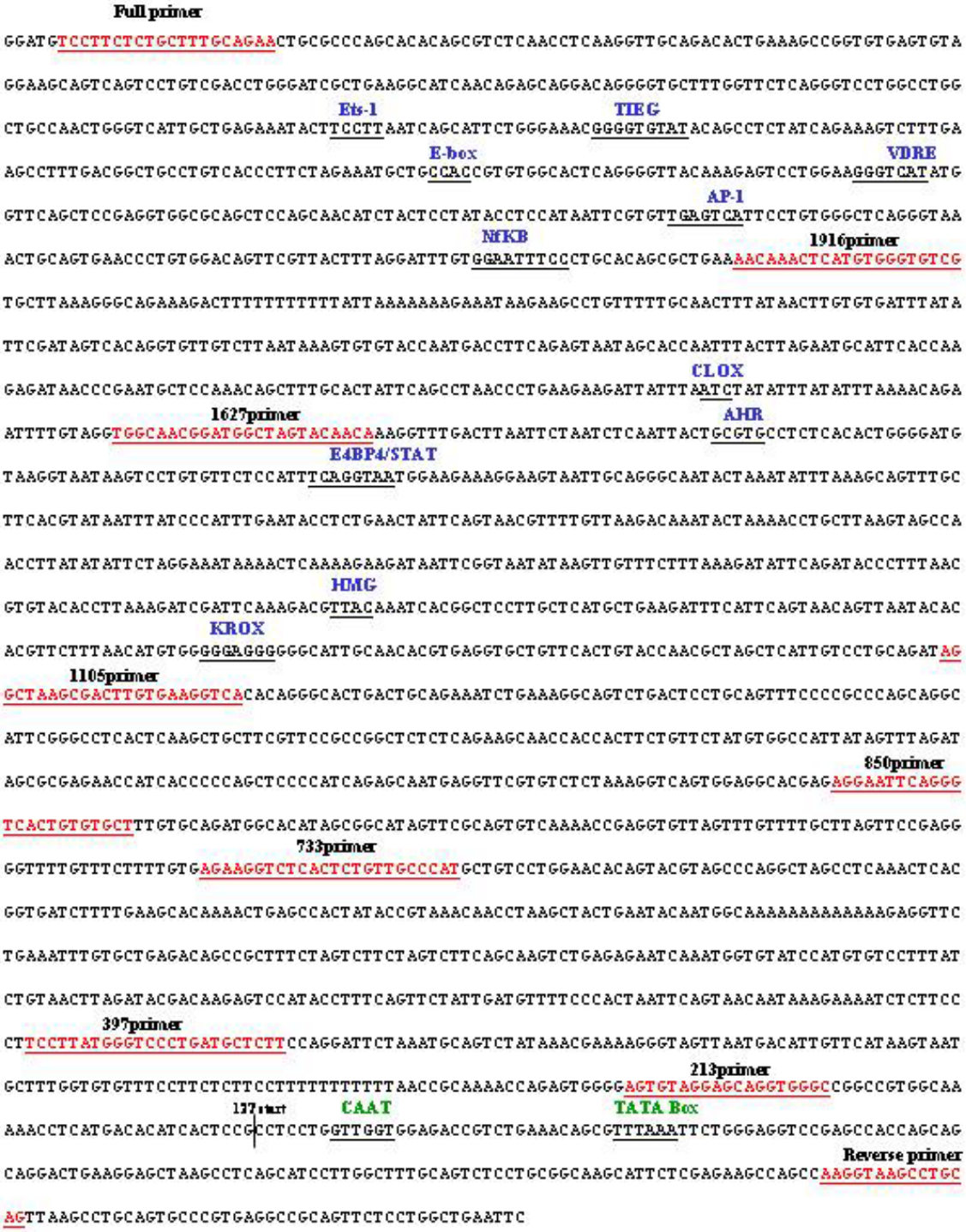
fractionated an a 6% nondenaturing polyacrylamide gel in 0.5X TBE buffer at room temperature for 1.5 hour at 100 V.

Results

Enhanced expression and secretion of osteopontin is a hallmark in the atherogenic phenotype [203]. Increased osteopontin mRNA levels have previously been described by our laboratory in an in vivo model of oxidative stress using vascular smooth muscles [198]. To evaluate the molecular mechanisms of osteopontin regulation, luciferase constructs containing the osteopontin promoter were generated and transfected into rat vSMCs. The full length OPN gene promoter sequence, genebank #AF017272, was subjected to transcription factor analysis using MatInspector ver 2.2 to find potential transcription factor binding sites responsive to oxidative stress. Several putative response elements that may participate in the redox-mediated were identified. PCR primers were designed for the OPN promoter to allow for the construction of a series of sequential deletion mutants to study the relative contribution of different sites within the promoter. The 5 primers along with the anchored 3 primer used in the creation of the constructs are delineated by an underscore in the promoter sequence shown in figure 13.

Figure 13. Full length rat OPN promoter.

Primer sequences used for the construction of the luciferase constructs are underlined and labeled in red. CAAT and TATA box are labeled in green downstream of the 127bp start site (vertical line). Select cis acting elements identified via MatInspector analysis are shown in blue, with their core binding sequence underlined in black.


Full primer
Ets-1
TIEG
E-box
VDRE
AP-1
NFKB
1916primer
CLOX
1627primer
AHR
E4BP4/STAT
HMG
KROX
1105primer
850primer
733primer
397primer
213primer
CAAT
TATA Box
Reverse primer

Eight different PCR-generated constructs of the osteopontin promoter were spliced into pGL3-basic, a luciferase reporter assay. The constructs created and spliced into the reporter vector were as follows; full length 2094bp, 1916bp, 1627bp, 1105bp, 850bp, 733bp, 397bp, and 213bp.

The constructs were then transfected into cells. After allowing 36 hours post transfection for the cells to recover, the cells we subjected to one of three treatments 30 minutes prior to measurements of luciferase activity. The treatments were as follows, control-water only, hydrogen peroxide treatment 0.001 mM , or 0.5 mM NAC pretreatment for 30 minutes after which the NAC was removed and treatments with hydrogen peroxide for 0.5 hours initiated. The results presented in figure 14 show, the response of the full length promoter to oxidative stress conditions was greatest relative to all of the constructs tested. The redox sensitive nature of this response was confirmed by the decrease in luciferase activity seen in the constructs pretreated with the antioxidant NAC. The region of the osteopontin promoter from 2094bp-1916bp contains oxidative stress responsive element(s), since deletion of the sequences inhibited the induction response greater than 150%. The minimal promoter, 213bp, displayed very low levels of activity, but some degree of redox-sensitivity was retained in this region.

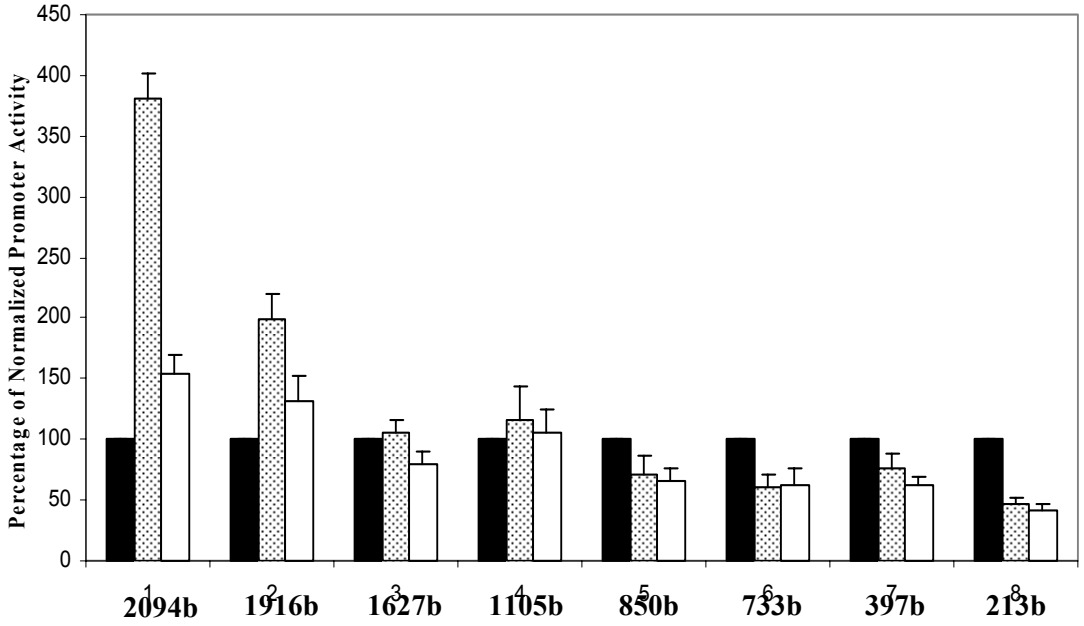


Figure 14. OPN promoter activity of full length and serial deletion constructs. Control and stressed cells were seeded at equivalent densities and allowed to recover for eight hours. Cells were then transfected with the respective OPN promoter constructs. Cells were co-transfected with a renilla construct to allow for normalization of the luciferase signal. Sequential deletions of the full length OPN promoter (2094bp) were transfected into vSMCs. Black bars represent control untreated cells, grey bars represent cells treated with hydrogen peroxide, clear bars represent cell pretreated with NAC prior to treatment with hydrogen peroxide. n>3.

Examination of the 178bp sequence contained within the full length promoter identified several cis-acting elements, including NF-κB and TIEG-1 binding sites. These cis-acting elements identified by computerized analysis were then subjected to further experimentation to examine their contributions to the oxidative responsiveness of the promoter. Site directed mutation analysis was performed on the two sites to determine whether or not they are contributory to the redox response. The first site examined was the NF-κB binding site. Previous experiments in this laboratory had identified the redox nature of this binding site in a phenotypically-modified cellular

model of oxidant induced injury (unpublished results). Site directed mutagenesis of the putative sites was performed using the QuikChange Site Directed Mutagenesis Kit. The mutations of the NF-κB binding site are detailed in figure 15a with the alterations in each element denoted by lower case letters. The mutation introduced into the NFKB binding site was determined in previous experiments to be sufficient to ablate NFKB binding at least 50% of the response seen with oxidant treated cells (data not shown). Site directed mutation analysis was also performed on the TIEG-1 binding site to identify point mutations in the nucleotide sequence that would cause a reduction in luciferase activity of the promoter. Mutation of a single nucleotide C to A outside of the consensus binding site did not have a significant effect on the luciferase activity of the promoter. Likewise, a two nucleotide mutation GG to TA in the consensus binding site did not significantly decrease the luciferase activity. However, mutation of the third G to A in the 5 end of the consensus binding site caused a 50% reduction of the luciferase activity of the full length promoter. From these data it can be concluded that the extreme 5′ end of the TIEG-1 DNA binding element does not contribute greatly to the binding of the zinc finger to this site (figure 15b).

A.

NF-κB -original **AGGATTTGTGGAATTTCCCTGCACAGC**

NF-κB -mutated **AGGATTTGaaGAATaaCaCTGCACAGC**

B.

GGAAACGGaGTGTATACAGCCTCT

GGAAACtaGGTGTATACAGCCTCT

GGAAAaGGGGTGTATACAGCCTCT

GGAAACGGGGTGTATACAGCCTCT

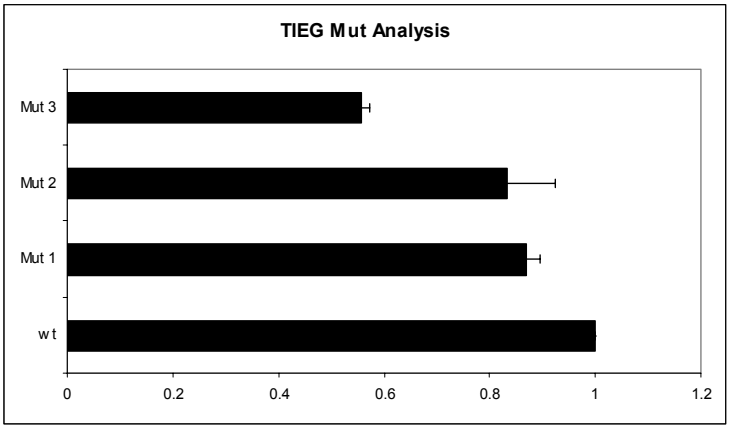


Figure 15. Site directed mutagenesis of the rat osteopontin promoter.
A) NF-κB binding site was identified in the full length promoter at 481-495bp from the 5′end of the OPN promoter (genebank#AF017274). Site directed mutagenesis was used to alter the consensus binding sequence (underlined). The resulting construct was then used for analysis in luciferase studies. B) Transcriptional binding site software also identified a TIEG-1 binding site 228-242bp from the 5′ end of the OPN promoter. The consensus sequence for TIEG-1 is underlined, with the changes induced via site directed mutagenesis shown in lower case. Several different mutations at different locations were tested. The changes resulting from mutation 3 (mut3) caused the greatest decrease in promoter activity under oxidative stress conditions. For this reason, mut3, was used for all TIEG-1 mutation luciferase experiments. n>3.

To examine the contributions of each of these elements to the oxidative response seen in the full length osteopontin promoter when cells are treated with hydrogen peroxide, a luciferase activity assay was conducted using vSMCs challenged with hydrogen peroxide. In this experiment, oxidant treatment of cells transfected with full

length osteopontin promoter caused a greater than five fold increase in luciferase activity compared to control. When cells containing the same construct were pretreated for thirty minutes with antioxidants before addition of hydrogen peroxide the increase in luciferase activity was reduced to levels approaching or below that which is seen cells transfected with wild type construct. This decrease in activity suggests that the luciferase activity seen in response to oxidant treatment is due in part to the presence of a redox sensitive element(s) within the osteopontin promoter. To identify these elements, constructs containing the mutated binding site for NFKB and TIEG-1 were analyzed. In constructs containing the mutated NFKB binding site a ~3 fold decrease in luciferase activity was seen upon treatment with hydrogen peroxide. Additionally, in the construct containing the mutated TIEG-1 binding site a decrease in relative luciferase activity was observed. This reduction however was only ~1.7 fold decrease of the levels seen in wild type cells treated with hydrogen peroxide. The minimal promoter 213 bp showed little activity (figure 16).

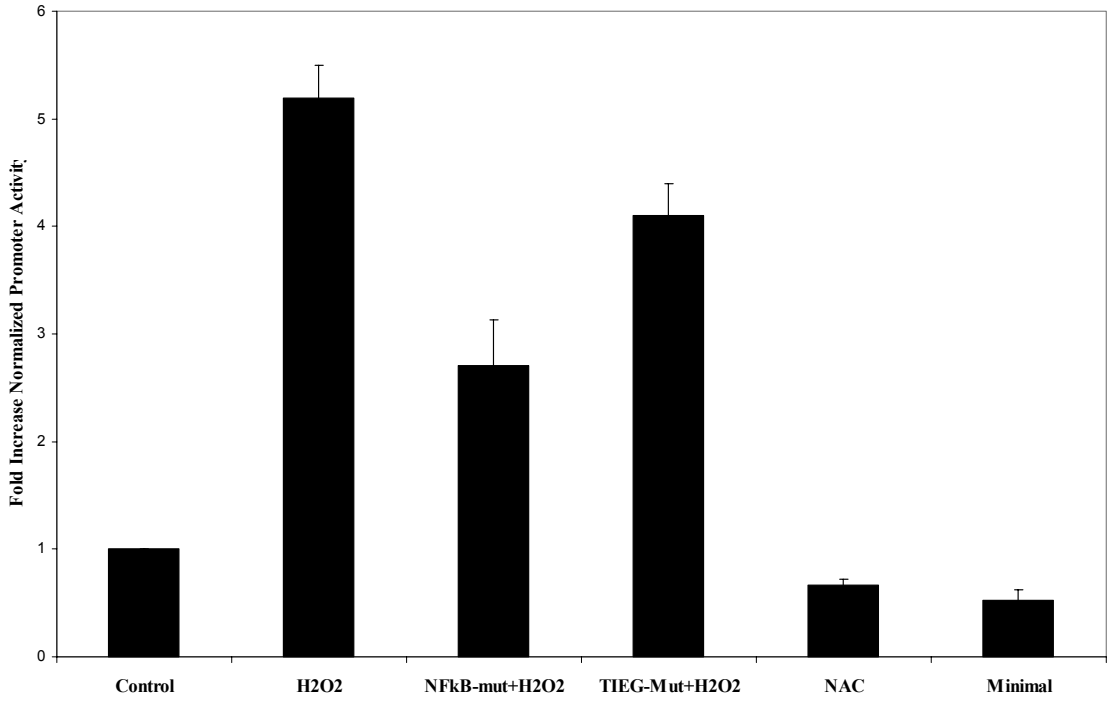


Figure 16. Analysis of site directed mutants of the OPN promoter.
Site directed mutations created in the NF-κB and TIEG-1 binding sites were evaluated using luciferase assays. The bars labeled control are cells containing the full length OPN promoter in the absence of oxidative stress. All results were normalized to control. H2O2 bar represents cells treated with hydrogen peroxide before measurement of luciferase activity. NAC is cells pretreated with antioxidant before treatment of hydrogen peroxide. NF-κB and TIEG-1 are full length promoters in which the respective binding sites were mutated. The minimal promoter is included as a control. n>3.

Transfection of vSMCs with increasing amounts of various promoter constructs yielded a dose-dependant activation of reporter activity. These dose dependence profiles demonstrate that increases in luciferase activity are a direct result of the activity of the promoter constructs. The data from this experiment, especially that at the 1000ng level, also shows that the NF-κB and TIEG-1 binding sites are acting in concert in causing increases in luciferase activity. When double mutants for both of these sites are

introduced into a single construct the increase seen in response to oxidative stress is completely negated (figure 17).

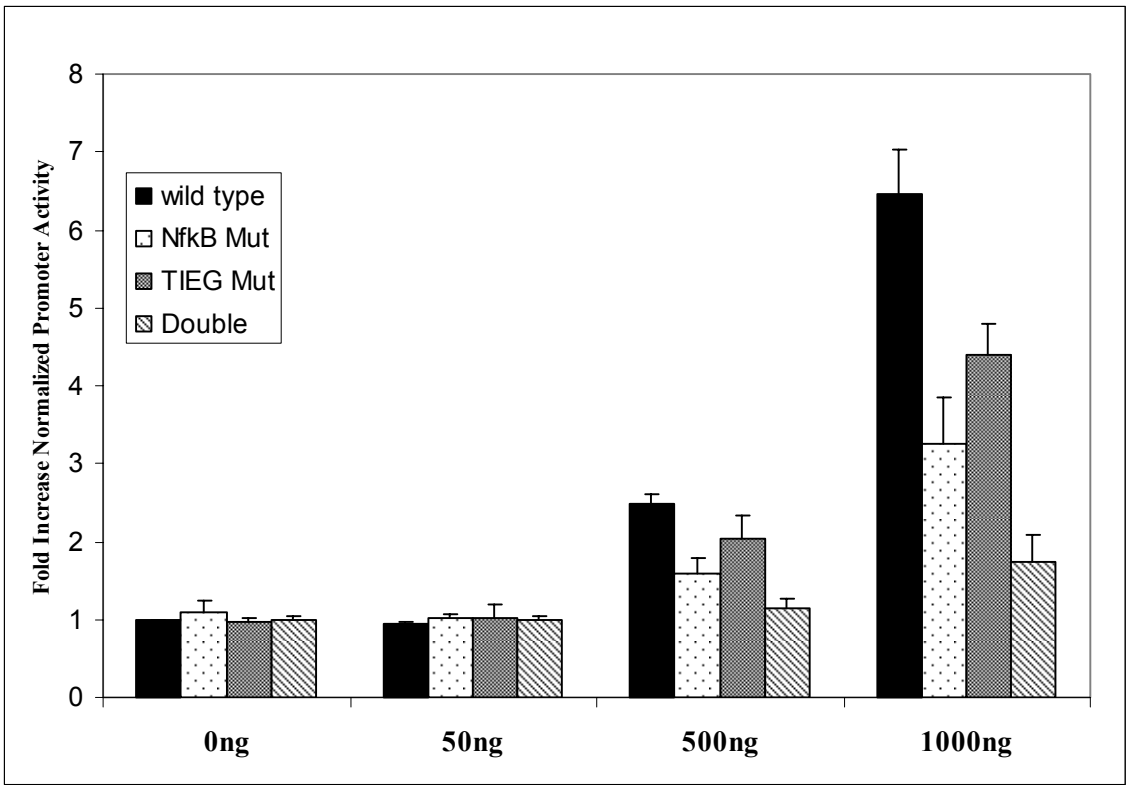


Figure 17. Concentration response for full length wild-type and mutant OPN promoter constructs.
Increasing amounts of the respective promoters 0ng-1000ng were transfected into cells seeded at equivalent densities. Cells were subjected to oxidative stress conditions via treatment with hydrogen peroxide and co-transfected with a renilla vector to allow for normalization of transfection efficiencies. NFKB-mut and TIEG-mut refer to full length vectors in which the respective cis acting elements have been mutated. Double refers to a full length OPN promoter in which both the NFKB and TIEG-1 binding sites have been mutated. n>3.

In order to determine the optimal concentration of hydrogen peroxide that will cause an increase in luciferase activity in cells transfected with the full length promoter,

a dose response experiment was performed. In this experiment, the full length promoter shown previously to be responsive to oxidative stress was examined in cells treated with (0.0001μM-1μM). A concentration of 0.001μM hydrogen peroxide was the optimal concentration to activate luciferase activity in full length promoter constructs (figure 18a). Higher concentration of hydrogen peroxide produced a reduction luciferase activities compared to the optimal concentration. This reduction may be due to the fact that at those levels hydrogen peroxide starts to become toxic. To determine the optimal conditions under which to perform the luciferase assays, a time course of hydrogen peroxide treatment was also undertaken. Time points from 0-15 minutes showed little, if any, increase in luciferase activity when cells were treated with an optimal concentration of hydrogen peroxide. At the 30 minute mark, there was a dramatic increase in luciferase activity. This increase continued into the 60 minute time point and then started to decrease by 120 minutes (Figure 18b).

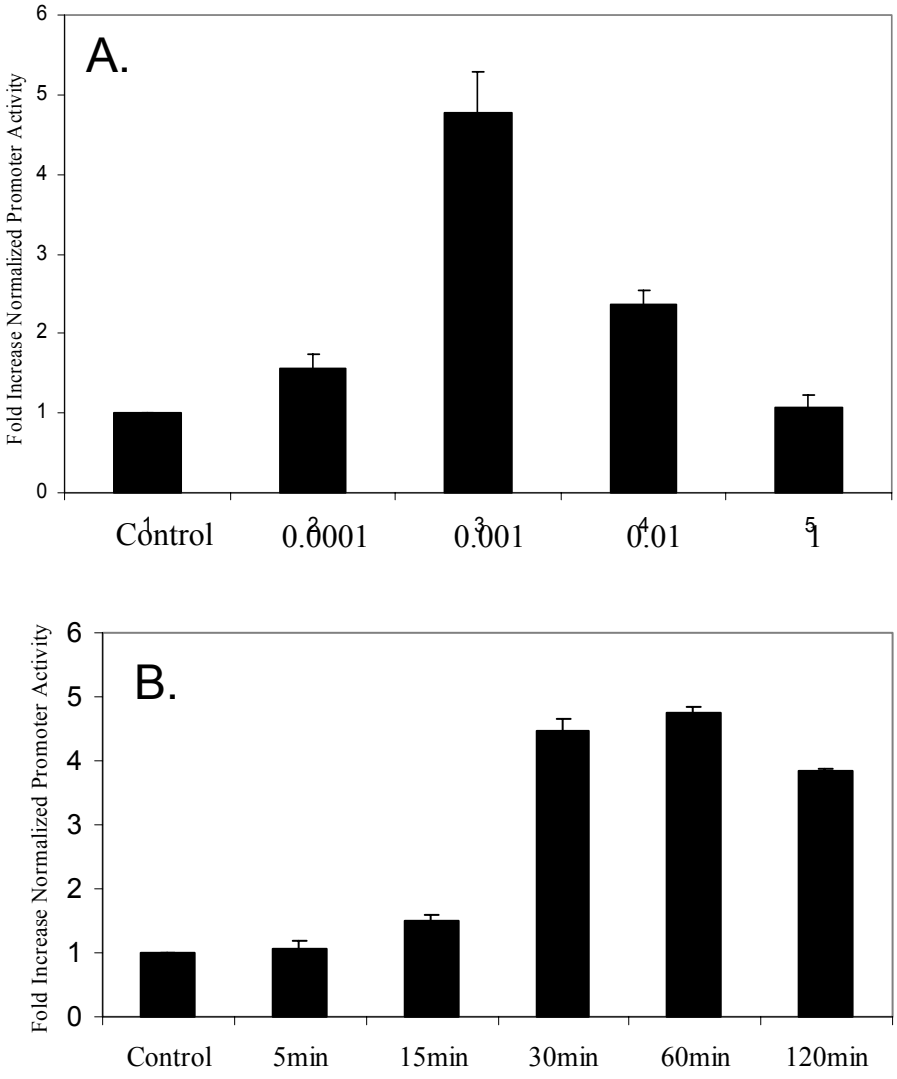


Figure 18. Dose and time course response of the full length OPN promoter in vSMCs.

A) vSMCs were treated with a range of hydrogen peroxide from 0(Control) to 1μM. B) Cells transfected with the full length OPN promoter were treated with 0.001μM hydrogen peroxide for varying time points from 0 minutes to 120. Cells were seeded at equivalent densities and co-transfected with a renilla construct to normalize for transfection efficiency. n>3.

From this set of experiments it was determined that treatment of vSMCs with 0.001μM hydrogen peroxide for 30 minutes elicits the greatest activation of the osteopontin promoter. Since experiments were conducted to identify the oxidative responsive elements within the osteopontin promoter, experiments were performed in the presence of antioxidant. NAC, N-acetylcysteine , is a sulfur containing free form amino acid, derived from the amino acid L-cysteine was chosen. NAC acts as a precursor of glutathione and proven to boost the intracellular production of glutathione [71]. To determine the optimal concentration and time for pretreatment with antioxidants, a series of experiments was performed to determine the dose and time response profile for antioxidant pretreatment. Figure 19a shows that cells treated with 0.001 μM hydrogen peroxide were afforded protection against oxidative stress only when pretreated with 0.5 mM NAC. Higher antioxidant concentrations did not afford any additional protection, this is due to the fact that NAC can become cytotoxic at these levels. Also, to determine the optimal time for the NAC pretreatment a time course study was undertaken. This set of experiments helped to determine the minimal length of time needed for NAC pretreatment to afford antioxidant protection. From this study it was determined that 30 minutes was the minimal amount of time needed in vSMCs to provide protection from oxidative stress (Fig 19b).

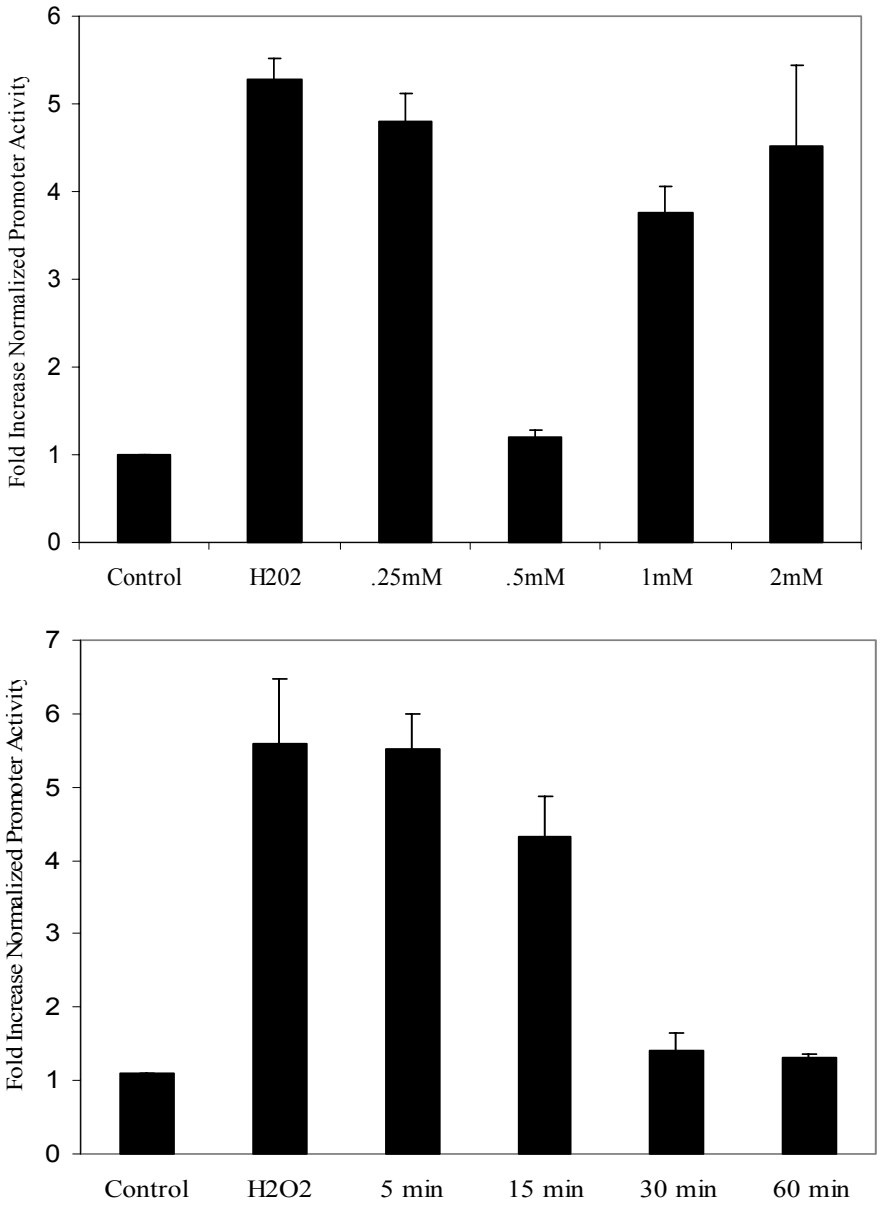


Figure 19. Confirmation of the oxidative stress responsiveness of the OPN promoter.

A) vSMCs seeded at equal densities were transfected with the full length OPN promoter and a renilla vector as control. Cells were subjected to three different conditions. Control- no treatment 2)H_2O_2 cells treated with 0.001 mM hydrogen peroxide for thirty minutes or 3) cell treated with a range of the antioxidant NAC (0.25 mM-2 mM). B) It was also determined via a time course study that pretreatment with 0.5 mM NAC for 30 minutes would afford the greatest protection from oxidative stress. n>3.

To confirm the true oxidative responsiveness of the promoter, a second antioxidant was used. PTCD, pyrrolidine dithiocarbamate, functions as a NFKB inhibitor as well as a hydroxyl radical scavenger. Pretreatment with 0.1mM PTDC caused the greatest decrease in luciferase activity in cells treated with hydrogen peroxide. In a time course study, it was determined that pretreatment of cells with PTDC for thirty minutes prior to addition of the oxidant would afford the greatest protection against oxidative stress and evidenced by the decrease in luciferase activity (Figures 20 a and b).

Next, experiments were conducted to examine the specificity and binding profiles of NF-κB in treated and untreated cells (Figure 21). Specifically, competition and supershift EMSAs were performed to examine these profiles. The profile of NF-κB binding in vSMCs produces 3 distinct complexes labeled C1-C3, arrows denote the location of the complexes in figure 21. To identify the composition of these complexes, a supershift assay was also performed with antibodies against RelA (p65). This antibody was added to Lanes 3,5,8,9,11 and 12. The antibody produced a supershift in each of these lanes. The supershift is denoted by (SS) in the figure. This supershifted band was the greatest in cells that were subjected to oxidative stress (lane 5). Cells that were pretreated with optimal level antioxidant, either 0.5 mM NAC or 0.1mM PTDC, showed a marked reduction in the supershift band intensities(lane 8 and 11). When a lower concentration of antioxidant was given to these cells, a concentration that has been shown to not afford protection, there was an increase in the supershift in the bands when compared to that of the cells treated with the optimal levels. This reduction produced a

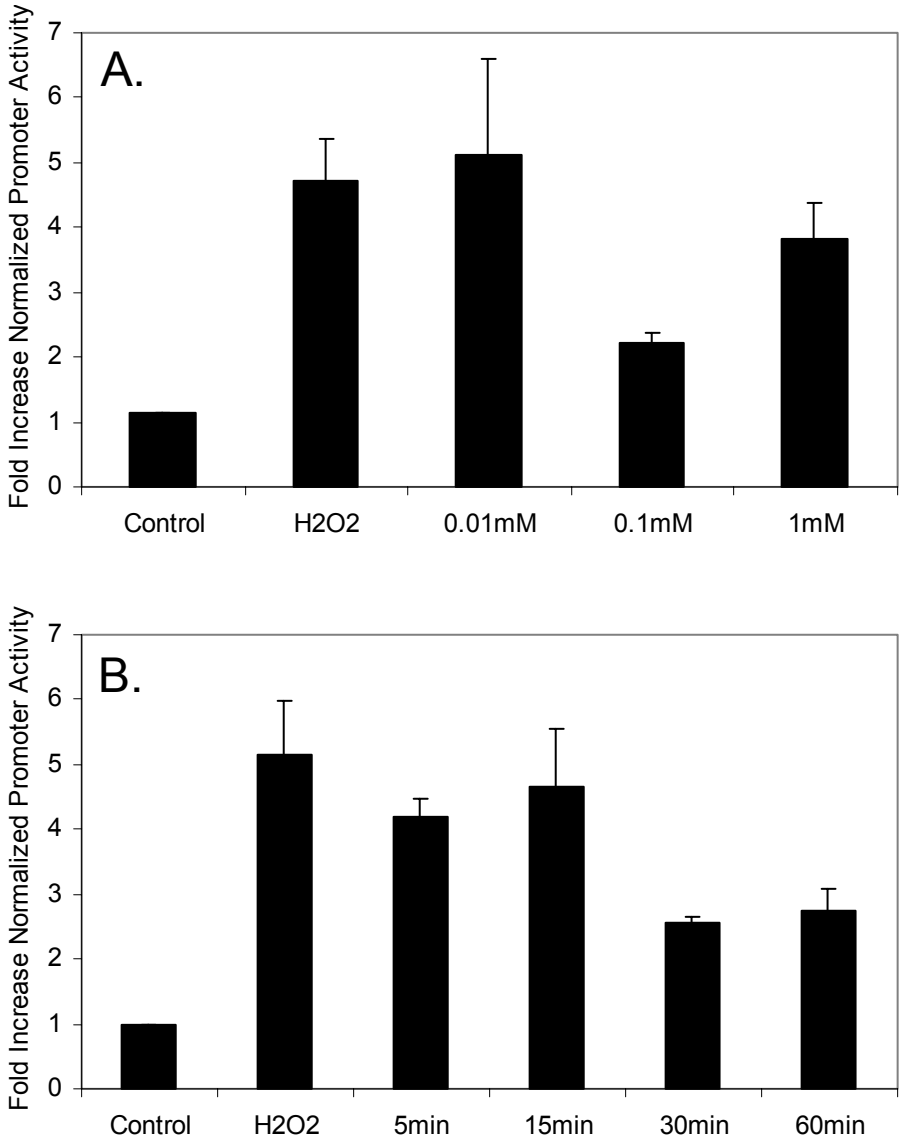


Figure 20. Effect of PTDC on promoter activity.
To further confirm that the luciferase activity of the osteopontin promoter was indeed being controlled via a redox sensitive mechanism, the activity of the promoter was tested in the presence on another antioxidant (PTDC). These date the dose response (A) and time course (B) indicate that the activity that is seem in the full length OPN promoter is indeed redox sensitive. n>3.

band that is similar in intensity to that seen in untreated cells. The identity of this supershift is confirmed by lanes 6, which has not antibody and did not produce a supershift. Sequence specificity of NF-κB binding was confirmed by running extracts in the presence of 10X cold oligonucleotides (Lanes 1, 2, 4, 7, 10 and 13)

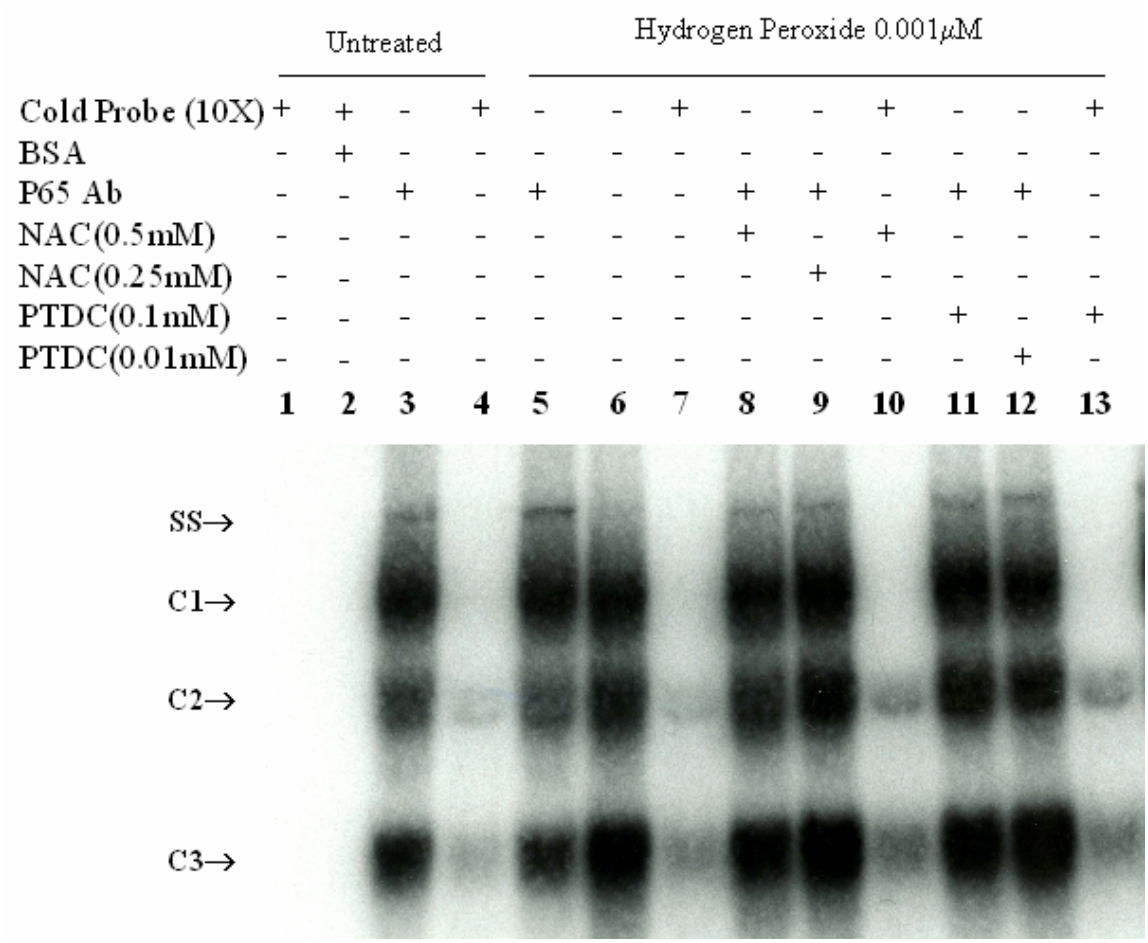


Figure 21. Specificity of NF-κB binding activity.
Competition and supershift EMSA were performed on extracts of vSMCs. The cells were either untreated or subjected to oxidative stress. Lanes 3,5,8,9,11 and 12 represent lanes in which anti- **NF-κB** p65 antibody was added and incubated with the extract after addition of the labeled oligonucleotide. Lanes 4,7,10 and 13 represent lanes in which the extract was incubated with 10 fold excess unlabeled oligonucleotide. Lanes 8,9,11 and 12 represents lanes in which cells we pretreated with antioxidants before the addition of hydrogen peroxide.

Discussion

Of relevance to the work presented here is NF-κB, a transcription factor which uses a helix-loop-helix motif to bind to DNA sequence elements in the promoter / enhancer region of target genes. DNA binding involves one of the alpha helices which positions itself in the major groove of the specific DNA sequence [204]. NF-κB is involved in the expression and regulation of a number of important cellular and physiological processes such as growth, development, apoptosis, immune and inflammatory response, and activation of various promoters. NFkappaB represents a group of structurally related and evolutionarily conserved proteins related to the proto-oncogene c-Rel. Five members have been identified in mammals including Rel (cRel), RelA (p65), RelB, NFkappaB1 (p50 and its precursor p105), and NFkappaB2 (p52 and its precursor p100) [205,206]. NFkappaB/Rel proteins exist as homo- or heterodimers to form transcriptionally competent or repressive complexes. Although most NFkappaB dimers are activators of transcription, the p50/50 and p52/52 homodimers can repress the transcription of their target genes [207,208]. The p50/p65 heterodimer of NF kappa B is the most abundant in cells. A critical component in NF kappa B regulation is the IkappaB Kinase (IKK) complex. In a majority of unstimulated cells, the NF kappa B transcription factors exist in their inactive form and are retained in the cytoplasm by the bound inhibitory I kappa B proteins. Upon stimulation by multiple inducers including viruses or cytokines, such as TNFalpha, IL-1, or PMA, I kappa B alpha is rapidly phosphorylated and degraded, resulting in the release of the NF kappa B complex, most commonly the p105/p65 heterodimer. The p105 subunit is cleaved into its active p50

form [209]. This cleavage exposes the NLS sequence on the p50 subunit. The p50/p65 heteroduplex then translocates to the nucleus where it activates gene transcription. NF kappa B induces the transcription of its own inhibitor, I kappa B alpha, causing an autoregulatory mechanism of NF kappa B activity and generating the inactive form of NF kappa B [210, 211]. The newly formed nuclear NF kappa B-I kappa B alpha complexes are then exported out to the cytoplasm, thereby reestablishing the cytoplasmic pool of inactive NF kappa B complexes primed for another round of activation to take place. The wide variety of genes regulated by NF kappa B includes those encoding cytokines, chemokines, adhesion molecules, acute phase proteins, and inducible effector enzymes.

Also identified in this study as contributing to the redox sensitivity of the osteopontin promoter activity was TIEG-1. Transforming growth factor-beta (TGFβ) and members of the TGFβ/Smad signaling pathway share similar anti-proliferative properties and are well-documented suppressors of growth [212,213]. The TGFβ signal transduction pathway is implicated in tumor development in several types of cancers, and the activity of this pathway is modulated by mutations of critical components such as the TGFβ receptors, Smad2, and Smad4, or over-expression of signaling inhibitors such as c-Ski, SnoN, Smad6, and Smad7 [214,215,216,217]. Transforming growth factorβ inducible early gene, TIEG, was discovered and characterized as a three-zinc finger containing nuclear, transcription factor [218,219,220]. TIEG was first identified as a primary response gene for TGFβ in human osteoblasts, that encodes a 480 amino acid (72 kDa) protein [218], TIEG is a Krüppel-like transcriptional factor that serves a

unique regulatory role in the TGFβ signal transduction pathway. TIEG over-expression mimics the actions of TGFβ in many cell types. For example, TIEG modulates markers of differentiation and gene expression, regulated cell proliferation, and induced apoptosis [212,219]. Whereas Smad proteins are grouped according to their ability to activate or repress gene expression, the two TIEGs identified thus far only repress gene expression. The TIEG NH_2-terminal region has three conserved motifs involved in repression, and four linker segments that separate the repressor motifs and the COOH-terminal DNA binding domain. Overexpression of TIEG1 in a pancreatic cell line leads to growth suppression and apoptosis [221]. Similar overexpression of TIEG2 suppresses epithelial cell proliferation. Transgenic mice expressing TIEG2 exhibit increased apoptosis and disorganized acinar cell organization--similar to the early pancreatic atrophy observed in TGF-ß transgenic mice [222]. It is unknown whether TIEGs bind to the TGF-ß receptor, as demonstrated for Smad proteins, nor is it known whether TIEG1 and TIEG2 share redundant properties or have specific roles in TGF-ß-mediated signaling [223]. These studies are beginning to fill a gap in the existing knowledge by characterizing those zinc finger proteins which participate in growth factor-induced cell proliferation, cell cycle arrest, death, and the modulation of neoplastic transformation [224]. The importance of this information is further emphasized by the fact that several therapeutic protocols currently exist which take advantage of controlling abnormal cell growth by interfering with growth factor signaling pathways.

The present study presents evidence that there exist in the 5 end of the osteopontin promoter elements that are redox sensitive in nature.

A progressive rise of oxidative stress due to altered reduction–oxidation (redox) homeostasis appears to be one of the hallmarks of the processes that regulate gene transcription in many diseases [225]. Reactive metabolites serve as signaling messengers for the evolution and perpetuation of the inflammatory process that is often associated with cell death and degeneration. Redox-sensitive transcription factors are often associated with the development and progression of many human disease states and inflammatory-related injury [226]. The present study examines the role of the redox-sensitive and oxygen-sensitive transcription factor NFκB in mediating oxidative injury. Changes in the pattern of gene expression through regulatory transcription factors are crucial components of the machinery that determines cellular responses to oxidative and redox perturbations [227].

CHAPTER IV

SUMMARY

At its core, the pathobiology of atherosclerosis and other inflammatory disorders is a result of, and intricately linked to, altered patterns of gene expression. A major finding in this dissertation is that OPN, a secreted glycoprotein that binds cellular integrins, is a major target of oxidative injury within the vascular wall. OPN participates in the pathogenesis of divergent diseases, such as tumor metastasis, diabetes and atherosclerosis [228], disorders which share a significant inflammatory component [229]. As such, OPN gene expression may serve as common denominator of the inflammatory response in somatic cells. The molecular mechanisms linking inflammation and OPN have not yet been fully elucidated. Evidence is presented here that redox mechanisms participate in the regulation of OPN gene expression in vSMCs. This finding is consistent with the mechanistic linkage between inflammation and redox stress , and the positive regulation of OPN gene by ROS [230,231].

Inflammation is associated with Inflammation is characterized by macrophage activation, production of cytokines, inflammatory mediators, chemokines, acute phase proteins and mast cell activation. All of these processes work in conjunction to activate and promote the inflammatory process. At a mechanistic level, the process of inflammation involves over-production of ROS that interact with cellular macromolecules to modulate the redox status in target cells and their surroundings. In the context of tissue repair, inflammation is a desirable process resulting in protection

against infectious agents and the propagation of pro-inflammatory responses in the context of human disease. By the same token, ROS can severely damage biological macromolecules by oxidative modification to become detrimental to preservation of overall cellular homeostasis and health [232]. Redox reactions have the capacity to disrupt cellular functions via free radical mediated-injury if they are not kept in check by the cellular machinery [233]. Whether positive or negative outcomes are realized during the course of inflammation is defined by the relative expression of enzymes that regulate the cellular response to oxidative stress. Many of these enzymes, such as glutathione transferase, quinone reductase, epoxide hydroxylase and heme oxygenase, neutralize the adverse effects of ROS and afford protection of the cell from the harmful effects of ROS [234].

Oxidative injury to cells within the vascular wall is a critical event in the pathogenesis of atherosclerosis. To study the cellular and molecular basis of this complex interaction, this laboratory has adopted an in vivo model of repeated cycles of oxidative injury by allylamine (AAM), a cardiovascular-specific toxicant that induces vascular lesions reminiscent of those seen in human atherosclerosis. AAM is metabolized within the vascular wall by a vascular specific enzyme, known as semicarbazide sensitive amine oxidase, to acrolein and hydrogen peroxide. These metabolites compromise redox homeostasis in vascular smooth muscle cells leading to oxidative stress, activation/ repression of redox-regulated genes, peroxidative injury, and cell death. To study these interactions, six week-old (175-180g) male Sprague-Dawley rats were gavaged daily with 35 or 70 mg/kg/day AAM or water as control (1ml/kg/day)

for twenty days. The induction of oxidative stress by chemical stress was confirmed on the basis of glutathione depletion, activation of NF-κB, and increased formation of isoprostane. Isoprostanes are chemically stable peroxidation products of arachadonic acid that have been identified as useful indicators of oxidative stress in vivo [160]. F2 isoprostanes are prostaglandin F2-like compounds unaffected by COX inhibitors [235]. An increase of 8-epi-PGF2α isoprostane is observed in animal models of oxidant injury [175], and in pathological processes such as atherosclerosis and cancer.

A notable difference between normal and oxidant-injured tissue was the expression of proteins not normally expressed within the cell. Many of the genes encoding these proteins were activated by transcription factors such as NF-κB [236]. NKFB is a central mediator of the immune and inflammatory response; and the activation and translocation of NF-κB leads to transcription of genes that mediate stress signaling [237]. In its latent form, NF-κB exists in the cytoplasm of unstimulated cells as a dimer bound to an inhibitory protein, IB. Upon activation by oxidative stress, NF-κB dissociates from the inhibitory IκB protein and translocates to the nucleus where it regulates the expression of genes involved in diverse cellular functions, including the adaptive cellular response to stress [177]. Recent in vitro studies have identified NF-κB as a key transcription factor involved in the regulation of proliferative phenotypes and integrin-coupled ECM signaling in vSMCs following oxidative stress. Of particular significance within this context is that NF-κB was markedly upregulated in stressed vessels, and that many of the genes regulated by vascular oxidative stress exhibited some level of regulation by NF-κB [238].

vSMCs are involved in a number of diseases including hypertension and atherosclerosis. In these pathological states, vSMCs undergo a phenotypic modulation from a contractile state where the expression of α-smooth muscle actin is high, to a proliferative state with different functional, morphological and biochemical characteristics. When vSMCs transition to atherogenic phenotypes during formation of neointimal lesions, a loss in the expression of α-smooth muscle actin is observed [178]. In our study, evidence was obtained that oxidative stress down-regulated α-smooth muscle actin expression. This phenotypic modulation was also coupled to loss of normal tissue architecture, increased thickness of the vessel wall and increased cell numbers. Collectively, these alterations are consistent with the conclusion that oxidative injury induces atherogenesis in vivo.

Microarray analysis allows for the screening of thousands of genes in a single experiment. This approach presents some pitfalls, mainly due to large data sets that must be extensively mined in order to glean useful information. K-means clustering is a commonly used method for finding relationships in these large data sets. Clustering analysis of the data indicated that allylamine treatment induces oxidative stress within the vessel wall in vivo, and identified distinct, as well as overlapping, transcriptional responses regulated by oxidative stress. These emerging functional relationships are consistent with existing biological knowledge databases [239], and identified novel gene networks involved in the adaptive response of the vessel wall to oxidant injury. Our results suggest that interactive gene networks that contribute to the progression of the atherogenic phenotype include: 1) adaptive response genes; 2) growth regulatory genes,

and 3) matrix-associated genes. These targets were identified based on spatio-temporal profiles of transcriptional expression in stressed versus normal tissue. The adaptive response, a.k.a. "stress response", includes the regulation of genes required for detoxification of reactive oxygen species. Initiation of oxidative stress is due to poor maintenance of the redox flux following peroxidative injury, and uncoupling of the respiratory chain during adaptation to the oxidant injury response [240]. Following oxidant injury, the differential regulation of growth regulatory and matrix-associated genes is likely part of the regulatory cascade that culminates in induction of atherogenic phenotypes [241]. Thus, if the role of interactive gene networks in a multi-factorial disease process such as atherosclerosis is to be elucidated, gene regulation networks involved in such diverse functions as growth control, matrix protein synthesis and deposition, and apoptosis must be considered.

In order to validate our genomic findings, and to define the molecular bases of the atherogenic response, OPN and α_1 integrin levels in vivo were probed at the protein level. The levels of these proteins were significantly increased in stressed vessels compared to controls. OPN is a secreted acidic phosphoprotein involved in ECM/integrin interactions and proliferative control of vSMCs [167]. Elevated levels of OPN have been observed during neointimal formation and atherosclerotic disease [162]. Because the proliferation of vSMCs is one of the key features of atherogenesis, OPN is an ideal marker of modified vSMC phenotypes. However, it is not yet clear if OPN expression is a cause, or a result, of atherosclerosis.

α_1 integrin expression was modulated by oxidative stress. Integrins are a family of heterodimeric transmembrane glycoproteins composed of α and β subunits that allow cells to identify changes in ECM composition and activate intracellular signaling pathways [181]. ECM remodeling, a critical step in the pathogenesis of atherosclerosis, is highly dependent on the expression of matrix components and their receptors. For instance, α_1 integrin is a collagen/laminin receptor implicated in cellular proliferation, abnormal laminin deposition and inflammatory responses [163]. In inflammation, the α_1 integrin is responsible for monocyte adhesion to damaged tissue. Blockade or deletion of the α_1 integrin inhibits accumulation of matrix proteins, and may be of therapeutic value in the management of inflammatory disorders [182]. Recent reports from several laboratories, including our own, have demonstrated that cellular adhesion molecules provide specificity for signaling during the atherogenic response. Although integrins independently afford intracellular signaling, it is the interaction of the ECM with the integrins that provides crucial signaling steps in the progression of proliferative phenotypes in atherosclerosis. $\alpha_1\beta_1$ integrin is the only collagen receptor that can activate the Shc/Grb2 pathway. α_1 integrin is unique in that it plays a central role in Shc mediated proliferation in response to changing conditions in the ECM, thus providing an autocrine pathway for proliferation [242]. Its role in oxidative injury-induced atherogenesis is intriguing and worthy of further investigation.

A change in vSMC phenotype is marked in part by differential regulation of alpha tropomyosin. The alpha-tropomyosin (Tpm1) gene generates different mRNAs that encode for striated and smooth muscle isoforms by alternative splicing [243].

Tropomyosin is a component of the contractile mechanism of the cell, and is present in both stress fibers and thin filaments. Changes in the tropomyosin profile of the cell would thus be indicative of a change in the morphology and functional characteristics of the cell. Tissue-specific regulation and developmental expression of smooth muscle cells is regulated by alternative splicing of duplicated isotype-specific exons [243]. The expression of an alternate form of the isoform for gene Tpm1 in vSMCs may be partially responsible for a shift from contractile to proliferative phenotypes [185]. In keeping with this observation, evidence was obtained that oxidative injury increases the levels of Tpm1within the vascular wall. Interestingly, Tpm1 expression was mainly localized to vSMCs in close proximity to the advential side of the vessel wall. While the significance of this finding is unknown, it suggests that the response of vSMCs within the vessel wall to oxidative injury is highly heterogenous. The localization of Tmp1 mRNA is intriguing in light of evidence implicating the adventitia in neointima formation in the rat carotid injury model [186].

Of interest was the finding that oxidative injury in vivo increased the expression of LINE. LINE (Long Interspersed Nuclear Elements) encodes a reverse transcriptase and perhaps other proteins [187]. Recent studies in this laboratory have shown that activation of LINE expression in vSMCs is mediated by activation of redox-regulated transcription factors [189]. As such, it has been hypothesized that activation of retrotransposons following oxidative injury may disrupt genomic stability and therefore, explain some of the phenotypic changes observed following oxidative injury [187,189].

In addition to causing cellular damage, low levels of ROS can initiate gene transcription [244]. Gene transcription involves the process of constructing a messenger RNA molecule using a DNA molecule as a template, with resulting transfer of genetic information to the messenger RNA.. Transcription is the first of the two-step protein biosynthesis process. Transcription is an enzymatic process that reads DNA and transcribes it into its complementary RNA sequence. Transcription ultimately leads to DNA translation. There is no proofreading or correction process; therefore, it has a lower copying fidelity than DNA replication. Transcription proceeds in a downstream direction (5' toward 3').The transcription process is divided in 3 stages: initiation, elongation and termination. In the simplest of terms, gene expression is the manifestation of the cellular genotype into a phenotype. This complex process involves the execution of the instructions held within the sequence of nucleotides making up the genome, namely transcription of the genes, translation of the mRNAs and function of the protein product. The study of gene expression is of prime importance to elucidate the nature and order of molecular genetic events and processes in cells which ultimately allow it to survive and propagate, and is an essential part of utilizing the genome sequence information [245]. Here we describe aspects of a redox-activated transcriptional mechanism that mediates expression of the OPN gene. The importance of redox mechanisms in regulating the expression of OPN is suggested based on the ability of hydrogen peroxide to activate gene expression, and the ability of antioxidants to completely ablate gene expression. The data summarize here indicate that transcriptional mechanisms represent an important molecular mechanism in the regulation of OPN under imposed by cellular

stress conditions. Analyses of deletion mutants of the OPN promoter defined the 5′ end of the OPN promoter as essential for inducibility by oxidizing agents. Western blot analysis and real-time PCR showed that treatment of vSMCs with different oxidants increased the expression of OPN and that this induction response is mediated by oxygen-derived free radicals. Together, the data indicate that regulation of OPN by oxidizing agents is a complex process that requires both NF-κB and TIEG-1, and that these binding sites act in concert to mediate the cellular response to oxidative stress.

It should be noted, however, that OPN is highly phosphorylated and glycosolated protein and these modifications are responsible for many aspects of the regulation of receptor recognition and affinity [246]. Neame and Butler have shown have shown that osteopontin is a multiply-phosphorylated glycoprotein which contains an RGD cell adhesion sequence and regions containing a high level of aspartic acid. Using mass spectrophotometry to analyze peptides, eleven sites were found that were variably phosphorylated [247].

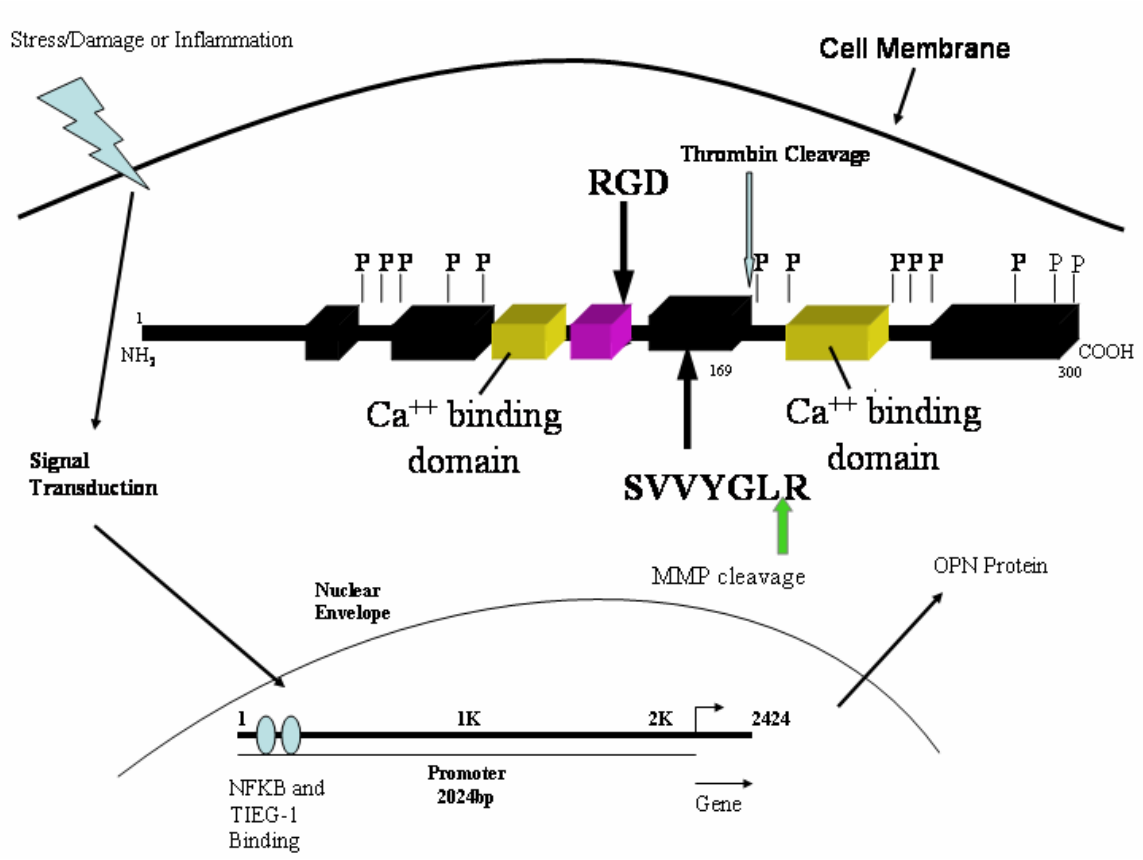


Figure 22. Promoters: where genes meet the environment.
Promoters make appropriate adaptive changes in gene expression to maintain cellular homeostasis

The form and function of a cell are determined largely by the control of relative expression of precise sets of genes at precise times in its development, and in precise positions within the tissues. The activity of a gene is mainly determined by the regulation of transcription initiation and not the rate at which transcription proceeds along, which is largely constant for all gene transcription. Evidence for this level of control comes from direct measurements of transcription from specific genes and of quantitative levels of transcription products. The activity of the gene promoter determines the abundance of the corresponding mRNA. The number of molecules of a

particular mRNA sequence is determined by the efficiency of the promoter and activating factors in assembling the initiation complex with RNA polymerase II. This determines how many transcripts are derived from the gene at any given point in time [248].

To this end, the Ramos laboratory has begun to identify "key" genes that are predictive of genomic behavior of disease phenotypes. Since the development of microarray technologies in the mid 1990's, there has been an enormous increase in gene expression data from many different model systems and organisms that is available to researchers. One problem that has arisen from increases in the amount of expression data is how to make sense of what is happening at the level of your gene of interest in a large 'sea' of genes. Emerging methodology is being designed to reconstruct how genes interact within the genomic context, and methods and algorithms are being developed that can best represent the intricate interconnections that exist between genes. The ability to create gene networks from experimentally-derived data facilitates a systematic review of function at the genetic and molecular level of the cells. In short, gene networks need to be developed that accurately describe these interactions. To this end, methodology has been applied to describe the co-expression of genes coupled to additive probabilistic relationships to identify gene sets that are predictive of the complex and intertwined biological relationships that exist in situ.

Of relevance to the work presented here is that in large scale computational genomic studies OPN has emerged as a central node in regulation of the cellular response to stress [249]. Several other methods of creating gene networks have also

identified OPN as a key gene [250,251]. OPN gene regulatory networks were verified via literature searches as having experimentally-documented interactions. It should come as no surprise that OPN emerged as a key predictor, especially when considering that OPN is seen as a target gene in many disease states such as arthritis, cancer, diabetes, and atherosclerosis.

As noted previously, oxidative stress and the mechanisms and pathways affected by it may be a common denominator that allows for OPN to be an effective predictor of, and a central target of gene behavior. Collectively, our findings emphasize the importance of oxidative stress in atherogenesis. A greater understanding of the underlying mechanisms and genetic networks responsible in the generation of ROS, along with a more detailed characterization of the signaling and transcriptional pathways affected by oxidative stress will, without a doubt, lead to the identification of novel molecular targets and ultimately more effective therapies for the reduction of ROS-induced vascular disorders.

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VITA

NAME: Charles Randal Partridge

EDUCATION Bachelor of Science (1994)
Major: Biology
Minor: Chemistry
Midwestern State University
Wichita Falls, Texas

SELECTED PUBLICATIONS

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