DIFFERENTIAL EXPRESSIONS OF CELL CYCLE REGULATORY PROTEINS AND ERK 1/2 CHARACTERIZE THE PROLIFERATIVE SMOOTH MUSCLE CELL PHENOTYPE INDUCED BY ALLYLAMINE

A Dissertation

by

SARAH ANNE LOUISE JONES

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

August 2003

Major Subject: Veterinary Physiology

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Approved as to style and content by:

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August 2003

Major Subject: Veterinary Physiology

ABSTRACT

Differential Expressions of Cell Cycle Regulatory Proteins and ERK 1/2 Characterize the Proliferative Smooth Muscle Cell Phenotype Induced by Allylamine. (August 2003)

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Chronic oxidative injury by allylamine induces proliferative vascular smooth muscle cell (vSMC) phenotypes in the rat aorta similar to those seen in rodent and human atherosclerotic lesions. In this study, we evaluate the potential role of cyclin dependent kinase inhibitors, p21 and p27, and extracellular regulated kinases (ERK1/2) to mediate the proliferative advantage of oxidatively stressed (*i.e.* allylamine injured) vSMC. Isolated rat aortic SMC from allylamine treated and control rats were cultured on different extracellular matrix (ECM) proteins. Following mitogen restriction, cultures were stimulated with serum with or without inhibitors of NF-κB or MEK. Western blot analysis was performed to identify protein differences between treatment groups. Basal levels of p21 were 1.6 fold higher in randomly cycling allylamine cells than control counterparts seeded on a plastic substrate, a difference lost when cells were seeded on collagen. p27 levels were comparable in both cell types irrespective of substrate. Basal levels of p21 and

p27 were 1.4 fold higher in G_0 synchronized allylamine cells compared with G_0 synchronized control cells seeded on a plastic substrate. Following cell cycle progression, differences in protein levels were not detected. Treatment with 100 nM pyrollidine dithiocarbamate (PDTC) resulted in significant decreases in p21 and p27 in allylamine cells versus control cells following serum stimulation for 9 hours. This decrease was even greater for p21 in allylamine cells when grown on collagen relative to control cells. Alterations in peak and temporal activation of ERK1/2 were observed in allylamine cells seeded on a plastic substrate as compared to control cells, following serum stimulation. Seeding on collagen decreased the enhanced peak phosphorylation of ERK1/2 and increased the sustained activity in allylamine cells compared with control counterparts. Inhibition of ERK1/2 activity resulted in reduced p21 expression in both cells types, but the response was markedly enhanced in allylamine cells, and preferentially observed on a restrictive collagen substrate. We conclude that induction of proliferative (*i.e.* atherogenic) phenotypes following repeated cycles of oxidative injury involves ERK1/2 activity and modulation of the cyclin dependent kinase inhibitors, p21 and p27, in a matrix-dependent manner.

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CHAPTER I

INTRODUCTION

1.1. Atherosclerosis

Atherosclerosis or " hardening of the arteries" is a systemic disease involving all of the major arteries supplying the heart, brain, kidneys and extremities. In the United States and most other Western countries, atherosclerosis is a leading cause of illness and death with carotid and coronary artery involvement resulting in strokes and heart attacks, respectively [1]. Atherosclerosis is a disease of the arterial intima resulting in the formation of atheromatous plaques followed by stenosis and occlusion of the vessel lumen. Development of atherosclerosis involves multiple changes in the vascular wall, including damage to endothelial cells and vascular smooth muscle cells (vSMC), influx of inflammatory cells to the damaged site, release of inflammatory mediators within the vessel wall, proliferation of vSMC in response to mediators, and accumulation of lipids and matrix proteins. While the contributing cell types and characteristics of the plaque are well characterized, molecular mechanisms responsible for the initiation and propagation of these pathologies remain elusive. Much of the early research in atherogenesis revolved around the cholesterol theory, or hypercholesterolemia, as the major risk factor in the development of atherosclerosis [2,3]. Elevated serum cholesterol, due to

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The journal used as a model for this dissertation is Cardiovascular Research.

consumption of diets rich in lipids or due to a hereditary defect of lipid metabolism, results in intracellular accumulation of lipids and formation of atherosclerotic plaques. Experimentally, increasing plasma cholesterol levels through dietary manipulation or utilizing naturally defective or genetically modified animals with alterations in lipoprotein metabolism have been reliable methods for induction of atherogenesis [2]. More recently, however, evidence for involvement of numerous factors, other than hypercholesterolemia, has been revealed [3,4]. It is now believed that there are multiple factors contributing to atherogenesis including serum cholesterol levels, hypertension, aging, obesity, oxidative stress, inflammation, cigarette smoking and other emerging risk factors.

Beyond cholesterol, there have been two major theories of atherogenesis, both originally proposed in the early '70s. The response-to-injury hypothesis involves injury to the endothelial cells lining the vessel lumen [5]. The endothelium is complex and dynamic responding to environmental stimuli and activating vasoactive substances that mediate vascular tone, structure and function, and influence vSMC growth and differentiation [6]. It is known that de-differentiation of vSMC from native contractile phenotypes to highly proliferative and synthetic phenotypes is a key step in atherogenesis. The release of growth factors and cytokines from injured endothelial cells and migrating inflammatory cells, such as monocytes and lymphocytes, promotes the proliferation of vSMC and alteration of extracellular matrix protein secretion [7].

The second hypothesis introduces atherosclerosis as a carcinogenic process whereby the atherosclerotic plaque is a benign neoplasm of the vessel wall stemming from the monoclonal expansion of a single mutated vSMC [8]. In this case, genetic reprogramming of vSMC is a hallmark of atherogenesis following injury or toxic insult. It is difficult to separate these hypotheses, as most likely it is a combination of events that ultimately leads to atherosclerosis. It is expected that if endothelial injury resulted in aberrant vSMC proliferation, following repair of the injury or removal of the injurious insult, the vessel wall would return to normal. However, this does not occur spontaneously supporting the progression of atherosclerosis as genetic reprogramming within the vSMC. As well, altered vSMC proliferation can occur *in vitro* in the absence of endothelial cells, macrophages and other non-cellular factors, implying that endothelial injury alone is unlikely to be the sole factor in atherogenesis. Aberrant vSMC proliferation, however, appears to be a consistent abnormality in atherogenesis.

1.2. Vascular Smooth Muscle Cell Growth and Differentiation

VSMC exist in a diverse range of phenotypes. In normal mature blood vessels, vSMC function to regulate vessel diameter and blood flow. In order to provide this function, the differentiated, or contractile, phenotype of vSMC is predominant. During vascular remodeling, vSMC alter their phenotype to a non-contractile or synthetic state resulting in generation of intimal vascular lesions [9]. These de-differentiated, or synthetic, vSMC have reduced expression of proteins

required for muscle cell contraction and have increased synthesis of extracellular matrix proteins. Extracellular matrix proteins such as fibronectin and laminin are known to help control the growth and phenotype of vSMC, with differential effects observed on these growth matrices [10]. Cell adhesion to fibronectin supports cell proliferation and the activation of focal adhesion kinase (FAK), while cell adhesion to laminin does not. Also, cell growth on fibronectin results in proliferation and low expression levels of smooth muscle myosin, whereas growth on laminin slows proliferation and increases expression levels of smooth muscle myosin. These findings indicate that integrin signaling pathways have a profound effect on vSMC proliferation and phenotype.

Several factors contribute to phenotype, growth, proliferation and migration of vSMC [11,12]. Those to be discussed in detail include integrin- and growth factor- mediated signaling and the coordinated effort of both signaling pathways.

1.3. Integrins and Integrin Mediated Signaling

Integrins are a large family of heterodimeric transmembrane receptors that link the extracellular matrix to the cytoskeleton. Integrin function is to integrate the

extracellular matrix to the interior cytoskeleton of the cell, hence the name. Integrins consist of noncovalently bound α- and β-subunits. To date, there exist 24 receptors, which are the result of heterodimeric combinations of α- and β-subunits. Currently, 18 α- and 8 β-subunits have been identified [13]. Each of the α- and β-subunits contains a large extracellular domain, a single membrane spanning region and a short cytoplasmic domain (Figure 1) [14]. The extracellular domains of the α and β subunits are non-covalently linked to form a globular head domain that binds specific components of the extracellular matrix with ligand specificity determined by a particular combination of α- and β- subunits [15]. Cytoplasmic domains of integrin subunits, particularly α-subunits, display highly divergent sequences allowing each αβ heterodimeric combination to have its own signaling properties [16,17]. For example, $\alpha_v\beta_3$ integrin interacts with a variety of extracellular matrix ligands, including vitronectin, fibronectin, and osteopontin (OPN); while $\alpha_5\beta_1$ shows ligand specificity exclusively for fibronectin; and $\alpha_1\beta_1$ has predominant ligand specificity for collagen [18].

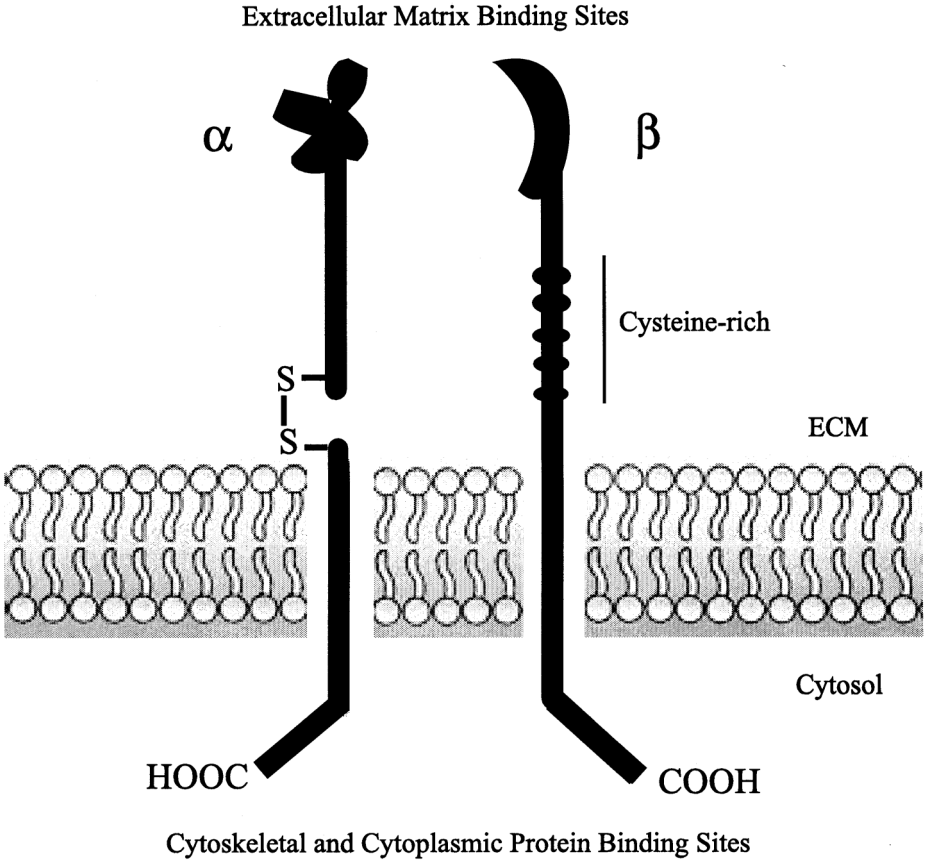

Figure 1: Subunit structures of a representative mammalian integrin receptor (fibronectin receptor). Following extracellular matrix recognition, the α and β subunits noncovalently bind forming a globular head structure. Within the cytosol, at the C-terminus of the protein, binding sites exist for cytoskeletal and cytoplasmic proteins. Once bound to cytoplasmic structures, further protein recruitment and phosphorylation cascades occur resulting in intracellular transmission of signals from the external cellular environment (ECM – extracellular matrix).

Following cellular adhesion and clustering, integrins recruit cytoskeletal and cytoplasmic proteins, allowing anchorage of the newly formed complexes to the

actin cytoskeleton. Local remodeling of the actin cytoskeleton leads to formation of focal adhesions. Focal adhesions not only supply a structural link between the extracellular matrix and the cytoskeleton, they are also important sites of signal transduction. Integrins, as a class of signaling receptors, do not share the typical kinase or phosphatase domains or G protein association that other signaling receptors have [19-21]. However, signal transduction properties of integrins are well established. It has been proposed that integrin signaling may occur through signal transmission during cytoskeletal reorganization, or it may in fact occur through protein phosphorylation cascades similar to other signaling receptors. Recent evidence points to the presence and contribution of both pathways, either alone or in combination, during integrin signal transduction [22,23].

There are many transmembrane proteins, including growth factor receptors, that interact with integrins [14,24]. The interaction of integrins with growth factor receptors may aid in recruitment of receptor clustering, may delay internalization of the receptor following ligand binding, or may prevent dephosphorylation thereby sustaining or augmenting receptor signaling efficiency [25]. It is clear that cell survival and proliferation require interaction and adhesion with the extracellular matrix. Neoplastic cells are altered to such an extent that they no longer require anchorage to the extracellular matrix for cell survival [13,15,26]. It is the coordinated efforts of integrin- and growth factor- mediated signaling that modulate cell cycle progression and thus cell growth and differentiation [15,27,28].

There are also many cytoplasmic proteins that associate with the cytoplasmic domains of integrins and with the focal adhesion complex. Many of these proteins are involved in downstream signaling cascades important in cell cycle progression, including Shc, Grb 2 and several protein kinases [14,29]. Of considerable importance is the presence of a specific tyrosine kinase known as pp125 focal adhesion kinase (FAK) that is localized to focal adhesions [30,31]. FAK becomes phosphorylated and activated when ligands bind to integrin receptors. Phosphorylated FAK then binds and activates downstream signaling cascades including Src, Grb2, and PI3-kinase. In addition to FAK, several other protein kinases participate in integrin-mediated signaling, including protein kinase C (PKC) and extracellular regulated kinases (ERK(s)) [32,33]. It is interesting to note that activation of ERK through integrin signaling is independent of ras which reveals an alternate ERK activation pathway from tyrosine kinase receptor activation following growth factor binding [34].

1.4. Extracellular Regulated Kinases (ERKs)

Intricately involved in regulation of vSMC phenotype is the activation of ERKs [35]. Alterations in intracellular signaling cascades following ERK activation are known to play a vital role in cell growth, differentiation and proliferation through modulation of gene expression. ERKs are known to be activated by growth factor binding to tyrosine kinase receptors as well as ligand binding to integrins [15,22,26,35]. The phenotype of vSMC is also largely dependent on the interaction of the cell with extracellular matrix proteins and extracellular environment.

Attachment of vSMC to fibronectin, an arginine-glycine-aspartate (RGD) sequence containing protein, promotes conversion of contractile vSMC to proliferative phenotypes [36]. As in most other cell types, modulation of vSMC growth and proliferation is highly dependent on ERK signaling [24]. Zhu and colleagues have demonstrated that the kinetics of ERK activation by growth factors differ from those observed following integrin signal transduction [37], perhaps indicating that discrete differences exist in intracellular signaling mediators and in nuclear translocation of activated ERKs.

Figure 2 outlines the proteins involved in activation of ERKs resulting in nuclear translocation where interaction with a number of transcription factors modulating gene expression occurs. Ligand binding to the growth factor receptor results in tyrosine kinase receptor dimerization and autophosphorylation. This phosphorylation event causes the activation of several intracellular signaling cascades including diacylglycerol (DAG), inositoltriphosphate (IP_3), PKC and the ras/raf/MAP kinase kinase (MEK) pathway leading to ERK activation.

Recent evidence has shown that not only is ERK activation pivotal to cell growth and differentiation, but prolonged ERK activation via integrin signaling (in the absence of mitogen stimulation) is associated with phenotypic modulation of vSMC when grown on fibronectin [11]. This prolonged ERK activation is also associated with increased nuclear translocation of activated ERK. As well, it has been shown that prolonged ERK activation alters cell cycle progression through modulation of cyclin D1 activity in late G_1 phase of the cell cycle [38-41].

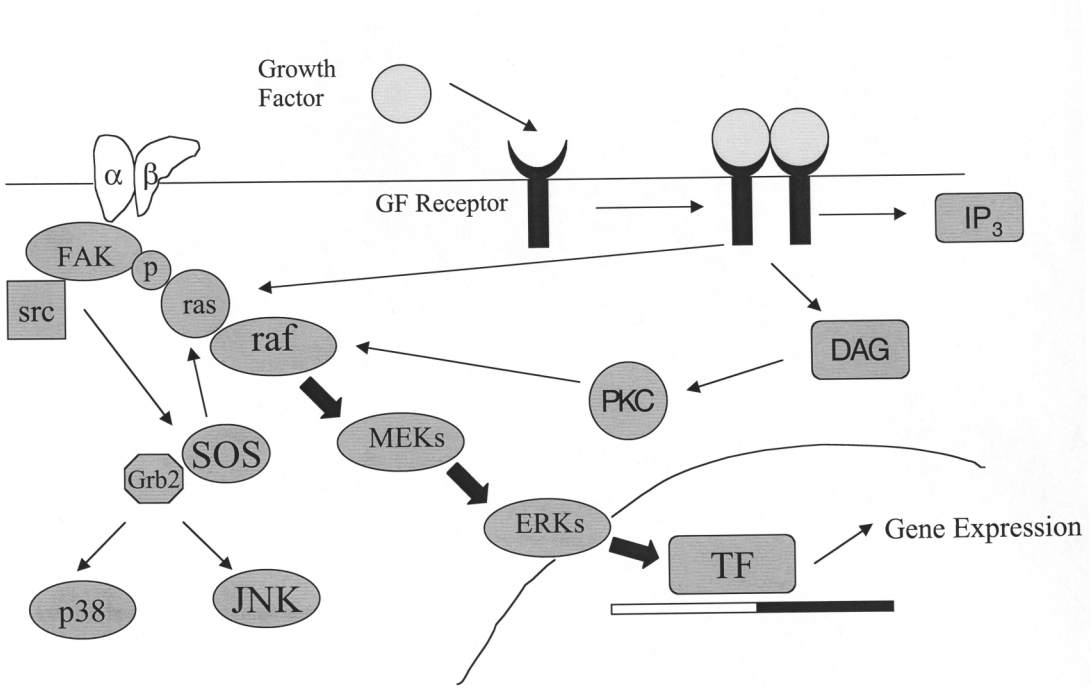

Figure 2: Activation of intracellular signaling cascades through integrin/extracellular matrix and growth factor receptor signaling. Growth factor binding to its receptor causes receptor dimerization and autophosphorylation, which activates a variety of signaling pathways including IP_3, DAG, PKC and activation of ras. Ligand binding to integrin receptors results in phosphorylation of FAK and the association of FAK with src. These phosphorylated complexes provide sites of attachment for Grb-2/SOS, a complex that initiates nucleotide exchange of inactive ras-GDP to active ras-GTP. Ras-GTP activates raf to begin a phosphorylation cascade involving MAP kinase kinases (MEKs) and ERKs. Once activated, ERKs translocate to the nucleus to activate various transcription factors (TF) modifying gene expression.

1.5. Cell Cycle Regulation

Modulation of cell cycle proteins is the final common pathway of cell mitosis, growth arrest, or apoptosis. There are several checkpoints in the cell cycle that allow a cell to pass through a quiescent state to mitosis and division (Figure 3). Within the G_1 phase of cell growth, the cell is preparing for DNA synthesis, a necessary step for cell division. Cyclins D and E and the cdk inhibitors p27 and p21

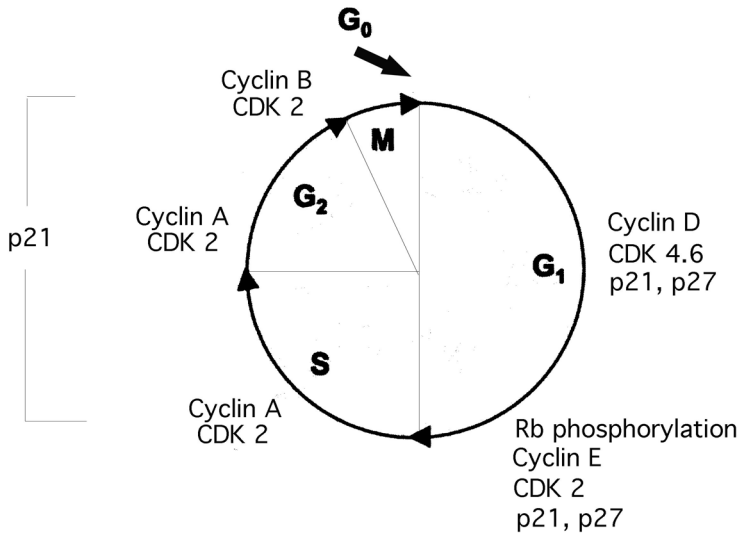

Figure 3: Phases of the cell cycle. Schematic representation of the cell cycle with notation of key regulatory proteins and the phase(s) in which they exercise their regulation.

are important components of the cellular machinery necessary for cells to proceed through G_1 to S phase. Alterations in the expression of cell cycle proteins are also

elicited by both growth factors and adhesion to the extracellular matrix [41-45]. Elevations in cyclin D1 levels are dependent on prolonged ERK1/2 activation in adhered cells [41]. Following cyclin/cdk complex formation, the phosphorylation status of key proteins, including the retinoblastoma protein (Rb), becomes altered allowing the cell to pass into S phase.

Integrins and growth factor receptors cooperate at several levels to ensure the proper control of cell proliferation. Both growth factor receptor binding and cell adhesion are required for transmitting signals to the ras/raf/MEK/ERK signaling pathway. Activation and nuclear translocation of ERKs result in an increased cyclin D1 transcription, and requires both growth factor- and integrin-mediated signal transduction. Cyclin D1 expression induces the activation of cdk4/6 kinases. Typically, the presence of the cdk inhibitors, p27and p21, inhibit cyclin D1/cdk4/6 complex formation thereby inhibiting progression of the cell through G_1 [13]. Integrin receptor activation functions to stimulate a degradation pathway that specifically degrades the negative cell cycle regulators p27 and p21, allowing the activation of cyclin E-cdk2 complexes. Activation of both cdk4/6- and cdk2-cyclin complexes are required for hyperphosphorylation of the retinoblastoma protein (Rb), causing the release of the transcription factor, E2F, which is required for increased transcription of cyclin A. Cyclin A-cdk2 complexes are required for entry into S phase (Figure 4) [13]. Through this coordinated effort of integrin- and growth factor-mediated signaling, a cell may progress through G_1 to S phase.

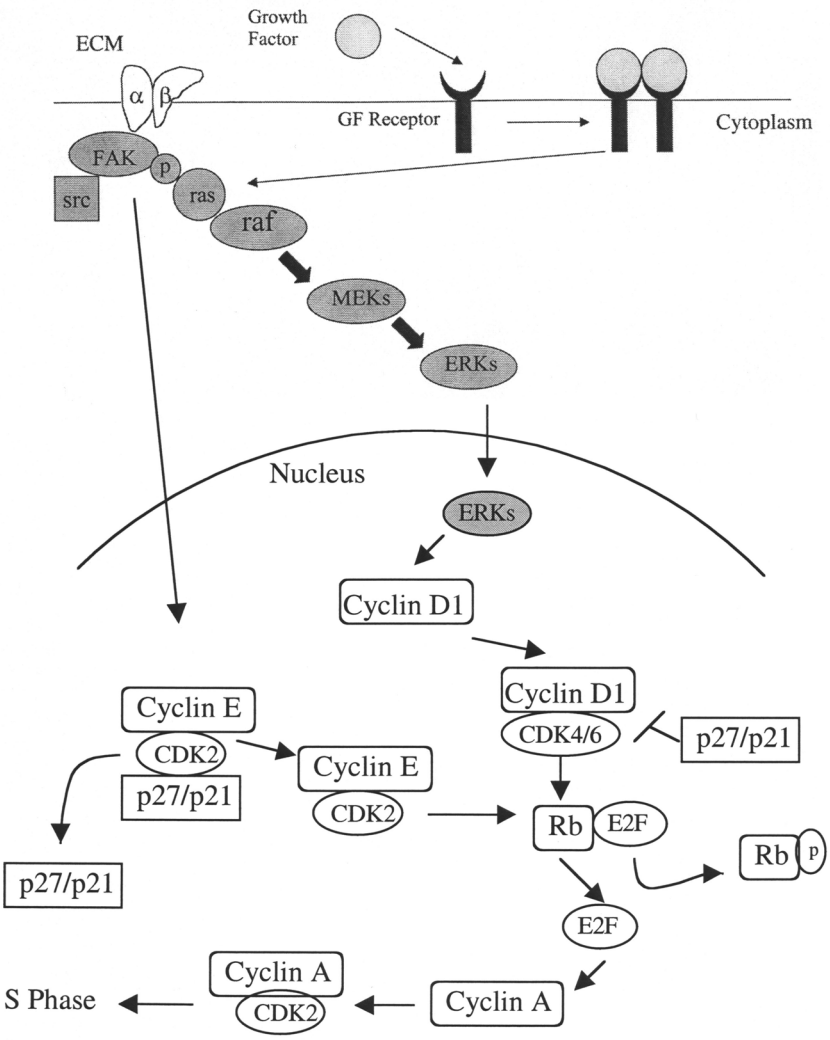

Figure 4: ERK regulation of cell cycle proteins following integrin/extracellular matrix and/or growth factor receptor activation. Cyclin D1 levels increase following nuclear translocation of activated ERKs. Complex formation between cyclin D1 and cdk4/6 results in phosphorylation of the retinoblastoma protein (Rb) which results in release of the transcription factor E2F. Binding of E2F to its DNA binding site results in increased transcription of cyclin A, which ultimately allows progression to S phase. Integrin activation also results in degradation of p27/p21 proteins that are bound to cyclin E, activating the cyclin E-CDK2 complex, which further phosphorylates Rb. Presence of p27 and p21 inhibits cell cycle progression through inactivation of cyclin/ CDK complexes.

Typically, cdk inhibitors inhibit the activity of cyclin/cdk complexes thereby negatively regulating cell cycle progression. [46]. However, at least one of the cdk inhibitors identified, p21, plays a dual role functioning as either an assembly factor, or an inhibitor, depending on the stoichiometry of the cyclin/cdk/p21 complex [47]. At low and intermediate concentrations, p21 functions as an assembly factor, while at higher concentrations it acts as an inhibitor of cell-cycle progression. Recently, it has been shown that in vSMC p21 is necessary for PDGF-mediated vSMC proliferation [48], thereby providing a framework for a dual role of p21 as both a growth promoter and inhibitory factor.

1.6. Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS) are highly reactive molecules, relative to molecular oxygen, that are generated during cell metabolism as partially reduced metabolites of oxygen. Many ROS possess unpaired electrons and thus are free radicals. These include molecules such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$) and lipid radicals. Other ROS, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl), are not free radicals but contribute to oxidant stress [49]. The production of one ROS may lead to the production of several others through radical chain reactions often involving lipid molecules in the cell membrane. Lipid radicals produced through chain reactions, and other ROS, affect cellular function in many ways including acting as cytotoxic

and mutagenic compounds and influencing intracellular signaling cascades that modulate cell growth, differentiation and survival [3]. One of the well known effects of ROS is the induction of oxidative damage to DNA resulting in strand breakage and base and nucleotide modification [50]. This ultimately may result in genetic mutations promoting the development of genotypically altered cells giving rise to the benign neoplasm theory of atherosclerosis.

Potential enzymatic sources of ROS include mitochondrial respiration, arachidonic acid metabolizing enzymes (lipoxygenase and cyclooxygenase), cytochrome p450s, xanthine oxidase, NAD(P)H oxidases, and NO synthase [49,51]. Along with enzymatic sources, ROS can also be produced exogenously from environmental chemicals, radiation, and ultraviolet light. ROS have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipid, proteins, and DNA. However, within the last few decades, accumulating evidence suggests that ROS are essential messengers in cell signaling and regulation [52]. It is essential, therefore, that ROS are maintained in a balanced fashion so as to avoid disruption of the physiological functions of ROS by pathological outcomes.

Fortunately, cells possess several antioxidant defense mechanisms that combat the excessive accumulation of ROS [53]. Antioxidants either detoxify ROS (glutathione (GSH) peroxidase, thioredoxin, superoxide dismutases, catalase), block free radical chain reactions (vitamins A and C) or bind transition metals (transferrin). Among the most important antioxidants is GSH (L-gamma-glutamyl-L

cysteinylglycine), which reduces peroxides by the action of glutathione peroxidase and is generated by NADPH-dependent glutathione reductase. Small changes in the ratio of GSH to GSSG (GSH disulfide) may have significant biological effects [54]. Thus oxidative stress can be defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury. Oxidative stress, or the shift in balance towards increased levels of ROS, has been implicated in a large number of human diseases, including atherosclerosis (Table 1).

Some of the potential targets of ROS are noted in Table 1. Of these targets, ultimate outcomes of ROS activation include cellular growth/hypertrophy, survival or apoptosis. The entire class of MAP kinases, including the stress-activated protein kinases (SAPKs - p38 and c-Jun-terminal kinase (JNKs)) and the extracellular signal-regulated kinases (ERKs) are influenced by ROS with variable outcomes depending on which MAP kinase is activated [55,56]. Akt is a kinase involved in

anti-apoptotic signaling that is regulated by ROS in angiotensin II-stimulated smooth muscle cells [57]. Also, various caspase cascades are activated by ROS with variable outcomes of cell survival or apoptosis [58]. In the above-mentioned examples, the targets outlined may not be direct targets of ROS. It is more likely that increased levels of ROS de-activate tyrosine phosphatases which allow tyrosine kinase activity to prevail leading to phosphorylation of their cellular targets, including the ERKs and SAPKs [59]. One of the final outcomes of intracellular signaling cascade activation is modulation of transcription factor binding to DNA.

NF-κB was the first transcription factor shown to respond directly to oxidative stress [60]. Included among many of the other transcription factors regulated by redox mechanisms are c-Fos, c-Jun, nrf-2 and AP-1 [61]. Many of these transcription factors are involved in cell growth and differentiation. Not only are ROS implicated in cell growth, differentiation, and survival, ROS can also elicit apoptosis and cell cycle arrest in vSMC [62,63]. Therefore, the response of vSMC to ROS is variable with paradoxical effects and is likely dependent on the dose and duration of exposure as well as the modulation of specific molecular targets.

TABLE 1

Sources of ROS, antioxidant defense mechanisms and potential targets.

ROS are endogenously generated inside cells by a variety of enzymes and are increased by exogenous agents. The levels of intracellular ROS are balanced by intracellular antioxidative defense mechanisms that consist of enzymatic and nonenzymatic parts. Potential signaling pathway targets of ROS are shown with cellular outcomes noted (Adapted from [59] and [64]).

Sources of ROS	
Endogenous	Exogenous
NAD(P)H oxidase	Environmental toxins
Mitochondria	Ionizing radiation
Peroxisomes	Ultraviolet light
Cytochrome P450	Electrical fields
Xanthine oxidase	Chemotherapeutics
Cyclooxygenase	Inflammatory cytokines
Lipoxygenase	
NO synthase	

Antioxidant Defense Mechanisms	
Enzymatic	Nonenzymatic
Superoxide dismutase	Glutathione
Catalase	Thioredoxin
Glutathione peroxidase	Vitamins C, A, E
	Transferrin

Targets of ROS		
Growth/Hypertrophy	Survival	Apoptosis
p38	ERKs	JNK
ERKs	Akt	Caspases
Akt	NF-κB	
	Caspases	

During the past decade reduction-oxidation (redox) reactions that generate ROS, including H_2O_2, O_2^- and OH^-, have been identified as important chemical mediators in the regulation of signal transduction processes involved in cell growth and differentiation. ROS ideally fulfill the prerequisites for intracellular signaling molecules since they are rapidly generated, highly diffusible, easily degraded and ubiquitously present in all cell types. For example, H_2O_2 acts as a signaling molecule through induction of the protooncogene c-fos resulting in vSMC growth. It has become clear that ROS stimulate early growth signals typical of mitogens, including induction of c-fos and c-myc mRNA expression [65].

A variety of cytokines and growth factors that bind to receptors of different classes have been reported to generate ROS in nonphagocytic cells. This ligand-mediated, or endogenous, production of ROS appears to regulate a large number of signaling pathways. For example, a number of growth factors that bind to tyrosine kinase receptors have been shown to generate intracellular ROS essential for mitogenic signaling. In smooth muscle cells, ligand binding (eg. platelet derived growth factor (PDGF)) to tyrosine kinase receptors results in the formation of H_20_2 which is required for PDGF-induced tyrosine phosphorylation, ERK activation and DNA synthesis [55]. Also, activation of G-protein coupled receptors, through angiotensin II binding, activates NAD(P)H oxidase dependent ROS production in smooth muscle cells causing activation of ERK and resultant cellular proliferation [56]. However, exogenous ROS may also directly interact and activate cell surface receptors, making kinase and phosphatase receptors targets of oxidative stress [52].

Oxidant stress has recently been evaluated as a key factor in the development of atherosclerosis and other cardiovascular diseases [51,66]. VSMC accumulation and hypertrophy are characteristic of atherosclerotic, restenotic and hypertensive vascular diseases. The net balance between cellular proliferation and apoptosis determines the extent of vSMC growth. Increased levels of ROS have been shown to shift vSMC from a contractile to a proliferative phenotype [9]. Evidence has also been presented that ROS are required for growth factor stimulated proliferation of smooth muscle [67]. The generation of reactive oxygen species (ROS) creates an imbalance in cellular redox status that contributes to induction of proliferative vSMC phenotypes [3]. Through activation of signaling cascades and redox-sensitive transcription factors, many genes with important functional roles in the physiology and pathophysiology of vascular cells are induced/repressed. With ROS having the capacity to activate pathways leading to growth/hypertrophy, cell survival or apoptosis/growth arrest [59], a balance in the production and removal of ROS is necessary to maintain normalcy in the vasculature.

1.7. Modulation of Vascular Smooth Muscle Cells by Allylamine

It is known that catecholamines cause a variety of vascular diseases [68]. Allylamine (3-aminopropene) is an aliphatic amine that is structurally similar to endogenous catecholamines, although much simpler as it lacks a benzene ring. Acting as a selective cardiovascular toxin, allylamine promotes the development of smooth muscle cell lesions similar to those found in atherosclerotic vessels, and has

been characterized as a model for chemically-induced atherosclerosis [69]. Allylamine has been used industrially in the past and has been known to be a cardiac and vascular toxin since the 1940's.

Earlier studies described the vascular lesions caused by allylamine to be similar to early atherosclerosis because of the intimal smooth muscle cell proliferation in smaller and medium-sized arteries [70]. More recent evidence indicates subtle nonlethal changes in the vSMC are mediated through toxic metabolites of allylamine resulting in medial hypertrophy and proliferation of vSMC [69]. Data has now accumulated indicating that allylamine's injurious effects on both vSMC and the cardiac myocyte are dependent upon its deamination by a form of amine oxidase, semicarbazide-sensitive amine oxidase (SSAO), a vascular specific enzyme expressed predominantly in vSMC [71,72]. One of the molecular mechanisms involved in allylamine toxicity is the generation of reactive oxygen species [69]. Oxidative damage to the vascular wall by allylamine is mediated by metabolic activation of the parent compound to acrolein and H_20_2 by SSAO (Figure 5). Both acrolein and hydrogen peroxide promote peroxidative injury, cellular damage and gene deregulation [61,73,74]. Smooth muscle cells derived from aortas of rats chronically exposed to allylamine maintain a proliferative advantage after serial propagation in vitro over cells derived from control rats [69]. It is proposed that phenotypic modulation of oxidatively stressed cells to a proliferative state involves genetic reprogramming of growth signaling induced by oxidative chemical injury.

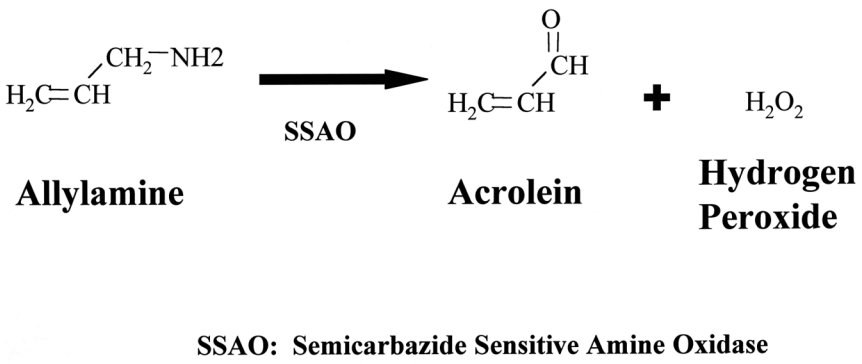

Figure 5: Metabolism of allylamine to acrolein and hydrogen peroxide. Acrolein and hydrogen peroxide induce cellular injury and repair pathways that result in modulation of vSMC from a contractile to a synthetic/proliferative phenotype.

Through activation of signaling cascades and redox-sensitive transcription factors, many genes with important functional roles in the physiology and pathophysiology of vascular cells are induced/repressed. As shown previously in this laboratory, vSMC that have undergone repeated cycles of oxidative chemical injury display increased rates of proliferation compared to controls [75,76]. This proliferative advantage is characterized by altered expression of integrin-associated proteins and extracellular matrix interactions. Previous studies have identified a key difference in secretion and extracellular matrix sequestration of several proteins, including OPN, in oxidatively stressed cells compared to control cells [74,75]. OPN, a secreted protein known to associate with the $\alpha_v\beta_3$ integrin, promotes both SMC attachment and migration [77,78]. When the α_v integrin subunit is blocked, allylamine cells lose their proliferative advantage relative to control cells, suggesting

that integrin mediated signaling may participate in the proliferative regulation of oxidatively stressed vSMC [75].

Along with alteration of OPN in allylamine cells, the transcription factor, AP-1, and NF-κB were increased in allylamine cells [75]. Inhibition of NF-κB by pyrrolidine dithiocarbamate (PDTC) selectively compromised proliferation of allylamine cells, while seeding of allylamine cells on a collagen matrix decreased inducible NF-κB binding to control cell levels following mitogen stimulation [Williams, ES and Ramos, KS, personal communication]. These results further implicate ECM interactions in the deregulation of vSMC proliferation following oxidative injury. Other alterations that are involved in the increased proliferative capacity of allylamine cells include enhanced phosphatidylinositol metabolism [69,79] and increased c-Ha-ras proto-oncongene expression [74,79].

Phenotypic modulation of vSMC by allylamine has been characterized, as outlined above. Yet, intracellular signaling pathways involved in the proliferative phenotype of oxidatively injured vSMC (allylamine cells) have not been clarified. Through evaluation of ERK activation and the expression profiles of cell cycle regulatory proteins, molecular mechanisms contributing to the proliferative vSMC phenotype induced by oxidative stress may be elucidated.

1.8. Research Objectives

The intent of this research is to characterize interactions between oxidative injury and key intracellular signaling pathways that may be involved in integrin-

extracellular matrix (ECM) coupled signaling in modulation of vSMC phenotype. Previous studies in this laboratory have shown a proliferative advantage of oxidatively injured vSMC over control counterparts when grown on a plastic substrate. This advantage is lost when cells are grown on collagen, a growth inhibitory substrate [76]. The intracellular signaling pathways involved in modification of oxidatively stressed cells have yet to be elucidated. Thus, this research will begin to delineate the signaling pathways that contribute to the altered proliferative rate of oxidatively injured vSMC. The hypotheses to be tested include: 1) Oxidatively injured vSMC will exhibit changes in expression of cell cycle regulatory proteins and growth matrix will modulate these alterations. 2) Matrix dependent regulation of phosphorylated extracellular regulated kinases (ERK1/2) will contribute to the proliferative advantage of oxidatively injured vSMC through modulation of cell cycle regulatory proteins. Four specific aims were examined, including:

1. Examination of matrix dependent alterations in the cell cycle regulatory proteins, p27 and p21, in random cycling, G_1 synchronized, and mitogen released allylamine cells compared with control counterparts.

Experiments were designed to determine the basal expression profiles of select cell cycle regulatory proteins in allylamine and control cells. Flow cytometry was utilized to determine cycling percentage of cells in various experimental

setups. The contribution of growth substrate to cell cycle protein expression levels was determined through seeding cells on plastic or collagen growth substrates. Protein levels were identified using SDS-PAGE followed by western blot analysis.

2. Characterization of ERK activation profiles in allylamine versus control cells when grown on both plastic and collagen.

To determine the involvement of the ERK signaling pathway in regulation of the proliferative phenotype of oxidatively stressed cells, experiments were conducted evaluating time dependent mitogen activation of ERKs in allylamine and control cells. Matrix dependency was also evaluated through growth on the different extracellular matrix substrates, plastic or collagen.

3. Determination of the contribution of ERK activation to the altered expression levels of cell cycle regulatory proteins in allylamine cells.

Through the use of the MEK1/2 inhibitor, U0126, alterations in cell cycle regulatory proteins were evaluated following inhibition of ERK activation. These studies were designed to assess the contribution of ERKs to differential cell cycle regulatory protein expression profiles in oxidatively stressed vSMC.

4. Investigation of the role of antioxidant administration (PDTC) in the alteration of cell cycle regulatory proteins.

The cell model utilized in these experiments is thought to result in atherogenesis due to oxidative stress following allylamine metabolism to acrolein and H_2O_2. Allylamine and control cells were grown in the presence and absence of PDTC to determine if this antioxidant would normalize cell cycle regulatory protein expression in oxidatively stressed vSMC.

CHAPTER II

MATERIALS AND METHODS

2.1. Reagents

Cell culture reagents were purchased from Gibco/Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Products from Sigma (St. Louis, MO) include NaCl, NaF, EDTA, Na pyrophosphate, Na orthovanadate, glycerol, and PDTC. Protease inhibitor cocktail, phosphatase inhibitor cocktail, dithiotreitol and propidium iodide were obtained from Calbiochem (San Diego, CA). RNase A was purchased from Worthington Biochemicals, (Lakewood, NJ). Bovine serum albumin (BSA) was purchased from Amersham Biosciences; USBiochemical (Piscataway, NJ). Control cell lysates including A431 EGF stimulated cell lysate and 3T3 nonstimulated cell lysate were purchased from Upstate Biotechnologies (Lake Placid, NY). U0126 (MEK1/2 inhibitor) was purchased from New England Biolabs (Beverly, MA). Supersignal®, Superblock® and Restore® were purchased from Pierce Chemical (Rockford, IL). Type I collagen (rat tail collagen) coated plates were obtained from Becton Dickinson (Franklin Lakes, NJ). All other reagents for SDS-PAGE and western blotting were from Bio-Rad (Hercules, CA).

Primary antibodies were purchased from New England Biolabs, Beverly, MA (rabbit polyclonal anti-phosphorylated ERK1/2), Upstate Biotechnologies, Lake Placid, NY (rabbit polyclonal anti-total ERK1/2), Advance Immunochemical

Incorporated, Long Beach, CA (mouse monoclonal anti-GAPDH) and Santa Cruz Biotechnologies, Inc., Santa Cruz, CA (rabbit polyclonal anti p21, p27, and cdk2). Secondary antibodies used were donkey anti-rabbit and donkey anti-mouse horseradish peroxidase conjugated IgGs obtained from Jackson (West Grove, PA).

2.2. Cell Culture

Vascular smooth muscle cells were isolated by successive enzymatic digestion of the aortas from adult male Sprague-Dawley rats gavaged with allylamine-HCl (70 mg/kg- allylamine cells) or tap water (control cells) daily for 20 days as previously described [69]. This dosing regimen is associated with permanent modulation of vSMC toward a highly proliferative, mitogen-responsive state. Subcultures were prepared by trypsinization of subconfluent cultures and maintained in Medium 199 supplemented with 10% FBS, and 1% penicillin/streptomycin in 5% CO_2:95% air at 37°C. Cell cultures between passage levels 20-24 were used in all experiments.

2.3. U0126 (MEK1/2 Inhibitor)

Cell cultures were grown to 70% confluence at which time they were washed twice with 1X phosphate buffered saline (PBS) followed by mitogen restriction (0.5% FBS in Medium 199) for 48 hours. Serum restricted cultures were then pretreated with U0126, an inhibitor of MEK1/2 activation (25 μM), in media containing 0.5% FBS for 30 min, followed by addition of media containing 10%

FBS and various concentrations of U0126 for 9 hr. At this time, cultures were harvested for protein extraction followed by SDS-PAGE/Western blot analysis.

2.4. Pyrrolidine Dithiocarbamate

Cell cultures were grown to 70% confluence at which time they were washed twice with 1X phosphate buffered saline (PBS) followed by mitogen restriction (0.1% FBS in Medium 199) for 48 hours. Synchronized cultures were then pretreated with PDTC (25 or 100 nM) in media containing 0.1% FBS for 2 hr, followed by addition of media containing 10% FBS and various concentrations of PDTC, an inhibitor of NF-κB activation [80], for 9 hr. At this time, cultures were harvested for protein extraction followed by SDS-PAGE/ Western blot analysis.

2.5. SDS-PAGE and Western Blot Analysis

Cells were lysed in buffer containing 20 mM Tris-Cl (pH 7.4), 50 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L EDTA, 20 mmol/L Na pyrophosphate, 1% Triton X-100, protease inhibitor cocktail containing 0.5 mmol/L AEBSF (4-(2-aminoethyl)benzene-sulfonylfluoride HCl), 150 nmol/L aprotinin, 2 μM E-64 protease inhibitor, 0.5 mmol/L EDTA and 1 μmol/L leupeptin and phosphatase inhibitor cocktail containing 2 mmol/L imidazole, 1 mmol/L NaF, 1.15 mmol/L Na molybdate, 1 mmol/L Na orthovanadate and 4 mmol/L Na tartrate dihydrate. The lysate was frozen, thawed and cleared by centrifugation. Protein concentration was determined using a BCA protein assay (Pierce Chemical, Rockford, IL).

Prior to electrophoresis, protein (2-10μg) was added to 2X sample buffer (0.5 mol/L Tris-Cl (pH 6.8), 75% glycerol, 50 mmol/L dithiothreitol, 0.05% bromophenol blue, and 5% SDS) and boiled for 5 minutes. Proteins were separated by SDS-PAGE using a 9.5-16% (w/v) gel. Proteins were electroblotted to a nitrocellulose membrane and blocked with 5% nonfat dry milk, washed and incubated with appropriate antibody. Blots were washed and incubated with horseradish peroxidase conjugated secondary antibody followed by Supersignal® reagent and exposed to x-ray film. Phosphorylated ERK1/2 antibodies were removed from the membranes with Restore® stripping reagent and reprobed with anti-total ERK1/2 antibody to verify loading. p21, p27,and cdk2 antibodies were removed from the membranes with Restore® stripping reagent and reprobed with mouse monoclonal anti-GAPDH antibody to verify loading. Densitometry was performed on signals detected on the developed film using Multianalyst® software (Bio-Rad, Hercules, CA).

2.6. Phosphorylated Antibodies

Due to the nature of phosphorylated antibodies and their specificity for phosphorylated residues, it was necessary to optimize for western blot analysis. In order to visualize phosphorylated ERK1/2, the phosphorylated antibody was diluted in Superblock® prior to membrane incubation. When 5% nonfat dry milk was utilized, signal was dramatically diminished due to the interaction of the phosphorylated milk proteins with the phosphorylated antibody. Also, it was necessary to filter the secondary antibody by diluting it in a small volume of

Superblock® followed by filtration through a 0.2 micron filter. The filtrate was diluted to its final concentration in 5% nonfat dry milk followed by membrane incubation. The remainder of the protocol was followed as outlined in section 2.5.

2.7. Flow Cytometry

Cell cultures were grown to 70% confluence followed by cell synchronization for 48 hr using media containing 0.1% or 0.5% FBS. At 3 hr intervals, cultures were harvested to determine cell cycle progression utilizing flow cytometry. Ethanol fixation and propidium iodide staining of cell suspensions was followed as outlined in *Cells: A Laboratory Manual* [81]. Briefly, cells were trypsinized and washed twice in 1XPBS + 1% BSA. Cells were counted and fixed in 5 ml of cold (-20 °C) ethanol. Propidium iodide staining was accomplished by centrifugation of the fixed cell suspension and removal of ethanol followed by washing twice with 1XPBS + 1% BSA. Cells were resuspended in 1XPBS +1% BSA. Propidium iodide (50 μg/ml prepared in 0.038M sodium citrate pH 7.0) and RNase A (1 mg/ml prepared in 10mM Tris-HCl, pH 7.5) were added to the resuspended cells. The cell suspension was incubated at 37 ° C for 30 minutes. Samples were immediately processed using a FACS Calibur apparatus (Becton Dickinson, Franklin Lakes, NJ) with ModFit software (Verity Software House, Topsham, ME) for data analysis.

2.8. Statistics

Results are expressed as the mean ± SE. Statistical significance was assessed by Student's unpaired two-tailed t test or analysis of variance followed by comparison of group averages by Fisher's LSD analysis, using the StatView statistical program (SAS Institutes Inc., Cary, North Carolina). Symbols denote statistically significant differences at the $p<0.05$ level.

CHAPTER III

RESULTS

3.1. Differential Regulation of p27 and p21 in Cycling Control and Allylamine Cells

Oxidatively stressed vSMC isolated from allylamine-treated rats are characterized by enhanced mitogenic sensitivity and increased rates of proliferation when seeded on plastic or fibronectin substrates, but not collagen [76]. To evaluate the involvement of cyclin dependent kinase inhibitors in the regulation of matrix-modulated proliferation, protein lysates were harvested from cycling control and allylamine cells. Flow cytometric analysis with propidium iodide staining was used to evaluate the percentage of cells in each phase of the cell cycle (Figure 6). The cell populations were determined to be randomly cycling cells (Table 2).

In cycling cell populations, no significant differences in p27 levels were observed under any of the experimental conditions examined. p21 protein was

increased in allylamine cells seeded on plastic compared to control cells (1.60 ± 0.12 fold increase), while comparable levels of p21 were observed in control and allylamine cells on a collagen substrate (Figure 7). Elevated p21 levels were observed in control cells grown on collagen compared to growth on plastic. The increase in the p21 allylamine/control ratio when cells were grown on a plastic substrate is significant ($p<0.05$) when compared to cells grown on a collagen substrate (Figure 8). These findings are consistent with the loss of proliferative advantage of allylamine cells compared to control cells when seeded on a collagen substrate [76]. Membranes were stripped and reprobed with anti-GAPDH, and anti-cdk2 to confirm equal protein loadings (Shown in Figure 7).

3.2. Differential Expression of p27 and p21 Following Serum Stimulation in G_0 Synchronized Control and Allylamine Cells

Next, p27 and p21 expression was examined in G_0 synchronized cells before and after challenge with serum to induce cell cycle progression. G_0 synchronization (0 hour) was achieved by serum restriction in 0.1% FBS for 48 hours, and confirmed with propidium iodide staining of fixed cell suspensions (Figure 9). Confirmation of

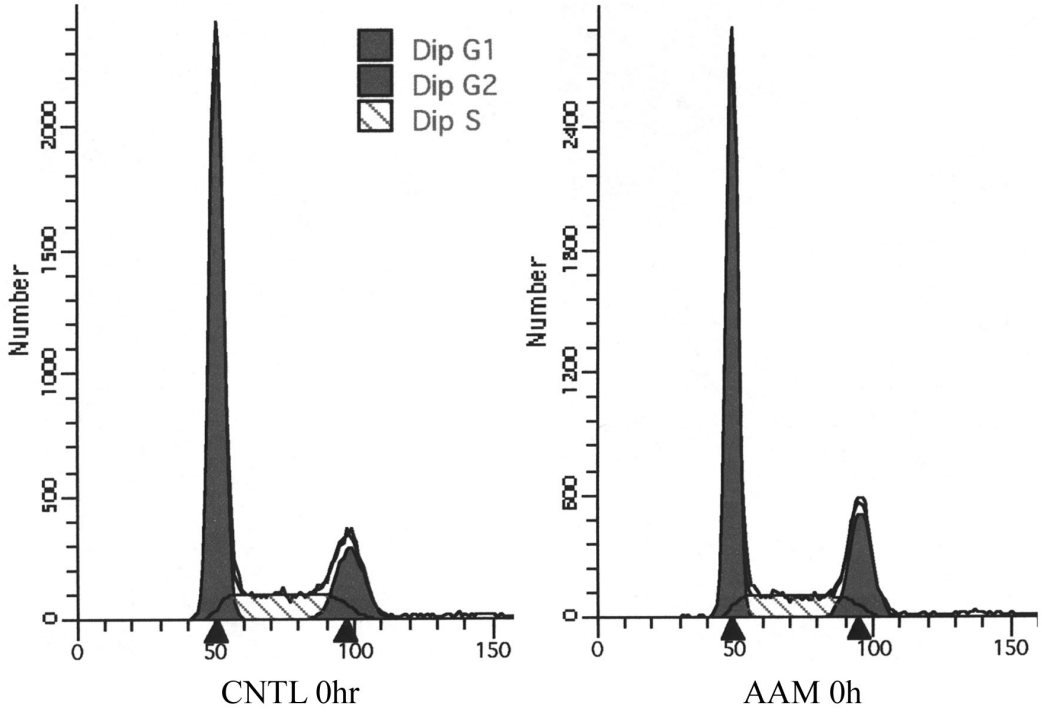

Figure 6: Flow cytometry of randomly cycling control and allylamine cells. Representative flow cytometry of propidium iodide stained fixed cells following 48 hr of serum deprivation (0.5%) showing inadequate cell synchronization (n=3).

TABLE 2

Inadequate G_0 synchronization in control and allylamine cells. Data is represented as average percentage of cells (SEM) (n=3).

Phase of Cell Cycle	Control % (SEM)	Allylamine % (SEM)
G_0/G_1	63.3 (1.4)	63.4 (5.7)
S	23.2 (1.9)	18.2 (1.9)

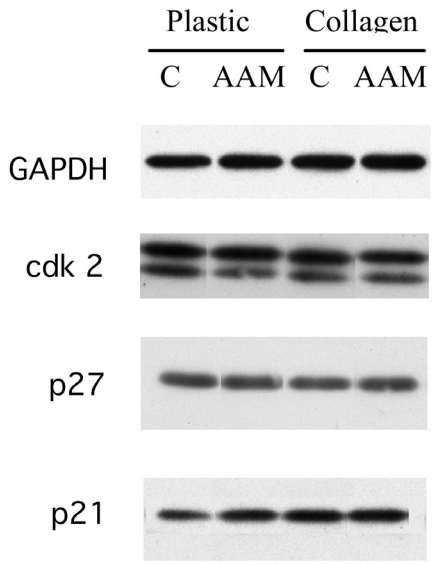

Figure 7: Differential regulation of p27 and p21 in cycling control and allylamine cells. Control (C) and allylamine (AAM) cells were grown to 70% confluence on plastic or collagen. Following serum deprivation (0.5% FBS) for 48 hours, cell lysates were prepared. Western blots of p27 and p21 proteins with control blots of GAPDH (p36) and cdk2 (p34) are depicted. These blots are representative of 3 or 4 replicates for p27 and p21, respectively, from at least two independent experiments.

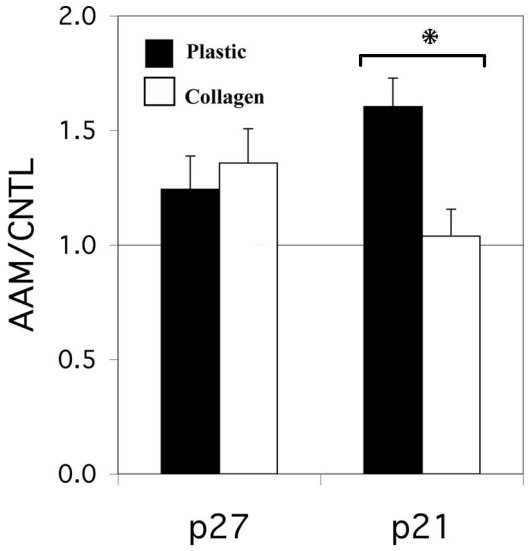

Figure 8: Collagen effects on p27 and p21 expression levels in randomly cycling control and allylamine cells. Graph of average fold differences of band densities between allylamine and control lysates for randomly cycling populations. This represents the average of 3 or 4 replicates for p27 and p21, respectively, from at least two independent experiments. Error bars represent standard error of the mean. ❋ $p<0.05$ for comparison of p21 plastic versus collagen.

cell cycle progression was accomplished by addition of 10% serum to G_0 synchronized cells for 18 hr, which reduced the G_0/G_1 population and caused proportional increases in S phase cells (Table 3).

p27 and p21 were higher (1.83 ± 0.36 and 1.77 ± 0.19 fold increase, respectively) in G_0 synchronized allylamine cells seeded on a plastic substrate relative to controls (Figure 10). When cells were grown on a collagen substrate, p27 and p21 in allylamine cells were comparable to levels in control cells. Levels of p27 and p21 for both cell types grown on collagen were elevated over levels observed

when control cells were grown on plastic. In both cell types, p27 levels decreased as cells progressed through G_1 when cells were grown on either substrate, but this difference was most pronounced on a collagen substrate. p21 levels increased as the cells cycled, irrespective of the growth substrate. Interestingly, no differences in the levels of p27 or p21 were observed between the two cell types following serum stimulation irrespective of the seeding substrate. The ratio of allylamine/control average band densities for p27 and p21 were significantly increased in G_0 synchronized cells when grown on plastic compared with growth on collagen or following 9 hours of mitogen stimulation (Figure 11). These results suggest that increased p27 and p21 in allylamine cells may play a role in regulation of the proliferative advantage of oxidatively injured vSMC on plastic.

TABLE 3

Confirmation of G_0 synchronization and cell cycle progression in control and allylamine cells. Data is represented as average percentage of cells (SEM) (n=3).

Phase of Cell Cycle	**Control**		**Allylamine**	
	0 h	18 h	0 h	18 h
G_0/G_1	75.2 (3.3)	36.0 (5.9)	73.3 (3.2)	46.3 (6.6)
S	16.6 (3.4)	62.9 (4.9)	12.2 (2.9)	53.5 (6.6)

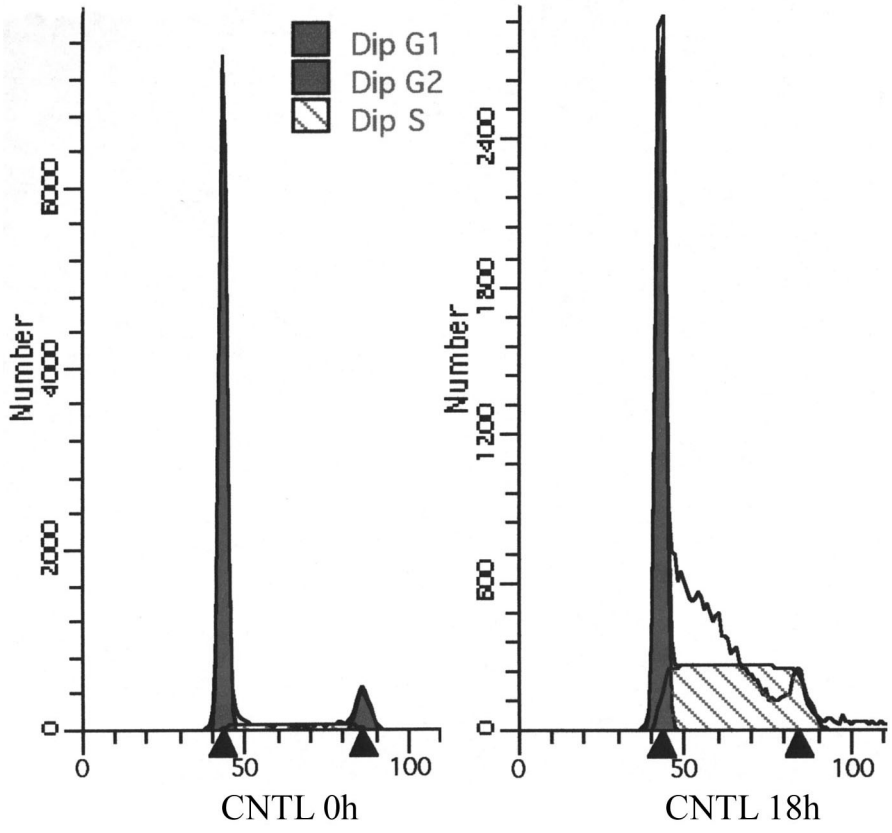

Figure 9: Flow cytometry of G_0 synchronized and cell cycle released control and allylamine cells. To determine cell cycle synchronization and progression, cells were harvested every 3 hrs following mitogen stimulation (10% FBS) in serum restricted (0.1% FBS) cultures. Ethanol fixation, propidium iodide staining and flow cytometry were performed. 0 hr time point represents cell populations following 48 hr of serum deprivation (0.1%). 18 hr time point represents mitogen stimulation (10%FBS) for 18 hr in serum restricted populations. The results shown are representative of 3 independent experiments.

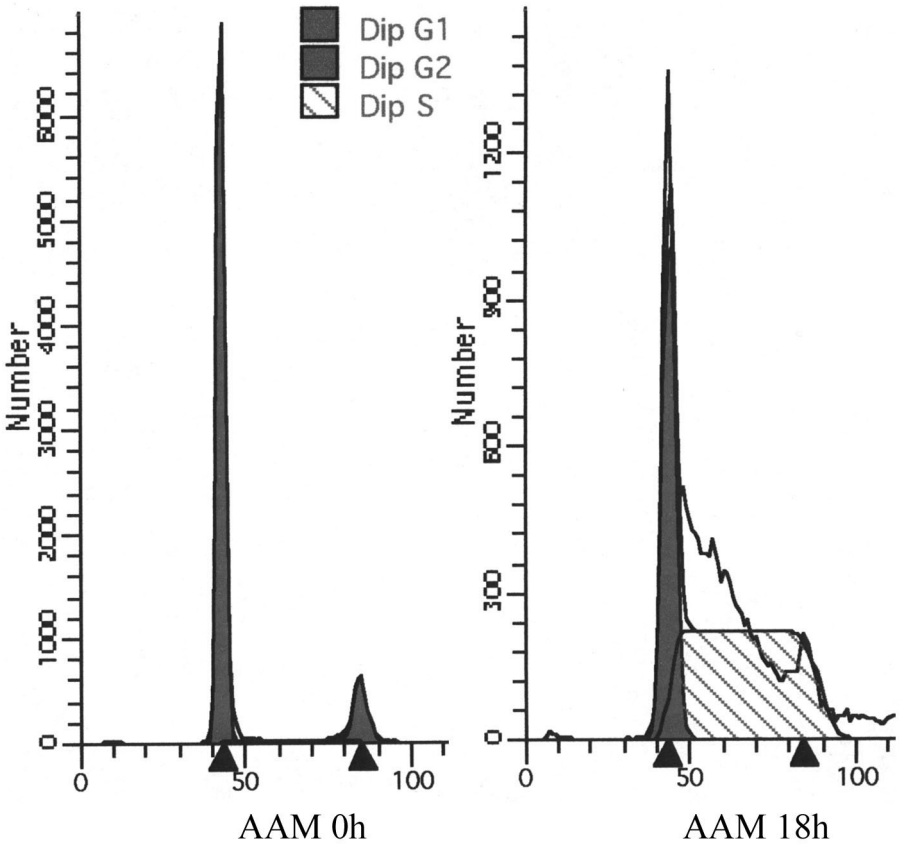

Figure 9: Continued

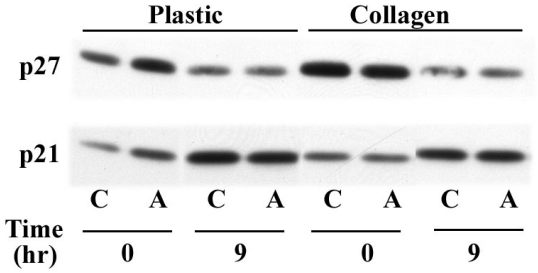

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Figure 10: Differential expression of p27 and p21 in G_0 synchronized and cell cycle released control and allylamine cells. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation (0.1% FBS) for 48 hours, cell lysates were prepared at 0 hr and 9 hr following serum stimulation. Western blots of p27 and p21 that are shown are representative of at least two independent experiments.

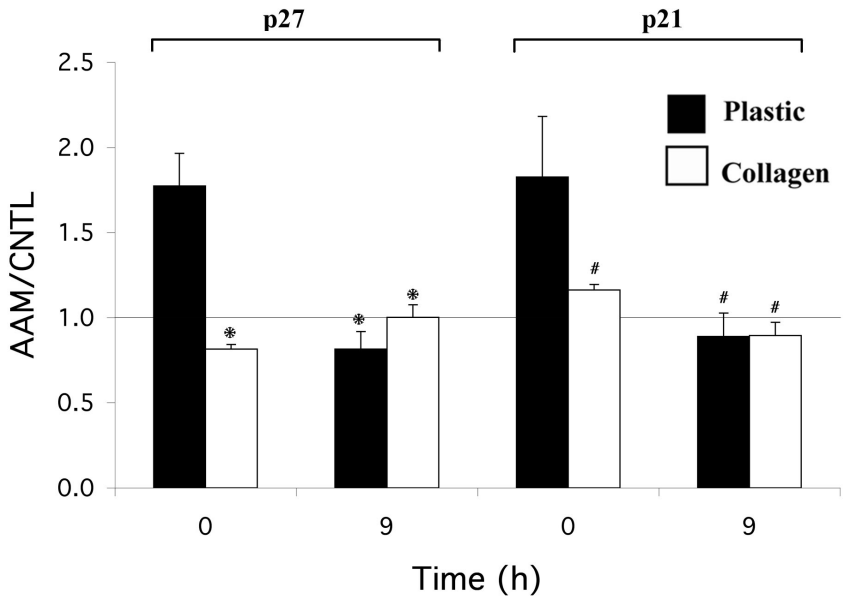

Figure 11: Effects of collagen and mitogen stimulation on p27 and p21 expression levels in control and allylamine cells. Graphic representation of average fold differences in band densities between serum restricted (0.1% FBS) allylamine and control lysates, grown on plastic or collagen, at 0 hr and 9 hr following mitogen stimulation (10% FBS). This represents the average of 4 or 3 replicates for p27 and p21, respectively, from at least two independent experiments. Error bars represent standard error of the mean. ❋ $p<0.05$ for comparison of p27 (plastic, 0h) versus other experimental groups. # $p<0.05$ for comparison of p21 (plastic, 0h) versus other experimental groups.

3.3. Effect of PDTC Treatment on p27 and p21 Expression in Control and Allylamine Cells

NF-κB is involved in the matrix-dependent regulation of the atherogenic vSMC phenotype following oxidative injury [76]. Inhibition of NF-κB by the antioxidant PDTC selectively nullifies the proliferative advantage of allylamine cells. To determine if a functional link exists between the p27 and p21 cdk inhibitors and NF-κB signaling in the regulation of vSMC proliferation, protein lysates were harvested from allylamine and control cells following mitogenic stimulation of growth arrested cultures seeded on plastic or collagen in the presence or absence of 25 and 100 nM PDTC. Similar to the effect of seeding cells on a non-permissive collagen substrate, 100 nM PDTC pre-treatment increased p27 and p21 levels in G_0 synchronized control cells seeded on a plastic substrate. There was no effect of PDTC pre-treatment on p27 or p21 levels in G_0 synchronized allylamine cells on either matrix and in G_0 synchronized control cells seeded on a collagen substrate (Figure 12).

Serum stimulation for 9 hr decreased p27 and increased p21 expression in both cell types grown on plastic or collagen. The decrease in p27 was further suppressed by 100 nM PDTC treatment when cells were grown on collagen. The higher concentration of PDTC did not have an effect on p21 levels in control and allylamine cells as cells progressed through the cell cycle. These results indicate oxidatively injured vSMC show upregulation of p27 and p21 levels under quiescent

conditions; following cell cycle progression, allylamine levels normalize to control levels when cells are grown on plastic. In contrast, growth on a collagen matrix results in comparable p27 and p21 levels in control and allylamine cells both in G_0 synchronized and cycling populations. Our results also show that following cell cycle progression, p27 protein decreases while p21 protein increases in control and oxidatively injured vSMC. This supports the hypothesis that p21 is growth promotive in oxidatively injured vSMC. On collagen, in the presence of PDTC and mitogen stimulation, p27 levels are further decreased in control and allylamine cells while PDTC and mitogen stimulation appear to increase p21 levels in both cell types.

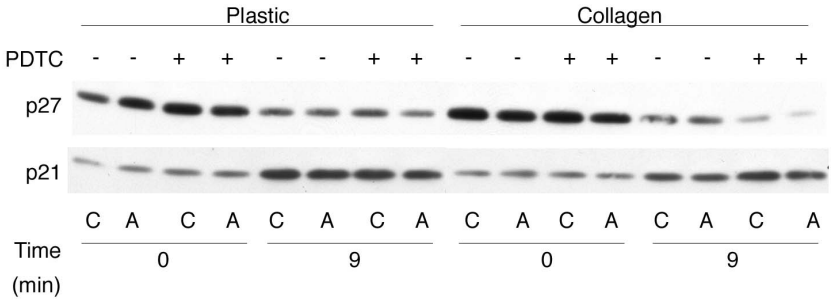

Figure 12: Effects of antioxidant administration on cdk inhibitors in control and allylamine cells. Cells were grown to 70% confluence on plastic or collagen. Following serum deprivation (0.1% FBS for 48 hours), 2 hr pretreatment (media change only or 100nM PDTC) was followed by serum stimulation with or without 100nM PDTC. Western blots of p27 and p21 that are shown are representative of at least two independent experiments.

Response differences between cell types was determined by evaluating the ratio of allylamine/control average band densities (Figures 13 and 14). When PDTC was administered at the higher concentration of 100 nM, the allylamine/control ratio for p21 and p27 was significantly increased ($p<0.05$) in G_0 synchronized cells grown on plastic. In cycling cells, in the presence of 100 nM PDTC, the ratio of allylamine/control for p27 protein was significantly decreased ($p<0.05$) in cells grown on both plastic and collagen compared to all other treatment groups (Figure 13). In contrast, this ratio for p21 protein was significantly decreased ($p<0.05$) when cells were grown on collagen only (Figure 14). As shown by this data, PDTC treatment in the presence of mitogen stimulation had a more pronounced effect on p21 expression in allylamine cells compared with control cells when grown on a collagen substrate. These results provide further evidence that altered p21 expression may provide the matrix dependent proliferative advantage of oxidatively injured vSMC.

When the lower concentration of PDTC (25 nM) was administered in a similar manner as outlined in the above experiments, smaller differences were observed (Figures 15 and 16). For p27, a significant decrease in the allylamine/control ratio was observed ($p<0.05$) when cells were grown on collagen in the presence of PDTC and mitogen stimulation compared with all other treatment groups except PDTC pre-treatment on both plastic and collagen (Figure 15). As well, a significant decrease ($p<0.05$) in the allylamine/control ratio was observed in

G_0 synchronized cells both in the presence and absence of PDTC when cells were grown on collagen. No other significant differences were observed for p27. p21 showed no significant differences between treatment groups following 25nM PDTC administration (Figure 16). However, a decreasing trend in the allylamine/control ratios following mitogen stimulation was observed following mitogen stimulation in the presence and absence of 25 nM PDTC, consistent with a dose dependence of the response to PDTC.

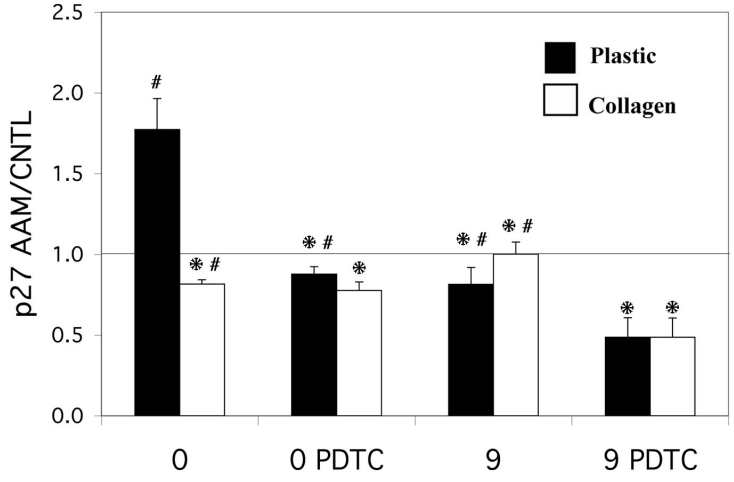

Figure 13: Effect of 100 nM PDTC treatment on p27 expression in control and allylamine cells. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation (0.1% FBS, 48 hours), 2 hr pretreatment (media change only or 100nM PDTC) was followed by serum stimulation with or without 100nM PDTC. Cell lysates were prepared at 0 hr (following pretreatment) and 9 hr following serum stimulation. Graphic representations of p27 densitometric band ratios of allylamine/control are shown. Graph represents the average of 4 replicates from at least two independent experiments. Error bars represent standard error of the mean. ❋ $p<0.05$ for comparison of plastic (0h) versus all other experimental groups. # $p<0.05$ for comparison of PDTC treated (9h, plastic) versus all other experimental groups.

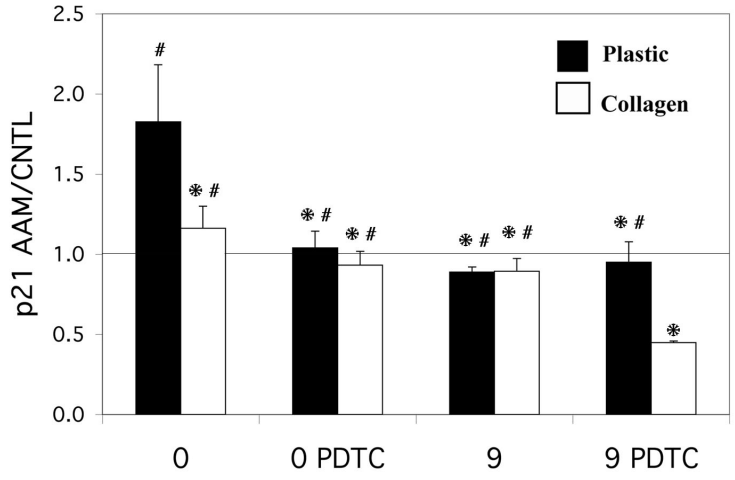

Figure 14: Effect of 100 nM PDTC treatment on p21 expression in control and allylamine cells. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation (0.1% FBS, 48 hours), 2 hr pretreatment (media change only or 100nM PDTC) was followed by serum stimulation with or without 100nM PDTC. Cell lysates were prepared at 0 hr (following pretreatment) and 9 hr following serum stimulation. Graphic representations of p21 densitometric band ratios of allylamine/control are shown. Graph represents the average of 3 replicates from at least two independent experiments. Error bars represent standard error of the mean. ❋ $p<0.05$ for comparison of plastic (0h) versus all other experimental groups. # $p<0.05$ for comparison of PDTC treated (9h, collagen) versus all other experimental groups.

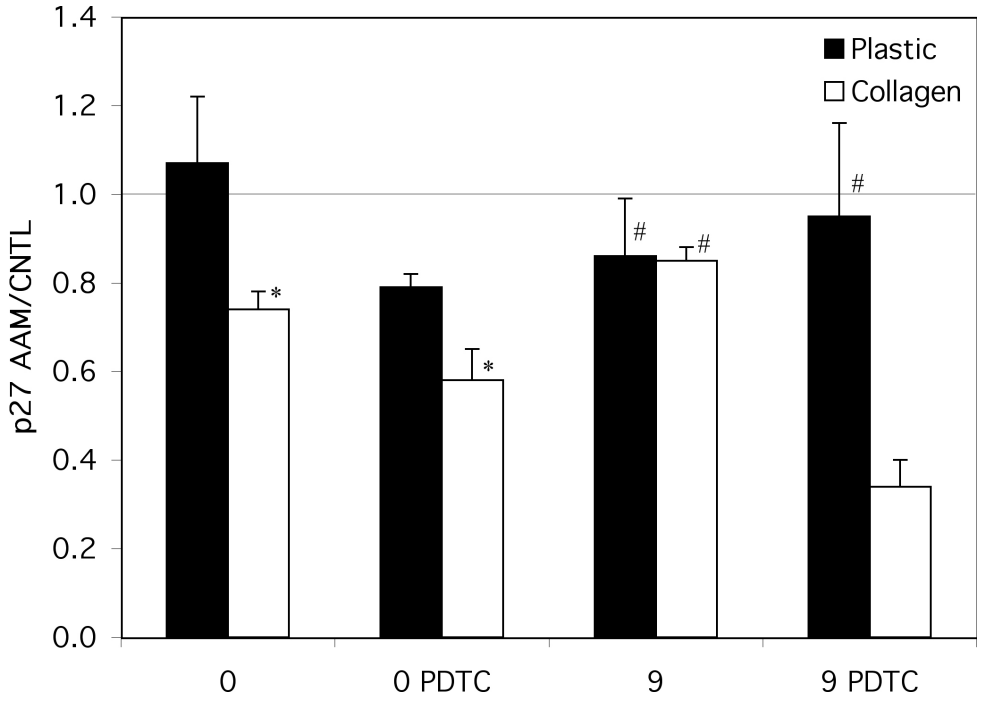

Figure 15: Effect of 25 nM PDTC treatment on p27 expression in control and allylamine cells. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation (0.1% FBS, 48 hours), 2 hr pretreatment (media change only or 25 nM PDTC) was followed by serum stimulation with or without 25 nM PDTC. Cell lysates were prepared at 0 hr (following pretreatment) and 9 hr following serum stimulation. Graphic representations of p27 densitometric band ratios of allylamine/control are shown. Graph represents the average of 4 replicates from at least two independent experiments. Error bars represent standard error of the mean. * $p<0.05$ for comparison of plastic (0h) versus collagen (0h) and PDTC treated (0h, collagen). # $p<0.05$ for comparison of PDTC treated (9h, collagen) versus plastic (9h), collagen (9h) and PDTC treated (9h, plastic).

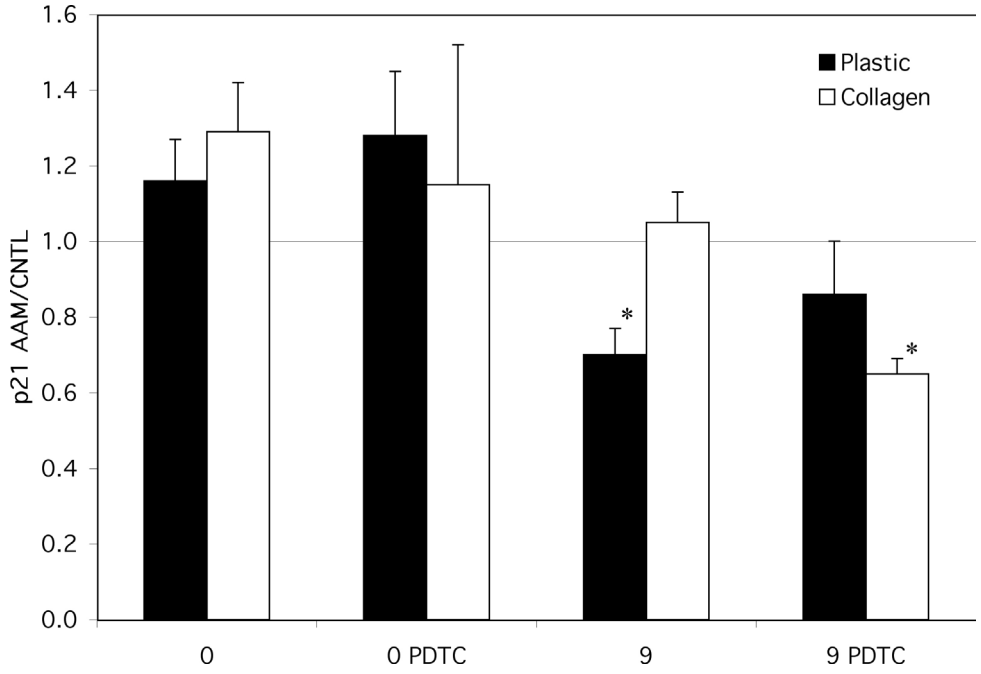

Figure 16: Effect of 25 nM PDTC treatment on p21 expression in control and allylamine cells. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation (0.1% FBS, 48 hours), 2 hr pretreatment (media change only or 25 nM PDTC) was followed by serum stimulation with or without 25 nM PDTC. Cell lysates were prepared at 0 hr (following pretreatment) and 9 hr following serum stimulation. Graphic representations of p21 densitometric band ratios of allylamine/control are shown. Graph represents the average of 3 replicates from at least two independent experiments. Error bars represent standard error of the mean. * $p<0.05$ for comparison of plastic (0h) versus collagen (0h) and PDTC treated (0h, collagen). * $p<0.05$ for comparison of collagen (0h) and PDTC treated (0h, plastic) with plastic (9h) and PDTC treated (9h, collagen).

3.4. Differential ERK1/2 Activation of Control and Allylamine Cells

The activation of ERK1/2 is an important signaling cascade for passage of a cell from G_1 to S phase of the cell cycle [41]. ERK1/2 activation is vital for continued cyclin D1 protein expression in late G_1 [38]. Cyclin D1 complexes with cdk4/6 for phosphorylation of the retinoblastoma protein followed by induction of E2F-regulated genes, including cyclin A [39]. These interactions allow transition of a cell from G_1 to S phase of the cell cycle. To determine the involvement of ERK1/2 activation in regulation of proliferation in vSMC, protein lysates were harvested from allylamine and control cells following time dependent FBS stimulation of mitogen restricted cultures grown on plastic and collagen. ERK1/2 activation peaked 15 min post-stimulation in both control and allylamine cells, as determined by western blot analysis using anti-phosphorylated ERK1/2 antibodies (Figure 17). Peak activation was increased in allylamine cells versus controls when cells were grown on plastic, and this difference was lost in cells grown on a collagen substrate. Comparable ERK1/2 loadings were confirmed by stripping and reprobing membranes with anti-total ERK1/2 (Fig 18).

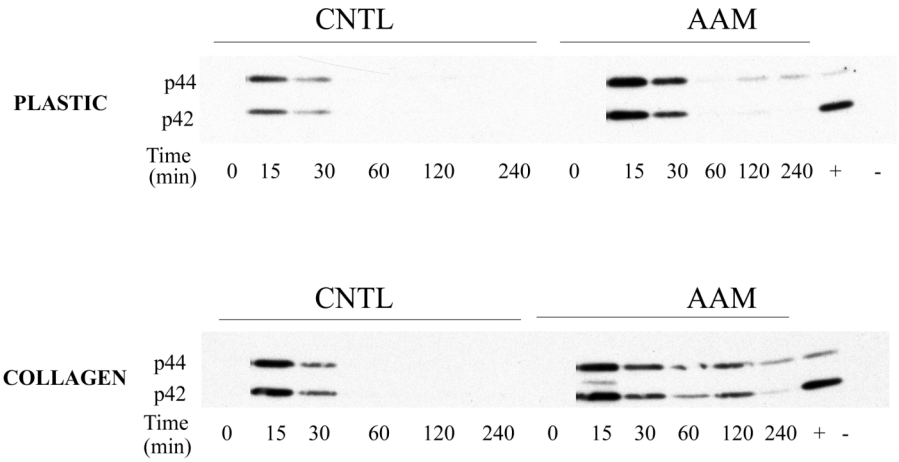

Figure 17: Differential ERK1/2 activation of control and allylamine cells. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation for 48 hours, cultures were stimulated with media containing 10% FBS. Cell lysates were prepared at 0, 15, 30, 60, 120, and 240 minutes following exposure to serum. (CNTL-control lysates; AAM-allylamine lysates; + positive control, serum stimulated 3T3 lysates; - negative control, nonstimulated 3T3 lysates). Immunoblots of anti-phosphorylated ERK1/2 on plastic and collagen are shown. Membrane was stripped and reprobed with anti-total ERK1/2 to verify equal ERK1/2 loadings (results shown in Figure 17).

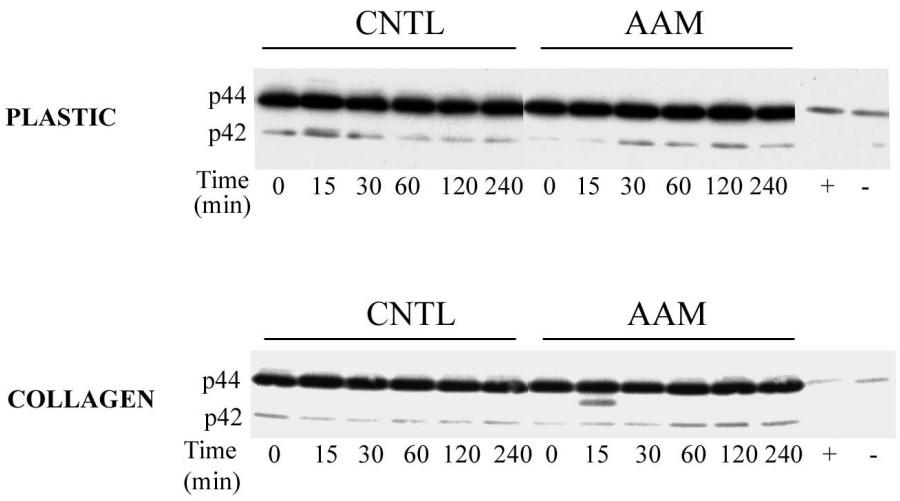

Figure 18: Confirmation of equal ERK1/2 protein loading. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation for 48 hours, cultures were stimulated with media containing 10% FBS. Cell lysates were prepared at 0, 15, 30, 60, 120, and 240 minutes following exposure to serum. (CNTL-control lysates; AAM-allylamine lysates; + positive control, serum stimulated 3T3 lysates; - negative control, nonstimulated 3T3 lysates). Immunoblots of anti-total ERK1/2 on plastic and collagen are shown.

As both the degree and duration of ERK1/2 activation are important in cell growth and proliferation, evaluation of ERK1/2 following prolonged mitogen stimulation was examined. Allylamine cells maintained increased ERK1/2 activation for a longer duration than control cells when grown on either plastic or collagen.

Further evaluation of the differential matrix and time dependent profile of ERK1/2 activation in allylamine and control cells consisted of examining band density ratios of allylamine/control cells (Figures 19 and 20). Growth on both plastic and collagen resulted in higher peak phosphorylation of ERK1/2 at 15 and 30 min (approximately 3.5 fold increase) followed by return to control levels at 60 min in allylamine cells compared to control cells. The second wave of activation, occurring at 120 and 240 min, was also higher in allylamine cells compared with control counterparts. However, this increase was not as dramatic as was observed when cells were grown on plastic (two-fold increase only) (Figure 19). In contrast, when cells were grown on collagen, the activation profile of ERK1/2 ratios showed a gradual increase with peak differences observed at 140 min (approximately 12-fold difference in allylamine over control ratios). Early ERK1/2 activation was still increased in allylamine versus control cells when grown on collagen, however, this difference was only slightly less than two fold (Figure 20). These data suggest that differences in ERK activation profiles play a key role in altered proliferation of oxidatively injured vSMC.

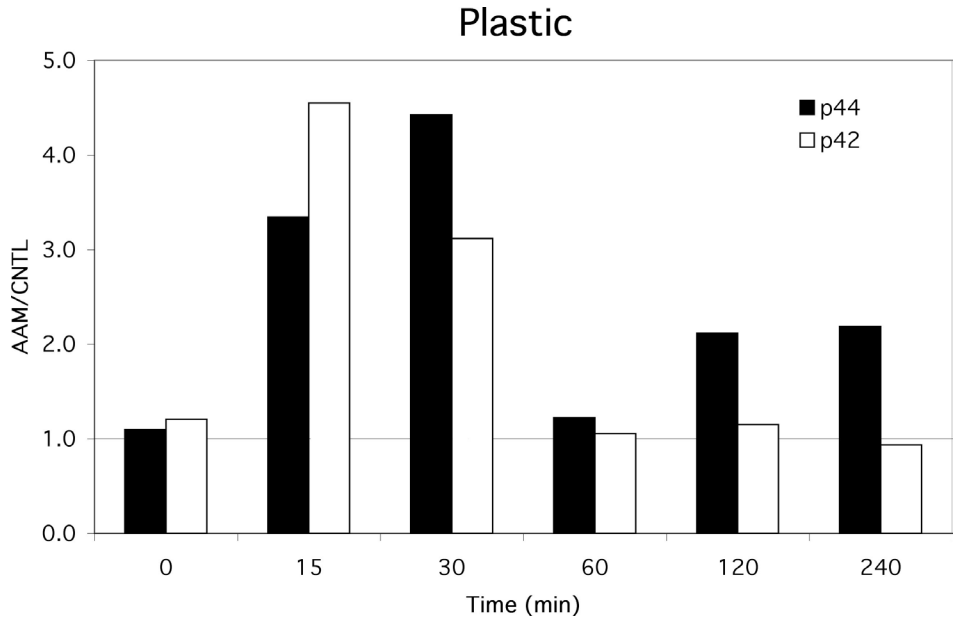

Figure 19: ERK1/2 activation profile of control and allylamine cells grown on plastic. Control and allylamine cells were grown to 70% confluence. Following serum deprivation for 48 hours, cultures were stimulated with media containing 10% FBS. Cell lysates were prepared at 0, 15, 30, 60, 120, and 240 minutes following exposure to serum. Graphic representations of phosphorylated p44 (ERK1) and phosphorylated p42 (ERK2) densitometric band ratios of allylamine/control are shown.

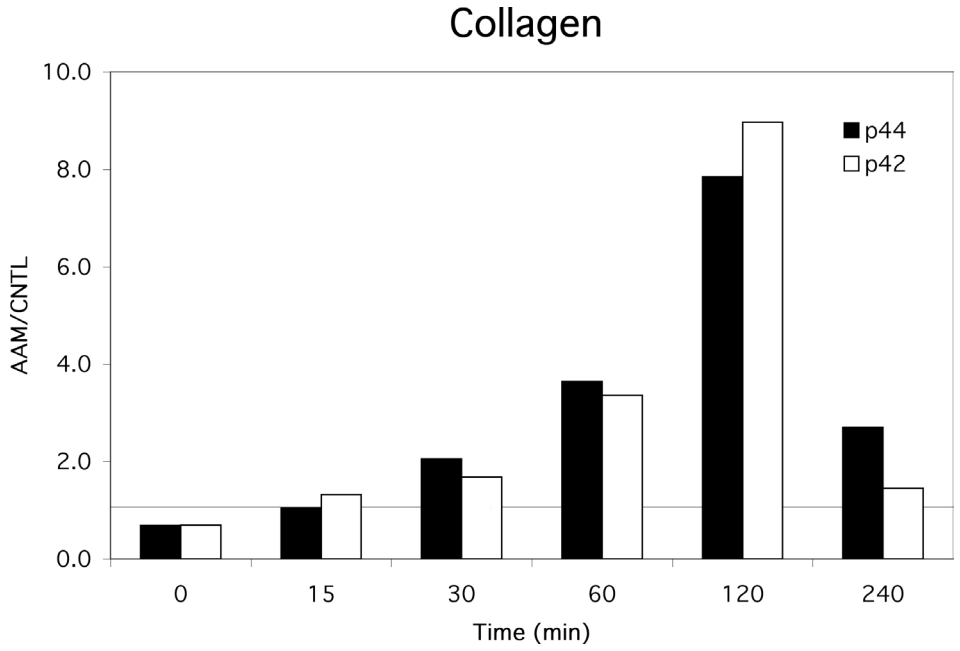

Figure 20: ERK1/2 activation profile of control and allylamine cells grown on collagen. Control and allylamine cells were grown to 70% confluence. Following serum deprivation for 48 hours, cultures were stimulated with media containing 10% FBS. Cell lysates were prepared at 0, 15, 30, 60, 120, and 240 minutes following exposure to serum. Graphic representations of phosphorylated p44 (ERK1) and phosphorylated p42 (ERK2) densitometric band ratios of allylamine/control are shown.

3.5. Effect of MEK1/2 Inhibition on p27 and p21 Expression in Control and Allylamine Cells

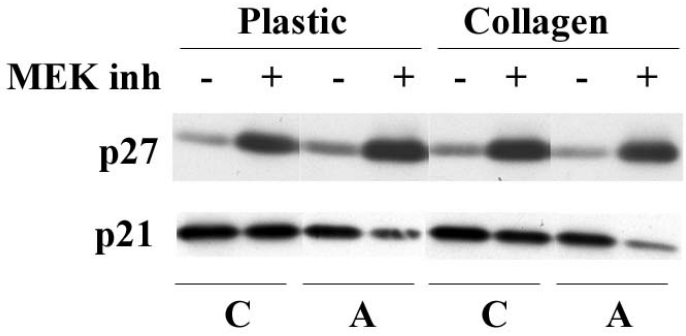

Figure 21: Effect of MEK1/2 inhibition on p27 and p21 expression in control and allylamine cells. Control and allylamine cells were grown to 70% confluence on plastic or collagen. Following serum deprivation for 48 hours, pretreatment for 30 min (media change only or 25μM U0126) was followed by serum stimulation with or without U0126 (25μM). Cell lysates were prepared at 9 hr following serum stimulation. Western blots of p27 and p21 protein lysates are shown and represent at least two independent experiments.

Given observed differences in matrix dependent alterations in peak ERK1/2 activation in allylamine cells, we investigated a possible functional link between ERK1/2 activation and cdk inhibitor expression. Protein lysates were harvested from allylamine and control cells seeded on either plastic or collagen following serum stimulation for 9 hours in the presence or absence of 25μM U0126, a MEK1/2 inhibitor. MEK1/2 inhibition increased p27 levels in both control and allylamine

cells irrespective of the seeding substrate (Figure 21). No differences in p27 induction were observed between the two cell types, suggesting that differences observed in p27 expression in oxidatively injured vSMC are not dependent on MAP kinase activation. In contrast, cell type-specific differences in p21 expression were observed following inhibition of MEK1/2 (Figure 22). U0126 reduced p21 expression in both cell types, but the response was markedly enhanced in allylamine cells, and preferentially observed on a restrictive collagen substrate (Figures 21 and 22). These data suggest the involvement of ERK1/2 activity in matrix dependent modulation of p21 levels in oxidatively injured vSMC.

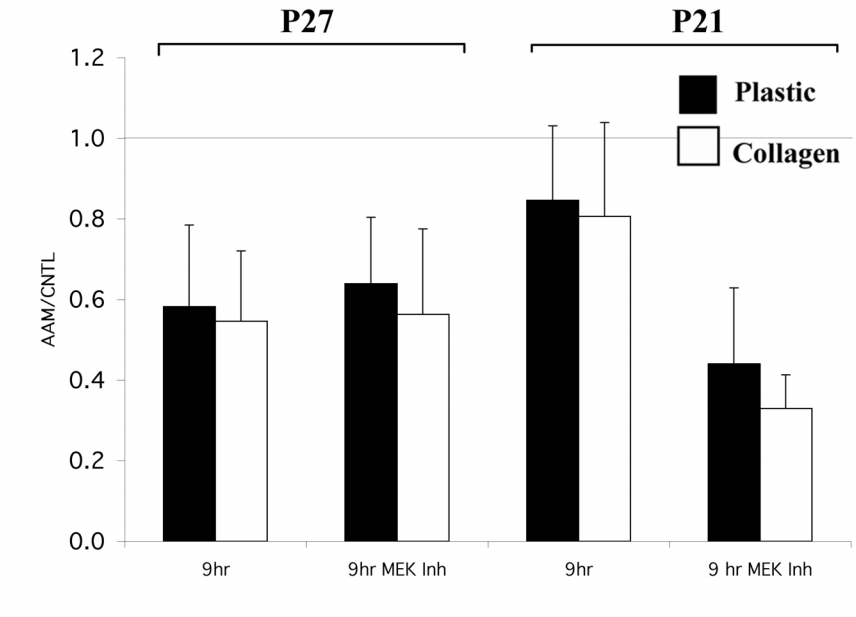

Figure 22: MEK1/2 inhibition by U0126 in control and allylamine cells. Graphic representation of fold differences in densitometric band ratios between serum restricted (0.1% FBS) allylamine and control lysates, grown on plastic or collagen (9 h) following mitogen stimulation (10% FBS) with or without U0126 (25 μM). Graphs represent the average of at least two independent experiments. Error bars represent standard error of the mean.

CHAPTER IV

DISCUSSION AND SUMMARY

4.1. p27 and p21 Expression in Allylamine and Control Cells

Phenotypic modulation of vSMC by chronic oxidative stress induced with allylamine differentially modulates profiles of surface integrin expression [75]. Expression of $\alpha 1$ and $\alpha 5$ integrin subunits are substantially reduced on allylamine cells, while expression of $\beta 3$ integrin is increased compared to control cells. Thus, the proliferative advantage seen in oxidatively stressed vSMC may be mediated by alterations in integrin-coupled signaling. This interpretation is supported by studies showing extracellular matrix-dependent differences in allylamine cell proliferation compared to control cells [76]. The proliferative advantage of allylamine cells is maintained when cells are grown on plastic, pronectin or fibronectin, but lost when grown on collagen, a ligand for the β_1 integrin subunit [76]. Consistent with these observations, Thyberg's group has previously shown that the substrate is a crucial factor in the progression of vSMC from contractile to synthetic/proliferative phenotypes [12]. Smooth muscle cells propagated on both laminin and collagen IV maintained contractile phenotypes longer than cells propagated on fibronectin and vitronectin, which promoted conversion to proliferative phenotypes.

To further explore the nature of integrin/ECM signaling in oxidatively stressed vSMC, we first evaluated the expression of the cdk inhibitors, p27 and p21,

in cycling allylamine and control vSMC cultured on plastic or collagen substrates. Alterations in cell cycle progression leading to increased proliferative rates are highly dependent on the activity of the cyclin dependent kinases. One mechanism of cdk regulation is through the binding of cdk inhibitors to the cyclin/cdk complex [82]. In mammalian cells, there are two families of cdk inhibitors known: the INK4 family, consisting of p16 and p15, and the Kip/Cip family including p27 and p21. The INK4 family is specific for inhibition of cdk4 and cdk 6 complexes thereby inhibiting cyclin D associated cdk activity; while p27 and p21 are general cdk inhibitors inactivating both cyclin D and cyclin E associated cdk activity [83]. p27 has been implicated in mediating TGF-β- and contact-induced inhibition of vSMC proliferation [84], as well as inhibiting vSMC and fibroblast proliferation and migration [85]. VSMC growth on polymerized collagen inhibits downregulation of p27, thereby inhibiting S-phase entry [86]. p27, therefore, acts primarily in the context of inhibition of cell cycle progression.

In contrast, p21 appears to have a multiplicity of functions in regulation of cell cycle control. p21 has dual functions as a cell cycle inhibitor [87,88], and as a permissive element for cell cycle progression in PDGF-stimulated vSMC [48]. Mechanisms through which p21 modulates cell cycle progression include the inhibition of cyclin/cdk complex phosphorylation, and direct inhibition of the transcription factor E2F. Permissive effects of p21 include promotion of cyclin/cdk complex assembly and nuclear localization through action as an adaptor protein, inhibition of apoptosis, and interaction with a number of transcription factors

including CEBP and NF-κB that are implicated in responses to oxidative stress [46,47,87].

In vSMC the process of growth inhibition is complex and can result in three outcomes depending on the combination of stimuli to which the cells are exposed: quiescence, hypertrophy or apoptosis [89]. For example, p27 induction after angiotensin treatment is associated with hypertrophy, whereas, serum-induced reduction in p27 is associated with proliferation [90]. These paradigms are indicative of the complex phenotypes that vSMC can exhibit both *in vivo* and *in vitro,* and the complexity of regulation of cdk inhibitors and their role in regulating vSMC phenotypic modulation.

Alterations in p27 and p21 expression have been implicated in the pathogenesis of vascular disease. VSMC ultimately undergo alterations in cell cycle progression leading to the vasculoproliferative response of many vascular diseases, including atherosclerosis [89]. Differential expression of p27 and p21 has been observed in human atherosclerotic arteries [91]. p27 was shown to be constitutively expressed in normal and atherosclerotic arteries, whereas p21 was elevated in human atherosclerotic arteries and undetectable in normal counterparts. These data are consistent with our observation that increased p21 plays a role in the proliferative advantage of oxidatively injured vSMC. It has been proposed that the cdk inhibitor p27 functions to inhibit cell proliferation during arterial repair, while p21 functions as a cofactor induced in the latter phases of arterial remodeling to cause G_1 arrest

[91]. As well, oxidative stress has been implicated in modulation of cell cycle regulation [63].

In our oxidative injury model of atherogenesis, we show that p27 levels were not altered irrespective of the growth substrate, implying that p27 has little effect on the proliferative advantage of allylamine cells compared with control cells. The increased expression of p21 observed in cycling allylamine cells seeded on a permissive plastic substrate provides evidence that p21 may act as a permissive element in vSMC cycle progression. Also, the normalization of p21 expression to control levels in allylamine cells seeded on a collagen substrate establishes the matrix dependency of p21 expression in randomly cycling oxidatively stressed cells.

In contrast to cycling cells, G_0 synchronized allylamine cells express higher levels of both p27 and p21 compared to control cells when grown on plastic. Both p27 and p21 allylamine/control ratios were increased in G_0 synchronized cells when grown on plastic compared with mitogen stimulation or growth on a collagen substrate. Cell cycle progression in both cell types resulted in decreased p27 levels on both substrates, with a more pronounced effect when cells were grown on collagen. These findings are consistent with the growth inhibitory function of p27. In contrast, following cell cycle progression, p21 protein expression increased in control and allylamine cells when grown on both matrices. We found no difference in p21 or p27 expression following 9 hours of serum stimulation between cell types irrespective of the growth matrix. This indicates that differences exist at the basal

level for cdk inhibitors in oxidatively injured vSMC compared to control cells, while mitogen stimulation results in similar responses for p27 and p21 in both cell types.

Mitogenic stimulation for both cell types on plastic or collagen results in decreased p27 expression and increased p21 expression. p27 levels are similar in both cell types during mitogen stimulation, and on both substrates, confirming that this cdk inhibitor is not involved in the proliferative advantage of oxidatively stressed vSMC. However, p21 expression shows less induction when cells are grown on a non-permissive substrate compared with growth on plastic providing further evidence of p21 as a positive regulator of growth in vSMC. The growth inhibitory effect of collagen in allylamine cells may occur post-9 hours of mitotic stimulation and can be explained through the differential effects of growth factors on phenotypic properties of vSMC [92]. For example, PDGF can initiate DNA synthesis, yet alone, does not provide adequate signals for completion of DNA synthesis and cell cycle progression. Other growth factors, such as EGF, are required for cell proliferation, consistent with previous studies from this laboratory showing that EGF synergizes with the oxidative injury phenotype [76].

4.2. ERK1/2 Activation Profiles in Allylamine and Control Cells

The role of ERK1/2 in mediating extracellular matrix modulation of growth properties in oxidatively stressed vSMC was also investigated in these studies. MAP kinases are intricately involved in modulation of growth, proliferation and differentiation by growth factors and integrins [15,22,26,35]. Thus, MAP kinases

may serve as points of integration for multiple signaling pathways, including those involved in matrix-regulated cell cycle activity and proliferation. In our model of oxidant induced vSMC injury, alterations of the extracellular matrix protein, OPN, play a key role in modulation of vSMC phenotype [75,78]. It has been reported that injury-induced OPN gene expression is dependent on activation of ERKs further implicating the coordinated effort of integrin- and growth factor-mediated signaling in modulation of vSMC phenotype [93]. It also has been reported that reactive oxygen species, such as H_2O_2 and $O_2^{\bullet -}$, differentially activate ERKs in vSMC [94] implying that the phenotypic modulation of vSMC through metabolic conversion of allylamine to reactive oxygen species may be dependent on ERK modulation. Not only is activation of ERKs crucial for cell growth, proliferation and differentiation, it is clear that the temporal activation profile of ERKs is also important in the cellular response [95,96]. The typical activation profile of ERK is biphasic: a rapid phase appearing within 15 minutes, followed by a sustained late phase of greater than 4 hours [96]. The sustained phase of ERK activation is an essential component for growth factor-induced cell cycle progression. When ERK activation is transient, the immediate early gene product, c-Fos, does not accumulate. However, sustained ERK activity ultimately allows phosphorylation of accumulated c-Fos. These temporal differences in protein alteration patterns provide a means through which cells interpret differences in ERK activation profiles [95]. Integrin-induced cell cycle progression is also an important determinant for ERK activation kinetics. Roovers and colleagues show that $\alpha_5\beta_1$ integrin sustains ERK1/2 activity resulting in

expression of cyclin D1 [41]. Loss of adhesion prevents cyclin D1 elevations thus progression through G_1 is blocked.

With this in mind, we evaluated the involvement of integrin/extracellular matrix signaling in modulation of ERK activation kinetics in our model of oxidatively injured vSMC. Our results show that activation of ERK1/2 is of greater magnitude and duration in allylamine cells compared to controls when seeded on both plastic and collagen substrates. Additionally, the kinetics of ERK1/2 activation are remarkably different between cell types and growth substrate. Growth on plastic shows a much greater early peak in ERK1/2 activation in allylamine cells compared to controls, while the sustained activity occurs, but not to the extent of the peak activity. In contrast, growth on collagen reveals a much lower early peak activity in allylamine cells compared to controls yet the sustained activity is dramatically enhanced over that seen in control cells. Others have shown that MAP kinase activation occurs through matrix/integrin signaling in adherent cells [32]. Morino et al. (25) showed that fibroblast adherence to fibronectin, a ligand for the β_1 integrin subunit, as well as β_1 crosslinking resulted in activation of MAP kinases. ERK activity plays an integral part in cell cycle regulation, specifically the passage from G_1 to S phase of the cell cycle [38,96]. Assoian et al. [39,44] have suggested that matrix:integrin binding with fibronectin or vitronectin in vSMC leads to sustained ERK activity. This activity leads to translocation of the active enzyme to the nucleus where it is required for synthesis of cyclin D1 and progression of the cell through the cell cycle. Thus, interactions of integrins, MAP kinase and cell cycle control proteins

may mediate dysregulated growth and differentiation of allylamine cells leading to induction of highly proliferative phenotypes. The early peak in ERK activation is likely a combined effect of growth and integrin/extracellular matrix signaling leading to ERK activation. These data are suggestive that ERK activation is an important contributing component of proliferative advantage and phenotypic alterations of oxidatively injured vSMC and that the collagen modulation of the proliferative advantage may be manifested through alteration of the activation profile of ERKs.

As seen for p21, peak ERK activation in vSMC was influenced by the extracellular matrix, thus implicating interactions between ERK signaling and p21 in the regulation of proliferation of vSMC. Altered integrin/matrix regulation of ERK and further downstream signaling events may contribute to induction of proliferative phenotypes following oxidative injury by allylamine. This interpretation is consistent with the results of studies showing that basal levels of cyclin D1 are increased in allylamine cells compared to control cells when cells are grown on plastic, but not collagen (Jones et al., unpublished). As cyclin D1 is an important cell cycle regulatory protein involved in transition from G_1 to S phase of the cell cycle, alterations in cell cycle regulatory proteins through ERK1/2 activation may be an important component of the proliferative dysregulation seen in oxidatively stressed vSMC.

4.3. ERK1/2 Contribution to p27 and p21 Expression in Allylamine and Control Cells

To evaluate the contribution of ERKs in modulation of p27 and p21 expression, we utilized the MEK1/2 inhibitor, U0126, to suppress ERK activity. Following inhibition of ERK activity, differential expression of p21, but not p27, was observed between allylamine and control cells. As cell cycle progression is blocked through MEK 1/2 inhibition, increases in p27 levels are expected. In this model, no differences were observed in induction of p27 between the two cell types grown on either substrate. Thus, the alterations in p27 expression in oxidatively injured cells are not dependent on ERK activation. On the other hand, decreases in p21 expression following MEK 1/2 inhibition were both cell and matrix dependent with allylamine cells and the restrictive substrate, collagen, showing the most depressed p21 levels. It can be deduced, therefore, that ERK activation and p21 induction are related and contribute to the matrix dependent, proliferative advantage observed in oxidatively injured vSMC.

4.4. Antioxidant Effects on p27 and p21 Expression in Allylamine and Control Cells

Among the complex interactions of p21 with a myriad of proteins are interactions with transcription factors C/EBP and NF-κB [87]. These factors have been implicated in oxidative stress signaling in vSMC [61]. To further evaluate the

effect of chronic oxidative injury on vSMC, we examined the influence of the NF-κB inhibitor PDTC in this cell system. PDTC can have a variety of effects on cultured cells including pro-oxidant and antioxidant effects, immunomodulatory actions, anti-apoptotic and apoptotic activity, as well as regulation of the activity of redox-sensitive transcription factors, such as AP-1 and NF-κB [97-100]. These effects appear to be concentration dependent as, for example, low PDTC concentrations increase AP-1 binding activity while higher PDTC concentrations inhibit NF-κB activity [100]. In our cell system of chronic oxidatively injured vSMC, 100 nM PDTC pretreatment had a greater effect on p27 and p21 levels in G_0 synchronized control cells grown on plastic than in any other treatment group. Additionally, following mitogen stimulation, p27 was further suppressed by the addition of PDTC, while PDTC had no effect on p21 levels when cells were grown on plastic. Compared with p27, a greater difference in p21 allylamine/control ratio at 0 hour versus 9 hour PDTC treatment on collagen versus plastic was also observed. Thus, high PDTC concentration suppresses the collagen/mitogen induction of p21 in allylamine cells but not the plastic/mitogen response; whereas, p27 shows enhanced suppression in allylamine cells compared to control cells on both substrates. Following administration of lower concentrations of PDTC (25 nM) less effect was observed. For p27, a significant decrease in the allylamine/control ratio was observed ($p<0.05$) when cells were grown on collagen in the presence of PDTC and mitogen stimulation compared with all other treatment groups except PDTC pre-treatment on both plastic and collagen. No other significant differences were

observed for p27. p21 showed no significant differences between treatment groups following 25nM PDTC administration. However, a decreasing trend in the allylamine/control ratios following mitogen stimulation was observed in the presence and absence of 25 nM PDTC. Influence of PDTC on this cell system thus appears to be dose dependent. Our results provide evidence that differential p21 expression in oxidatively injured vSMC can be influenced by PDTC and the growth substrate.

4.5. Implications of Current Research

One of the key features in the pathogenesis of atherosclerosis and restenosis is phenotypic alteration of vSMC from contractile to synthetic phenotypes, including increased proliferative capacity. Recently, the feasibility of targeting cell cycle specific proteins as a therapeutic option to minimize or prevent this alteration has gained credence. Several of the methods which target cell cycle regulatory proteins in vSMC that are currently under evaluation include: cell cycle modulating drug therapy, oligodeoxynucleotide therapy (antisense and decoy strategies), adenoviral mediated gene therapy, and liposomal-mediated gene therapy [88]. For example, the drug tranilast interferes with p21-dependent vSMC hyperplasia in mice [101], while short term therapy with pravastatin increases p27 expression resulting in inhibition of vSMC growth and induction of apoptosis [102]. The success of pharmacological and genetic approaches to vasculoproliferative therapy depends on the ability of the drug to reach affected areas. There are many routes through which antiproliferative agents may be administered (outlined by Sriram and Patterson) [89]. One of the most

promising methods is through the use of drug-coated stents [103-106]. These therapies appear promising in the prevention of re-stenosis following angioplasty or in restarting proliferation in ischemia-damaged cardiac myocytes. Our results offer further evidence that, in the future, pharmacological or genetic manipulation of p21 may be an alternative or existing therapy for atherosclerosis induced by oxidative injury.

4.6. Future Directions

The data presented in this dissertation suggest that integrin/extracellular matrix signaling plays a crucial role in the development of the proliferative phenotype of oxidatively injured vSMC. Through alteration of ERK1 activation and downstream cdk inhibitors, p27 and p21, oxidatively injured vSMC show upregulation of cell cycle progression that is ablated on a collagen matrix. Several issues remain to be addressed to fully define the molecular mechanism regulating the proliferative advantage seen in chronically oxidatively injured vSMC. Screening of a variety of cell cycle regulatory proteins, including cyclin D1, and cyclin E was attempted. However, difficulties arose in both variability of the cell cultures and purchased antibodies. It would be prudent, therefore, to further evaluate these proteins and others, including the retinoblastoma protein, both through western analysis and mRNA expression. This would provide further insight into alterations of cell cycle progression in oxidatively injured vSMC.

One of the main issues that arose in performing the experiments within this dissertation was the behavior of the allylamine and control cells. Culture of vSMC alters their phenotype from contractile to synthetic. In our experiments, we are investigating the mechanisms that modulate phenotype following oxidative injury in vSMC. In spite of cell culture conditions, allylamine cells maintained a proliferative advantage over control counterparts following serial propagation [69]. However, passage levels 20-25 were utilized in these experiments. The underlying effects of such high passage numbers in defining the mechanisms altering vSMC phenotype are unknown. Thus, further studies evaluating alterations in cdk inhibitor expression in alternative oxidative injury models needs to be addressed.

By utilizing naïve vSMC, the effect of acute administration of oxidants such as allylamine and its reactive oxygen species metabolites, acrolein and H_2O_2, may be evaluated. This would provide a means to study acute oxidative alteration of cell cycle control and ERK activity. In the cellular model used for these studies, allylamine cells have been genetically altered by chronic administration of allylamine. Evaluation of matrix-dependent alterations of ERKs and cdk inhibitors in an acute oxidative injury model may provide additional support for the integrin/extracellular matrix- and growth factor- induced modulation of ERK activation and cdk inhibitor expression in oxidatively injured vSMC. Supplementary studies involving transfection of p21 sense and antisense oligonucleotides into naïve vSMC would offer further evidence of the importance of p21 regulation in the matrix-dependent proliferative phenotype. Matrix protein secretion has been altered

through transfection of antisense p21 oligonucleotides [107]. Oligonucleotide transfection studies can be performed to target the cdk inhibitor, p21, thereby evaluating its requirement in cell cycle progression following perturbations of integrin/extracellular matrix- and growth factor- dependent signaling cascades. This provides an alternative protocol for evaluation of integrin/extracellular matrix signaling and its effects on vSMC proliferation through modulation of cell cycle regulatory proteins.

Finally, integral to the results presented in this dissertation, further experiments addressing cellular localization of activated ERKs, p27, and p21 will clarify mechanisms through which these proteins interfere with cell cycle progression in oxidatively injured vSMC. It is not only the expression levels of cell cycle regulatory proteins that are important for modulatory activity, but post-translational events such as the subcellular localization of proteins are relevant to their functions [108-110]. Given that allylamine injured vSMC display an altered kinetic profile of ERK activity, the events leading up to the proliferative advantage acquired by allylamine cells may be the result of alterations not only in ERK kinetics, but also in the cellular localization of activated ERKs. The cellular location of cdk inhibitors is an important component permitting the functional activities of these proteins. For example, contact inhibition in melanoma cells induced growth arrest through sustained nuclear localization of p21 [108]. As well, cytoplasmic relocalization of p27 in breast cancer cells inactivates the growth inhibitory properties of this protein [109]. Additionally, in differentiating myeloid cells, the

subcellular localization of p27 changes from nuclear to predominantly cytoplasmic and finally to perinuclear localization at progressive stages of hematopoiesis [110]. Thus, matrix-dependent altered cellular location of ERK1/2 and p21 represents an attractive hypothesis to explain the differences existing between proliferative rates of control an allylamine cells.

4.7. Summary

In summary, evidence is presented here that chronic oxidative stress alters expression of p27 and p21 in a matrix dependent manner. Antioxidant administration enhances the matrix dependent suppression of cdk inhibitors in vSMC that have undergone repeated cycles of oxidative injury. Differential ERK activation profiles and differential expression of cdk inhibitors, p27 and p21, in allylamine cells grown on plastic versus collagen indicate discrete alterations of integrin/ECM signaling in oxidatively injured vSMC. Thus, altered integrin/matrix regulation of cdk inhibitors (p27 and p21), ERKs, and further downstream signaling events may contribute to acquisition of proliferative phenotypes following oxidative injury by allylamine.

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