Angiotensin II-induced Apoptosis in Vascular Smooth Muscle Cells: 
Differential Effects in Human Saphenous Vein and 
Human Internal Mammary Artery

BY
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A THESIS

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Major Advisor

Dean
Abstract

Coronary artery bypass grafting serves as a viable treatment for arterial vascular occlusions. However, the likelihood for developing restenosis following coronary artery bypass grafting surgery remains a major threat. Subsequently, assessing the physiological differences of conduit bypass vessels has become a crucial topic for vascular research. The exact mechanisms underlying restenosis development are unknown, but many mediators, including angiotensin II, have been implicated.

The hypothesized will be examined that angiotensin II induces differential responses in the vascular smooth muscle cells (VSMCs) of human saphenous vein (SV) and human internal mammary artery (IMA). To test this hypothesis, VSMCs were isolated from surgical specimens of SV and IMA obtained from patients who had undergone coronary artery bypass graft surgery. SV and IMA VSMCs were serum starved for synchronization and treated with various concentrations of angiotensin II. Apoptosis was measured using annexin V-FITC and propidium iodide followed by flow cytometric analysis. Protein expression of Bax, Bcl-2, and phospho-p38 MAP kinase was examined by Western Blot.

The data revealed three major findings: (1) Angiotensin II induced significant apoptosis in VSMCs of the SV following 8 hours of treatment, whereas there was no pro-apoptotic effect of angiotensin II on VSMCs of the IMA. The effect of angiotensin II to induce apoptosis in SV VSMCs was supported by the up regulation of a pro-apoptotic protein, Bax, and decrease in the expression of a pro-survival protein, Bcl-2. Angiotensin
II had no effect on Bax protein expression in IMA VSMC, but increased Bcl-2 protein expression. (2) Angiotensin II induced the phosphorylation of p38 MAP kinase in SV VSMCs, whereas this effect was not observed in IMA VSMC, and (3) inhibition of p38 MAP kinase using SB 203580 decreased angiotensin II-induced apoptosis in SV VSMCs, thereby suggesting a linkage between p38 MAP kinase and angiotensin II-induced apoptosis. The current research demonstrated a differential response to angiotensin II-induced apoptosis between VSMCs of SV in comparison to IMA. The clinical significance of these findings warrants further investigations.
Acknowledgments

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Most importantly, I thank my parents for their eternal support throughout my education and guidance as my role models and best friends, and I am thankful for their sincerity, determination, and integrity.
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Abbreviations

ACE: angiotensin converting enzyme
Ang-II: angiotensin II
AT\(_n\): angiotensin II type \(n\) receptor (\(n\) specified as 1, 2, or 4)
BCA: bicinchoninic acid
CAD: coronary artery disease
CASMC: coronary artery smooth muscle cells
CD95: Fas ligand receptor
CVD: cardiovascular disease
Cyt-C: cytochrome-C
DNA: deoxyribonucleic acid
EGFR: epidermal growth factor receptor
ELISA: enzyme linked immunosorbent assay
ERK1/2: extracellular signal-regulated protein kinase 1 and 2
FADD: Fas-associated death domain
FASL: Fas ligand
FITC: fluorescein isothiocyanate
HDL: high-density lipoprotein
IH: intimal hyperplasia
IMA: internal mammary artery
IRS: insulin receptor signaling
JAK/STAT: Janus Kinases / Signal Transducers and Activators of Transcription
### Abbreviations (continued)

<table>
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<th>Abbreviation</th>
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<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PI3 kinase</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
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<tr>
<td>SB 203580</td>
<td>p38 MAPK pathway inhibitor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>VSMC</td>
<td>vascular smooth muscle cells</td>
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(A) Cardiovascular Disease

(1) Background

For over one century, cardiovascular disease (CVD) has been the leading cause of mortality within the United States, contributing to 38 percent or 1 in 2.6 deaths in 2002 (Figure 1). Surpassing many diseases which currently gain much attention in modern medical research, cardiovascular disease was the primary cause over 1,400,000 deaths, equating to an average of 1 death every 34 seconds. In 2005, the American Heart Association estimated the costs associated with cardiovascular diseases totaling $343.5 billion (American Heart Association 2005b). Although many factors contributing to cardiovascular diseases are indirectly controllable, such as regulating blood pressure and body weight, a great number of uncontrollable factors make cardiovascular disease a viable threat, including increasing age, gender, race, and heredity (Table 1) (American Heart Association 2005c). Evidently, not only are cardiovascular diseases costly to society, but the factors contributing to cardiovascular diseases are often not recognized. Therefore, targeting the molecular processes contributing to cardiovascular diseases has become a focal point for much of cardiovascular research.
Causes of Death in the United States

Cardiovascular Diseases -
Cancers -
Pulmonary Diseases -
Unintentional Injuries -
Pneumonia and Influenza -
Diabetes Mellitus -
AIDS / HIV -
Suicide -
Homicide -
Other -

Rate per 100,000 Population

Figure 1 – Comparison of Leading Causes of Death in the United States.
In the United States, cardiovascular disease (CVD) has been the leading cause of death for over one century. All data are age-adjusted for total U.S. Population (U.S. National Center for Health Statistics).
Risk Factors Contributing to Cardiovascular Diseases

<table>
<thead>
<tr>
<th>Controllable</th>
<th>Uncontrollable</th>
</tr>
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<tr>
<td>High Blood Pressure</td>
<td>Increasing Age</td>
</tr>
<tr>
<td>Tobacco Smoke</td>
<td>Male Sex</td>
</tr>
<tr>
<td>High Blood Cholesterol</td>
<td>Race</td>
</tr>
<tr>
<td>Low-Density Lipoproteins (LDLs)</td>
<td>Heredity / Family History</td>
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<tr>
<td>High-Density Lipoproteins (HDLs)</td>
<td></td>
</tr>
<tr>
<td>High-Serum Triglycerides</td>
<td></td>
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<tr>
<td>Body Weight</td>
<td></td>
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<tr>
<td>Diabetes Mellitus</td>
<td></td>
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Table 1 – Risk Factors Contributing to Cardiovascular Diseases.

According to the American Heart Association in a 2005 survey, the leading risk factors contributing to cardiovascular disease may be classified as controllable or uncontrollable to patients. Moreover, the more risks a person develops, the greater chances are that he or she will develop cardiovascular disease (American Heart Association 2005c).
(2) **Coronary Artery Disease**

Coronary artery disease (CAD), referring to an occlusion of the coronary artery, is the most common form of cardiovascular disease as well as one of the leading causes of morbidity and mortality within the United States. Treatment for arterial occlusion resulting from coronary artery disease is often alleviated through coronary artery bypass grafting. Although coronary artery bypass grafting is commonly practiced as a viable treatment for coronary artery disease, the invasive nature of this technique ironically leads to complications, such as restenosis or an occlusion to the grafted vessel functioning as a bypass conduit.

(B) **Coronary Artery Bypass Grafting**

(1) **Background**

Patients suffering from coronary artery disease are now faced with a number of treatment options, including coronary artery bypass grafting, medical therapy, and percutaneous coronary interventions through the use of bare-metal or drug-eluting stents (Ali and Davidoff 2006). Although all treatment options carry benefits and risks, coronary artery bypass grafting is most commonly prescribed for patients experiencing cardiovascular diseases with coronary artery disease. In the late 1960s, the successful introduction of the human saphenous vein (SV) to function as a bypass conduit to divert blood around occluded coronary arteries initiated an era of possibilities for treating CAD. Since its initial implantation in 1967, the human saphenous vein continues to be the most commonly used vessel to
function as a bypass conduit for patients experiencing CAD. One of the greatest limitations to SV bypass grafting, however, is the possible and likely development of intimal hyperplasia (IH) resulting in arterial occlusions. Fortunately, the use of the internal mammary artery (IMA) as a coronary artery bypass, although more technically challenging to graft, offers patients better opportunities for successful bypass recoveries due to higher patency rates in comparison to the SV (Loop et al. 1986).

Although functionally similar as bypass conduits in coronary artery bypass surgery, the SV and IMA inherently differ in response to growth factors, cytokines, and hormones (Frischknecht et al. 2006). For example, stimulation with growth factors such as platelet-derived growth factor (PDGF) generated significantly different results between both vessels in vascular smooth muscle cell (VSMC) proliferation, cell cycle progression, and cell death through apoptosis (Frischknecht et al. 2006). This finding suggests that VSMCs from the SV and IMA differ in their intrinsic properties. Since both SV and IMA vessels are commonly used in coronary artery bypass surgery, a sufficient understanding about the responses of these conduit vessels to biological stimuli is necessary in sustaining long-term treatment for cardiovascular diseases such as CAD. Therefore, assessing the balance between proliferation and apoptosis between both vessels may yield valuable information toward successfully using either vessel in coronary artery bypass surgery and combating complications including restenosis.
(2) Saphenous Vein

In May 1967, the first saphenous vein (SV) implantation for a coronary bypass in a human was performed by Dr. Garrett and colleagues (Garrett, Dennis, and DeBakey 1973). Nearly half a century later, the SV continues to remain a common conduit for coronary artery bypass procedures. Although this promising discovery may effectively provide treatment for patients experiencing restenosis, the SV inherently poses many challenges as a bypass conduit to the coronary artery. During the first year after bypass surgery, up to 15% of SV grafts occlude, and further revascularization is required for 19% of patients by 10 years and 31% of patients by 12 years after bypass surgery (Cameron, Davis, and Rogers 1995; Weintraub et al. 1994). Furthermore, patients undergoing additional bypass surgeries due to graft occlusion or rejection are subjected to greater morbidity and mortality rates (Hannan et al. 1990).

The complex mechanisms contributing to SV graft rejection are believed to involve thrombosis and an increase in the deposit of the extracellular matrix (Motwani and Topol 1998). Additionally, an imbalance between VSMC proliferation and apoptosis is supported as a factor leading to vascular occlusion although the exact parameters of apoptosis are not completely understood (Geng and Libby 2002; Isner et al. 1995; Kockx et al. 1994). Possibly due to an imbalance between VSMC proliferation and apoptosis, the processes of intimal hyperplasia and atherosclerosis may contribute to the development of restenosis and coronary

(3) Internal Mammary Artery

The necessity for more effective vessels than the SV to function as bypass conduits has also led to the discovery and use of the internal mammary artery (IMA) as a bypass graft. The IMA is preferably used as a bypass conduit when technically feasible due to its remarkable patency compared to the SV (Loop et al. 1986). In comparison with patients who received IMA grafts, patients with SV grafts were found to have 1.41 times the risk for late myocardial infarction, 1.25 times the risk for hospitalization due to cardiac-related irregularities, and 2 times the risk for bypass reoperation (Loop et al. 1986). The IMA, therefore, serves as the preferable bypass conduit although the SV is most commonly utilized in bypass surgery.

(C) Restenosis

(1) Background

The pathophysiology of restenosis describes a complex cascade initiated through an injury to the endothelial cell lining, which stimulates macrophage aggregation, platelet aggregation, fibrin deposition, and VSMC migration and proliferation (Carter et al. 1994; Libby and Simon 2001). In addition to VSMC proliferation, apoptosis has also been connected to the development of restenosis
(Malik et al. 1998; Cheng et al. 1995). Although the responsiveness of vessels toward developing restenosis varies depending upon the type of vessel used in coronary artery bypass grafting, restenosis is a common outcome following the invasive technique of bypass surgery. Furthermore, restenosis may occur over a time period of months to years, which generates challenges for treatment such as further operation to alleviate the occluded vessel in some patients. Therefore, hallmark processes contributing to restenosis, mainly intimal hyperplasia and atherosclerosis, are valuable targets for therapeutic intervention.

(2) Intimal Hyperplasia

Intimal hyperplasia (IH), a major mechanism contributing to restenosis, refers to the complex accumulation of new VSMC and the synthesis of extracellular matrix in intimal compartment of blood vessels. Unfortunately, the invasive techniques involved with coronary artery bypass grafting commonly result with IH. Several molecular cascades contribute to the complex development of IH, such as leukocyte-endothelial interactions that stimulate coagulation cascades leading to thrombus formation (Mitra, Gangahar, and Agrawal 2006). Additionally, VSMC proliferation and migration is thought to promote lesion formation (Mitra, Gangahar, and Agrawal 2006). While VSMC proliferation is believed to be a key event, apoptosis has also been detected as a contributing factor to the development of IH (Hayakawa et al. 1999). Although research has proposed valuable explanations, a complete understanding about the mechanisms governing IH is undefined.
Atherosclerosis, also a significant contributor to restenosis, is a clinical term describing the building up of arterial plaque resulting from the accumulation of fatty substances, cholesterol, cellular waste products, and various clotting materials in the epithelial lining of an artery (American Heart Association 2005a). Over time, atherosclerosis slowly causes the thickening, narrowing, and hardening of arteries, resulting with an increased susceptibility to arterial blockage by the rupturing of accumulated plaques. Myocardial infarction, stroke, and sudden cardiac death are all fatal consequences resulting from progressive atherosclerosis (Ross 1999).

Atherosclerosis is believed to be initiated through an initial injury to the epithelial lining of cardiovascular vessels which leads to cascade of events to facilitate atherosclerotic plaque formation. Additionally, plaque formation is promoted through the infiltration of monocytes, lipoproteins, and development of the foam cell, a hallmark for atherosclerosis. Atherosclerosis may also be characterized by the proliferation of VSMCs and increased extracellular matrix deposition, leading toward the development of an atheroma with a rich fibrous cap. Apoptosis, a term describing the process of programmed cell death, is also thought to play a crucial role in facilitating the rupture of the fibrous cap of atherosclerotic plaques leading toward stroke or myocardial infarction (American Heart Association 2005b; Isner et al. 1995). Consequently, regulating the development of
atherosclerosis may be mediated through targeting and therapeutically altering the balance between vascular smooth muscle cell proliferation and apoptosis.

(D) Apoptosis

(1) Background

In 1964, the term “programmed cell death” was introduced, suggesting that cell death follows a sequence of events leading toward self-destruction (Lockshin and Williams 1965). However, the term apoptosis was eventually used to describe programmed cell death. Apoptosis, a Greek term, has the literal meaning of “falling off” or “dropping off,” analogous to leaves falling from trees (Gewies 2003). Similar to leaves falling from trees, apoptosis is a naturally occurring event that is responsible for important roles such as regulating stable cell populations (Gewies 2003; Isner et al. 1995). Apoptosis, however, has also been identified as contributing to vascular diseases, including restenosis, atherosclerosis, and intimal hyperplasia (Bennett and Boyle 1998; Isner et al. 1995; Geng and Libby 2002). Thus, an understanding regarding apoptosis potentially has broad implications for treating cardiovascular diseases.

Kerr first described cell death in 1965 as consisting of two distinct classifications, apoptosis and necrosis, by differences in morphology (Kerr 1965). Apoptotic cells can morphologically be identified as dying through a highly regulated and coordinated process characterized by cell shrinking, deformation and...
loss of contact with adjacent cells, chromatin condensation, blebbing, plasma
membrane budding, and cell fragmentation into compact structures called apoptotic
bodies (Table 2) (Gewies 2003; Geng and Libby 2002). Apoptosis rarely elicits an
inflammatory response (Geng and Libby 2002). Cell death by necrosis, however,
occurs in a disorganized process indicated through cell swelling, loss of plasma
membrane, and the uncontrollable loss of cellular contents into the surrounding
environment to induce an inflammatory response (Table 2) (Gewies 2003).
Morphological Characteristic | Apoptosis | Necrosis  
--- | --- | --- 
Stimuli | External and Internal | External  
Number of Dying Cells | Individual | Group  
Death Process | Programmed | Random  
Cell Body | Shrinking | Swelling  
Plasma Membrane | Intact | Permeable  
Mitochondria | Intact; Releases Cytochrome-C | Broken  
Caspase Activity | Early Activation | Uncertain  
Inflammatory Response | Rare | Common  

*Table 2 — Comparison of Cellular Death by Apoptosis versus Necrosis.*

Cell death can be classified on the basis of morphological differences. The distinct processes of apoptosis and necrosis may be classified by morphological characteristics.
(2) Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway begins in the extracellular environment of a cell when a cellular stimulus or stimuli determine that a cell must die (Figure 2a). The extrinsic pathway is initiated with the binding of the Fas ligand (FasL) to its corresponding Fas ligand receptor (CD95), which results with the association and activation of additional complex of receptors known as the Fas ligand death domain (FADD) receptor family (Baker and Reddy 1998; Upstate Millipore 2006). Following receptor association and activation, the FADD receptor recruits the inactive procaspase-8/10 to the complex of death receptors, and the inactive form of procaspase-8/10 undergoes autocatalysis to become the activated caspase-8/10. Following activation, caspase-8 cleaves procaspase-3. Similar to procaspase-8/10, procaspase-3 next undergoes auto catalysis into the activated caspase-3. With the activation of caspase-3, the principle effector caspase of apoptosis responsible for the merging of both extrinsic and intrinsic pathways, subsequent steps in the extrinsic pathway cascade continue and ultimately conclude with apoptosis (Figure 2a) (Baker and Reddy 1998; Upstate Millipore 2006).
(3) Intrinsic Apoptotic Pathway

Unlike the extrinsic apoptotic pathway, the intrinsic apoptotic pathway is initiated through an internal cellular injury (Figure 2b). An internal cellular injury may activate several pro-apoptotic molecules and overcome the efforts of pro-survival molecules (Upstate Millipore 2006). More specifically, molecules belonging to the Bcl-2 protein family are categorized as either pro-apoptotic or pro-survival depending on activation or opposition to the intrinsic apoptotic pathway. For example, mitochondrial damage can initiate the intrinsic apoptotic pathway cascade, whereby the pro-apoptotic molecules overcome pro-survival molecules and release cytochrome C (Cyt-C) from the mitochondria. Cytochrome C subsequently continues a cascade of events to cleave procaspase-9 into the active caspase-9. Next, caspase-9 activates caspase-3, which terminates with apoptosis of the injured cell (Figure 2b) (Upstate Millipore 2006).
Figure 2: The Extrinsic (a) and Intrinsic (b) Apoptotic Pathways.

The apoptotic pathway consists of two divisions: the extrinsic division (a) and intrinsic division (b). While the extrinsic pathway is initiated by external stimuli such as the Fas ligand (FasL), the intrinsic pathway is initiated through internal cellular injuries. Following initiation, cascades of events lead to the convergence of extrinsic and intrinsic pathways at caspase 3, which ultimately result with apoptosis.
Members of the Bcl-2 protein family function to regulate apoptosis in mammalian cells. Categorization of the Bcl-2 protein family is based molecular classification as either pro-apoptotic or pro-survival qualities (Table 3) (Adams and Cory 1998). The pro-survival subgroup consists of approximately five members, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A1. Although the mechanism by which Bcl-2 pro-survival proteins inhibit pro-apoptotic proteins remains unclear, pro-survival molecules are believed to heterodimerize with certain pro-apoptotic proteins, causing a neutralization of apoptotic effects (Adams and Cory 1998; Minn et al. 1999; Oltvai, Milliman, and Korsmeyer 1993; Bennett 1999). Members of the Bcl-2 pro-apoptotic subgroup promote apoptosis and include Bax, Bak, Bad, Bik, Hrk, Bid, and Bcl-xs. Similarly to the Bcl-2 pro-survival proteins, the pro-apoptotic proteins are also thought to heterodimerize and neutralize opposing pro-survival proteins (Oltvai, Milliman, and Korsmeyer 1993; Bennett 1999; Bennett 1999). For example, Bad promotes apoptosis by displacing Bax from Bcl-2 and Bcl-xL (Yang et al. 1995). Ultimately, the propagation of the apoptotic pathway can be attributed to in imbalance of pro-apoptotic over pro-survival proteins (Adams and Cory 1998).
Table 3: Bcl-2 Protein Family Mediation over Apoptosis.

The Bcl-2 protein family functions to regulate the intrinsic apoptotic pathway via mediators which favor either pro-apoptotic or pro-survival (anti-apoptotic) reactivity. The pro-apoptotic mediators of apoptosis include Bax, Bak, Bad, Bik, Hrk, Bid, Bcl-xs, while the pro-survival mediators of apoptosis include Bcl-2, Bcl-xL, Mcl-2, Bcl-w, and A1. Ultimately, the propagation of the intrinsic apoptotic pathway can be attributed to an imbalance of pro-apoptotic over pro-survival mediators (Adams and Cory 1998).
(5) Actions of Apoptosis Promote Cardiovascular Diseases

An imbalance between pro-apoptotic and pro-survival proteins of the Bcl-2 family has been linked to the development of many cardiovascular diseases through promoting atherosclerosis (Kockx et al. 1998; Geng and Libby 2002; Bennett and Boyle 1998; Best et al. 1999) and intimal hyperplasia (Hayakawa et al. 1999). Therefore, the studies assessing Bcl-2 proteins, mainly the pro-survival protein Bcl-2 and pro-apoptotic protein Bax, may provide insight to the importance of the Bcl-2 protein family in mediating cardiovascular diseases.

(a) Apoptosis and Atherosclerosis

Although proliferation has been traditionally supported as the dominant mechanism associated with atherosclerosis, evidence now additionally supports apoptosis as contributing to atherosclerosis (Kockx et al. 1998; Geng and Libby 2002; Bennett 1999; Best et al. 1999). Apoptosis may promote the development of the lipid core center of an atherosclerotic plaque, a majority of which consists of dead cells (Geng and Libby 2002). Furthermore, apoptosis has also been hypothesized to affect the VSMCs responsible for stabilizing the fibrous cap of atherosclerotic plaques. Specifically, apoptosis may weaken and destabilize VSMCs associated with the fibrous cap of atherosclerotic plaques, thereby encouraging plaque rupture (Davies et al. 1994; Bennett and Boyle 1998). Data suggests that an imbalance between the pro-apoptotic Bax protein and pro-survival Bcl-2
protein increases apoptotic activity leading toward plaque rupture (Hayakawa et al. 1999).

(b) Apoptosis and Intimal Hyperplasia

Contradictory to apoptosis promoting atherosclerosis, the process of programmed cell death may serve as a protective measure against intimal hyperplasia. One proposed mechanism suggests that apoptosis may limit intimal hyperplasia through reducing foam cell formation, which is a factor normally promoting atherosclerosis (Geng and Libby 2002; Bennett and Boyle 1998). Consequently, the role of apoptosis on intimal hyperplasia formation requires further investigation to determine the many uncertainties surrounding this relationship.

(E) Angiotensin II

(1) Background

Angiotensin II (Ang-II), the major peptide hormone of the renin-angiotensin system (RAS), is considered a significant agent in contributing to the development of several cardiovascular diseases, including atherosclerosis, intimal hyperplasia, and restenosis. Angiotensin II functions as a potent vasoactive agent with a wide range of acute and chronic reactions. Acute stimulation with angiotensin II has been found to regulate vasoconstriction, salt/water homeostasis, and blood pressure (Mehta and Griendling 2006; Suzuki et al. 2002). Additionally, chronic stimulation
with angiotensin II has been implicated to mediate VSMC proliferation and apoptosis, ultimately leading toward vascular changes such as cardiac remodeling, restenosis, atherosclerosis, and intimal hyperplasia (Suzuki et al. 2002; Li et al. 2006a). Although angiotensin II has been traditionally regarded to induce proliferation in VSMCs, the exact mechanisms and action of angiotensin II on VSMCs vary by cell origin and remain unclear.

(2) Synthesis: The Renin-Angiotensin System

The renin-angiotensin system (RAS) is the term used to describe the primary processes through which angiotensin II is synthesized. Traditionally, angiotensin II synthesis has been described as an endocrine function whereby renin of the renal system acts on angiotensinogen of the hepatic system to produce the inactive angiotensin I for transportation via plasma (Figure 3) (Hardman JG, Limbird LE, and Gilman AG 2001). Once in contact with angiotensin converting enzyme (ACE), angiotensin I is cleaved into the activated angiotensin II (Figure 3) (Hardman JG, Limbird LE, and Gilman AG 2001). However, alternative means for synthesis have been established whereby angiotensin II may be produced directly by the conversion of angiotensinogen through interaction with tissue plasminogen activator, and cathepsin G, or angiotensin II synthesis may occur through the hydrolysis of angiotensin I by chymase and cathepsin G (Johnston and Risvanis 1997). Irrespective of the pathway involved in the synthesis of angiotensin II, angiotensin II reacts as a potent vasoactive substance with a wide range of physiological actions.
Figure 3: Renin-Angiotensin System (RAS).

The renin-angiotensin system synthesizes angiotensin II through the cleavage of the hepatic system-based angiotensinogen by renin from the renal system into angiotensin I. Next, angiotensin I is cleaved into its active substrate, angiotensin II, by the angiotensin converting enzyme (ACE).
(3) AT₁ and AT₂ Angiotensin II Receptors

Angiotensin II primarily interacts with the angiotensin II type 1 receptor (AT₁), which is strongly expressed on nearly all organs, including the liver, adrenals, brain, lung, kidney, heart, and VSMCs (Mehta and Griendling 2006). The AT₁ is classified as a seven-transmembrane G-coupled protein receptor comprised of an extracellular domain capable of binding angiotensin II and an internal domain responsible for signal transduction via the phosphorylation of amino acid residues on the receptor’s cytoplasmic tail. Once bound to AT₁, angiotensin II specifically induces the tyrosine kinase phosphorylation of serine/threonine residues on the receptor’s cytoplasmic tail to initiate cellular signaling cascades since AT₁ lacks intrinsic tyrosine kinase activity (Mehta and Griendling 2006; Taniguchi 1995).

Regulation over the effects of angiotensin II may be mediated through altering AT₁ receptor expression and desensitization. During acute exposure to angiotensin II, increasing concentrations of angiotensin II lead to the up regulation of AT₁ receptor expression; conversely, chronic exposure to angiotensin II results with the down regulation of AT₁ receptor expression (Mehta and Griendling 2006). Receptor desensitization also serves as a means for regulating the effects of angiotensin II on the AT₁ receptor. After stimulation between angiotensin II and its targeted tissue, the tissue may become desensitized to prevent further stimulation, resulting with the endocytosis of AT₁ receptors (Mehta and Griendling 2006).
Consequently, many proteins play a role in the process of receptor desensitization, resulting with a highly coordinated process for regulating angiotensin II.

Two additional cell surface receptors, AT\textsubscript{2} and AT\textsubscript{4}, are also expressed for angiotensin II. Although less is known about AT\textsubscript{4} functionality, AT\textsubscript{2} has been hypothesized to oppose AT\textsubscript{1} as well as exert anti-proliferative actions (Suzuki et al. 2002; Horiuchi, Akishita, and Dzau 1998; Mehta and Griendling 2006). Similar to the AT\textsubscript{1}, the AT\textsubscript{2} is classified as a seven-transmembrane G-coupled protein receptor with an extracellular domain capable of binding angiotensin II and an internal domain responsible for signal transduction via the phosphorylation of amino acid residues on the receptor’s cytoplasmic tail (Mehta and Griendling 2006). The AT\textsubscript{2} is highly expressed in fetal most fetal tissues, but expression rapidly declines shortly after birth (Shanmugam, Corvol, and Gasc 1996). Additionally, AT\textsubscript{2} expression has been detected in low populations in the kidney, lung, and liver, but the functional role between angiotensin II and AT\textsubscript{2} interaction in these tissues remains unclear. Similar to regulation over the AT\textsubscript{1}, the AT\textsubscript{2} may be regulated through the up regulation of receptor expression as well as receptor desensitization. While AT\textsubscript{2} functionality is not completely understood, the exact mechanisms responsible for AT\textsubscript{2} mediation continue to remain unclear as well.
(4) Signaling Pathways

Upon the binding of angiotensin II and corresponding receptor, several signaling pathways may become activated to regulate a wide range of angiotensin II-mediated physiological effects. The primary pathways activated by angiotensin II may be classified as four categories: (1) non-receptor tyrosine kinase pathways (including the Src, JAK/STAT, FAK, and Pyk2 pathways), (2) receptor tyrosine kinases pathways [including the platelet derived growth factor (PDGF), epidermal growth factor receptor (EGFR), and insulin receptor signaling (IRS) pathways], (3) G-protein coupled receptor pathway, and (4) mitogen-activating protein kinase (MAPK) pathways.

One of the most significant signaling pathways mediated by angiotensin II is the G-protein coupled pathway (Mehta and Griendling 2006). The G-protein coupled pathway is chiefly responsible for mediating vasoconstriction. Mechanistically, G-protein pathway initiation begins through the AT$_1$ receptor coupling with a series of G-protein complexes, which in turn signal downstream effectors through a cascade of events to activate phospholipase C (PLC). With the activation of PLC, inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) are produced and ultimately lead to an efflux of calcium into the cytoplasm, thus enhancing actin-myosin interaction and smooth muscle cell contraction (Mehta and Griendling 2006).
In addition to the G-protein coupled pathway, angiotensin II is responsible for activating the mitogen activated protein kinase (MAPK) pathway, which has been implicated to function in VSMC differentiation, migration, proliferation, and apoptosis (Sugden and Clerk 1997; Deschesnes et al. 2001; Grethe et al. 2004; Taniyama et al. 2004). MAPK pathway activation occurs through the activation of various kinases, including the extracellular signal-regulated kinase (ERK1/2), JNK, and p38 MAPK. Consequently, each of these kinases differently affects the unique outcome of MAPK pathway signaling.

Of great importance to this study, angiotensin II-induced phosphorylation of p38 MAP kinase has been associated with apoptosis (Sugden and Clerk 1997; Deschesnes et al. 2001; Grethe and Porn-Ares 2006; Taniyama et al. 2004). The significant molecule acting on the p38 MAP kinase pathway is the phosphorylated form of p38 MAP kinase, known as phospho-p38 MAP kinase, which has been linked to mediating VSMC cell survival, apoptosis, differentiation (Mehta and Griendling 2006). In addition, angiotensin II-induced phosphorylation of p38 MAP kinase can lead to the activation of the Akt kinase, which in return can affect glucose metabolism and protein synthesis (Nakagami et al. 2001). Consequently, the wide range of effects resulting from the angiotensin II-induced phosphorylation of p38 MAP kinase remains unclear.
(5) Role in Cardiovascular Disease and Atherosclerosis

Angiotensin II has been suggested to play a functional role in biological responses that elicit atherosclerosis and related cardiovascular diseases. Angiotensin II-induced cellular signaling cascades ultimately have been shown to promote vascular remodeling through the migration, hypertrophy, and proliferation of VSMCs (Cai, Lanting, and Natarajan 2004). Additionally, experimental evidence suggests that the activity of angiotensin II may be involved in altering the structure of the arterial wall, which may lead to atherosclerosis (Ferrario et al. 2004; Fernstrom, Farmer, and Ali 2005). Recent studies also demonstrate that angiotensin II encourages atherosclerosis by promoting monocyte migration, which may facilitate atherosclerosis by increasing monocyte-macrophage differentiation, foam cell formation, and extensive VSMC proliferation (Cai, Lanting, and Natarajan 2004). More specifically, angiotensin II has been shown to induce the macrophage-mediated oxidation of low-density lipoproteins (LDLs) and macrophage cholesterol biosynthesis, which are processes that lead to foam cell formation, an important hallmark in atherosclerosis development (Keidar et al. 1999; Keidar et al. 1995; Limor et al. 2005). In summary, evidence supports angiotensin II as an agent capable of inducing transmembrane signaling cascades leading toward the development of cardiovascular diseases and atherosclerosis.
Differential Responses in Angiotensin II-Induced Vascular Smooth Muscle Cells

Angiotensin II has traditionally been classified as a potent growth factor with ties to promoting VSMC proliferation in cardiovascular diseases such as atherosclerosis (Cai, Lanting, and Natarajan 2004). The role of angiotensin II as a pro-apoptotic agent, however, presents many challenges to previous studies. Nonetheless, recent evidence has been supportive that angiotensin II may not only induce VSMC proliferation, but angiotensin II may also exhibit additional actions by modulating VSMC apoptosis (Kockx et al. 1994; Li et al. 2006a).

(a) Angiotensin II-Induced Proliferation

Angiotensin II has been defined dominantly as a growth and proinflammatory agent capable of accelerating the development of atherosclerosis and cardiovascular diseases. Research supports angiotensin II as a significant agent for inducing proliferation in aortic VSMCs (Mangiarua, Galagedera, and Eastham 2001; Katz 1990). In atherosclerotic lesions, angiotensin II has been identified as an agent inducing aortic VSMC proliferation (Cai, Lanting, and Natarajan 2004). Additionally, the proliferative effects of angiotensin II were observed through the formation of the neointima in the rat aortic wall following balloon-catheter injury (Viswanathan et al. 1992). Proposed mechanisms for angiotensin II-induced VSMC proliferation are chiefly based on angiotensin II inducing proliferation through the \( \text{AT}_1 \) receptor (Viswanathan et al. 1992), while the \( \text{AT}_2 \) receptor is hypothesized as an \( \text{AT}_1 \) receptor antagonist (Sayeski et al.
With much support, angiotensin II is viewed as a potent growth agent capable of inducing VSMC proliferation.

(b) Angiotensin II-Induced Apoptosis

In addition to the well-documented effects of angiotensin II promoting growth through VSMC proliferation, research is rising in support of angiotensin II-induced apoptosis in VSMCs (Kockx et al. 1994; Li et al. 2006a; Li et al. 2006a). In VSMCs as well as endothelial cells and monocytes, angiotensin II-induced apoptosis has been detected to occur through an AT$_2$ receptor-mediated mechanism (Ono and Ishimitsu 2002; Jugdutt and Menon 2004; Touyz and Berry 2002). Additionally, angiotensin II-induced apoptosis in human VSMCs has been reported to activate the extrinsic apoptotic signaling pathway via the up regulation of FasL expression (Li et al. 2006a). However, more studies are needed to fully understand the mechanisms by which angiotensin II induces VSMC apoptosis.
(1) Background

The mitogen-activated protein kinase (MAPK) pathway has been indicated as a contributing factor to the induction of apoptosis associated with cardiovascular diseases (Deschesnes et al. 2001; Grethe et al. 2004; Taniyama et al. 2004). The MAP kinase pathway is composed serine and threonine kinases, which are activated in response to extracellular stimuli to induce dual phosphorylation on threonine and tyrosine residues (Cornelissen, Armstrong, and Holt 2004). Pathway activation can be activated through a range of stimuli, including G-protein coupled receptors, Fas ligands, and inflammatory cytokines, and MAP kinase pathway activation may commonly lead to the induction of apoptosis (Figure 4) (Herlaar and Brown 1999; Deschesnes et al. 2001). Specifically, regulation over the p38 MAP kinase pathway has been accomplished through the over expression of p38 MAP kinase as well as p38 MAP kinase inhibition using SB 203580 (Cornelissen, Armstrong, and Holt 2004; Grethe et al. 2004). Therefore, mediation via the over expression or inhibition of p38 MAP kinase serves as an important means for assessing the functional role of the p38 MAP kinase as related to cardiovascular disease and the induction of apoptosis.
Figure 4: p38 MAP Kinase Pathway Overview.

With a wide range of stimuli capable of kinase phosphorylation, the p38 MAP kinase pathway has been linked to cytokine production as well as the induction of apoptosis in VSMC. G-protein coupled receptors, inflammatory cytokines, the Fas ligand, and TNF have been reported to phosphorylate p38 MAP kinase.
(2) Role in Cardiovascular Diseases

The pathogenesis of several cardiovascular diseases has been linked to the p38 MAP kinase pathway. In rabbit VSMCs, balloon-induced vascular injury has demonstrated a sustained phosphorylation of p38 MAP kinase leading to intimal hyperplasia and vascular remodeling, while p38 MAP kinase inhibition has limited these vascular responses (Ju et al. 2002). Additional studies further support the inhibition of p38 MAP kinase leading to reduced intimal hyperplasia formation (Ohashi et al. 2000). Consequently, inhibiting the p38 MAP kinase may be a viable means for mediating restenosis and related cardiovascular diseases.

(3) Role in Apoptosis Induction

Although supported as contributing to the development of cardiovascular diseases, the association of p38 MAP kinase phosphorylation with apoptosis is of paramount importance in determining mechanisms underlying cardiovascular diseases. In human aortic endothelial cells, the p38 MAP kinase pathway was found to mediate doxorubicin-induced apoptosis, while pathway inhibition significantly decreased p38 MAP kinase-mediated apoptosis (Grethe et al. 2006). Furthermore, p38 MAP kinase phosphorylation induced by tumor necrosis factor (TNF) lead to the activation of caspase-3 and consequential induction of apoptosis (Grethe and Porn-Ares 2006). In the presence of p38 MAP kinase inhibitor, however, TNF-induced apoptosis via the p38 MAP kinase pathway became significantly attenuated (Grethe et al. 2004). In another study with human endothelial VSMCs, p38 MAP kinase was found to mediate TNF-induced
apoptosis through the phosphorylation of the pro-apoptotic protein, Bad, and the down regulation of the anti-apoptotic molecule, Bcl-xL (Grethe and Porn-Ares 2006). Consistent with previous studies, inhibition of p38 MAP kinase phosphorylation increased Bad protein expression while down regulating Bcl-xL expression. Thus, the role of p38 MAP kinase has been indicated in apoptotic cascades leading to the development of cardiovascular diseases.

(4) Angiotensin II Activation of p38 MAP Kinase Pathway in VSMC

Although the functional role of angiotensin II underlying proliferation in VSMCs is well documented, angiotensin II-induced VSMC apoptosis remains unclear. Angiotensin II-induced phosphorylation of p38 MAP kinase has been observed in human coronary artery smooth muscle cells (CASMC). Specifically, angiotensin II was found to negatively regulate DNA synthesis across the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathway for DNA synthesis through stimulating p38 MAP kinase (Kintscher et al. 2003). Furthermore, inhibition of the p38 MAP kinase resulted with DNA synthesis. These results, consequently, suggest the function role of the p38 MAP kinase pathway as an anti-proliferative and pro-apoptotic mediator for angiotensin II-induced signal transduction in VSMCs.

In another study, the well known phosphatidylinositol 3-kinase (PI3 kinase)/Akt proliferative pathway was suggested to be mediated by the p38 MAP kinase pathway in angiotensin II-induced VSMCs. Additionally, angiotensin II-
induced phosphorylation of p38 MAP kinase was attenuated with the addition of a p38 MAP kinase inhibitor (Taniyama et al. 2004). Thus, these data suggest the proliferative-inducing characteristics of angiotensin II may also be antagonized through the activation of the apoptotic p38 MAP kinase pathway in VSMCs.
Angiotensin II induces differential responses in the vascular smooth muscle cells (VSMCs) of human saphenous vein (SV) and human internal mammary artery (IMA). This hypothesis will be tested with the following three specific aims. In these studies, VSMCs were isolated from surgical specimens of SV and IMA obtained from patients who had undergone coronary artery bypass graft surgery. Three aims will be addressed:

1. **To assess the apoptosis of VSMCs in response to angiotensin II:** A time-course and dose-response assessment of apoptosis was measured using annexin V-FITC and propidium iodide followed by flow cytometric analysis to assess and compare the percentage of SV and IMA VSMCs undergoing apoptosis against a control (untreated) group. Also, protein expression of the pro-apoptotic protein, Bax, and the antagonistic pro-survival protein, Bcl-2, was examined by Western Blot to furthermore assess apoptosis.

2. **To examine the transmembrane signaling pathway involved in angiotensin II-mediated apoptosis of VSMCs:** Pathways previously implicated with angiotensin II-induced VSMC signaling as well as apoptosis was assessed for activation. Of interest to this study, activation and the role of p38 MAP kinase pathway was examined through detecting the phosphorylation of the p38 MAP kinase in response to angiotensin II.
To examine the effect of p38 MAPK inhibition on angiotensin II-induced apoptosis of VSMCs: Having established pathway(s) activated in response to angiotensin II, a pathway inhibitor(s) was used to detect the functional role of the pathway(s) on the induction of apoptosis. Specifically, the p38 MAP kinase, which was determined to be phosphorylated by angiotensin II, was inhibited, and VSMC apoptosis was measured using annexin V-FITC and propidium iodide followed by flow cytometric analysis.
II. MATERIALS AND METHODS

A. Human Saphenous Vein (SV) and Internal Mammary Artery (IMA) specimens and VSMC culture.

Surgical specimens of human SV and IMA were obtained from patients (mean age of 69.5 ± 2.7 yrs.) who had undergone coronary artery bypass graft surgery. The Institutional Review Board of Creighton University approved the proposed research and the informed consent was obtained from patients. Samples were collected freshly from the patients undergoing the surgery at the Nebraska Heart Institute, Lincoln, NE, preserved in a custodial solution, and immediately transported to the research laboratory. VSMCs from the samples were isolated under sterile conditions by gently mincing each vessel into small segments, approximately 1 cm² in surface area, and digesting segments with a digestion solution containing trypsin, elastase, collagenase, and Dulbecco’s modified Eagle’s medium (MediaTech, Inc., Herndon, VA) in a 37 °C water bath for 30 minutes. The isolated VSMCs were removed by centrifugation at 900 g for 10 minutes at 4 °C, suspended in Smooth Muscle Cell Media (ScienCell Research Laboratories, San Diego, CA), and incubated at 37 °C for 14-21 days. Once VSMCs achieved approximately 70-90% confluence with spindle-shaped morphology of hill-and-valley patterns, cells were passaged and used between passages four and seven. The purity of the isolated VSMCs was tested with positive immunostaining for smooth muscle α-actin and caldesmon.
B. **BrdU Colorimetric Cell Proliferation ELISA Assay**

VSMC proliferation can be quantified through using immunofluorescence to monitor DNA synthesis occurring during the synthesis phase (s-phase) of the cell cycle. Specifically, 5-bromodeoxyuridine (BrdU), an analogue to thymidine, can be incorporated into the DNA of proliferating cells in place of thymidine (Gratzner and Leif 1981). After its incorporation, a second fluorescent label with a high affinity for BrdU can be added to the proliferating VSMCs, and BrdU can be detected via a colorimetric ELISA immunoassay (Gratzner and Leif 1981). Therefore, VSMC proliferation was quantified with a colorimetric ELISA immunoassay to detect any significant changes in optical density (O.D.) between the angiotensin II-stimulated groups and the control groups.

A BrdU Colorimetric Cell Proliferation ELISA immunoassay kit (Rouche Applied Science, Indianapolis, IN) was used to quantify cell proliferation based on the measurement of BrdU incorporation during DNA synthesis. Cells were seeded into 96-well microplates at $5 \times 10^3$ cells/well (Coulter Particle Counter, Beckman Coulter, Inc., Fullerton, CA), serum starved for 24 hours, and stimulated using $1 \times 10^{-6}$, $1 \times 10^{-8}$, and $1 \times 10^{-10}$ M concentrations of angiotensin II (Sigma Aldrich Company, St. Louis, MO). Simultaneous to angiotensin II stimulation, cells were labeled with BrdU labeling solution (Rouche Applied Science, Indianapolis, IN) and incubated at 37 °C for 4, 8, 12, and 24 hours. Cells were centrifuged, and labeling medium was removed by gently tapping the 96-well microplates. FixDenat (Rouche Applied Science, Indianapolis, IN) was added to each well for
30 minutes at approximately 25 °C and removed. Cells were incubated with anti-BrdU-POD peroxidase substrate working solution (Rouche Applied Sciences, Indianapolis, IN) at room temperature for 90 minutes, removed, washed three times with Washing Solution (Rouche Applied Science, Indianapolis, IN). Cells were incubated with Substrate Solution (Rouche Applied Science, Indianapolis, IN) at room temperature for 20-30 minutes, followed by the addition of stopping solution (1M H₂SO₄) to each well for approximately 1 minute, and absorbance was measured at 450 nm (Bio-Rad Laboratories Microplate Reader 3550 with Microplate Manager 4.0 software, Bio-Rad Laboratories Research, Hercules, CA). VSMC proliferation was quantified and reported in optical density (O.D.).

C. Annexin V-FITC Apoptosis Assay

Apoptosis is a highly coordinated event associated with the homeostasis of cell populations, cellular growth and development, and the development of diseases. Apoptosis is characterized by a series of morphological changes, including the loss of the asymmetric plasma membrane and attachment, condensation of the nucleus, and DNA fragmentation. During the early phases of apoptosis, the loss of the asymmetric plasma membrane occurs. Specifically, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet to the outer leaflet of the plasma membrane. Consequently, PS exposure on the outer leaflet of the plasma membrane can be detected with Annexin V, which has a high affinity for PS (Vermes et al. 1995). Therefore, apoptosis can be characterized as early-phase apoptosis with the binding of annexin V only to
externalized PS (noted as annexin V + PI −). Following the translocation of PS to the outer leaflet of the plasma membrane, the apoptotic process continues into a late apoptotic phase, which can be characterized by the cleavage and fragmentation of DNA. To differentiate early-phase from late-phase apoptosis, propidium iodide (PI) is used to bind to nucleic acids once the plasma membrane is lost (Vermes et al. 1995). Conversely, late-phase apoptosis consisting of externalized PS in addition to fragmented DNA can be identified with the binding of both annexin V and PI (noted as annexin V + PI +.)

Therefore, apoptosis was assessed through the total of both early-phase apoptotic cells (annexin V + PI −) and late-phase apoptotic cells (annexin V + PI +). Apoptosis was expressed as a percentage of the total number of VSMCs measured through flow cytometric analysis.

An annexin V-FITC detection kit (BD Biosciences, San Jose, CA) was used to analyze phospholipid, phosphatidylserine (PS), externalization from the inner to outer cell membrane to quantify the percentage of cells undergoing apoptosis. SV and IMA VSMCs between passages four and seven were serum starved with Dulbecco’s modified Eagle’s medium for 24 hours, stimulated with 1 x 10⁶, 1 x 10⁸, and 1 x 10¹⁰ M concentrations of angiotensin II (Sigma Aldrich Company, St. Louis, MO) for zero (control), 4, 8, 12, and 24 hours. Cells were detached from culturing flasks using trypsin (Sigma Aldrich Company, St. Louis, MO), followed by the immediate addition of trypsin neutralizing solution (Sigma Aldrich
Company, St. Louis, MO), washed twice with cold PBS, and resuspended in 1x Annexin V Binding Buffer (BD Biosciences, San Jose, CA). Cells populations were counted using a Beckman Coulter Particle Counter (Beckman Coulter, Inc., Fullerton, CA). A total number of 3 x 10^5 cells were incubated with annexin V-FITC and propidium iodide (PI) (BD Biosciences, San Jose, CA) for 15 minutes at 37 °C. Cells labeled with Annexin V-FITC and propidium iodide were analyzed with flow cytometry (Becton Dickinson FACS Calibur), and 10,000 events were recorded. Data was analyzed using Cell Quest Pro software (BD Biosciences, San Jose, CA).

**D. Western Blot Assay**

Human SV and IMA VSMCs were analyzed between passages four and seven. For Bax, Bcl-2, and GAPDH analysis, VSMCs were serum starved with Dulbecco’s modified Eagle’s medium for 24 hours prior to all experiments for synchronization. To block the p38 MAP kinase pathway, VSMCs were incubated with 10 μM SB 203580 inhibitor (Sigma Aldrich Company, St. Louis, MO) for 30 minutes prior to angiotensin II stimulation. VSMCs were stimulated with 1 x 10^{-6} M concentration of angiotensin II (Sigma Aldrich Company, St. Louis, MO) and incubated at 37 °C for 4, 8, 12, and 24 hours. Similarly, for phospho-p38 MAP kinase, VSMC were stimulated with 1 x 10^{-6} M concentration of angiotensin II (Sigma Aldrich Company, St. Louis, MO) and incubated at 37 °C for 5, 15, 30, 60, and 120 minutes. Following stimulation with angiotensin II, VSMCs were detached from culturing flasks using typsin (Sigma Aldrich Company, St. Louis,
MO), followed by the immediate addition of trypsin neutralizing solution (Sigma Aldrich Company, St. Louis, MO). Subsequently, VSMCs were sonicated in cell lysis buffer containing 1 mL RIPA buffer (Sigma Aldrich Company, St. Louis, MO) and 10 μL protease inhibitor cocktail (Pierce Laboratories, Rockford, IL) on ice, and cell lysates were centrifuged at 4°C for 10 minutes at 14,000 RPM. The protein concentration of the supernatant was quantified using the Bicinchoninic Acid (BCA) Method (Pierce Laboratories, Rockford, IL). Protein samples were adjusted with a sample buffer (950 μL Laemmli Sample Buffer (Sigma Aldrich Company, St. Louis, MO) and 50 μL 2-mercapto-ethanol (Sigma Aldrich Company, St. Louis, MO) accordingly to equalize concentrations, and protein samples were heated at 95°C for 5 minutes. Protein samples were then loaded, equal in concentration (50-80 μg protein) per lane, onto a 4-20% Tris-Cl gel (Sigma Aldrich Company, St. Louis, MO) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Next, non-specific binding of the primary antibody was blocked using 5% blocking grade non-fat milk (Bio-Rad Laboratories, Hercules, CA) for 60 minutes at room temperature or overnight at 4°C. Membranes were then incubated 1:1,000 dilution with primary antibodies overnight at 4°C against Bax (eBioScience, San Diego, CA), Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and phospho-p38 MAP kinase (Cell Signaling Technology, Inc, Danvers, MA) in 5% blocking grade non-fat milk. Protein bands were visualized using a UVP Bioimaging System (UVP, Inc., Upland, CA) and quantified for
density using LabWorks Image Acquisition and Analysis, version 4.6 software (UVP, Inc., Upland, CA).

E. DNA / Cell Cycle Analysis using Vindelov’s Reagent

Human SV and IMA VSMCs were collected, counted (Coulter Particle Counter, Beckman Coulter, Inc., Fullerton, CA), and \(1 \times 10^6\) cells were placed in a 12 x 75 mm flow cytometry tube. VSMCs were centrifuged at 250 g for 5 minutes followed by supernatant removal by suction. Next, VSMCs were resuspended in 1 mL Vindelov’s reagent (100mL Tris Buffered Saline, Ribonuclease A (Sigma Aldrich Company, St. Louis, MO), propidium iodide (Sigma Aldrich Company, St. Louis, MO) and incubated at 4°C for 18 hours. Subsequently, VSMCs were analyzed using flow cytometry (Becton Dickinson FACS Calibur) with Cell Quest Pro software (BD Biosciences, San Jose, CA) and found to be 88.75% synchronized in the Go phase of the cell cycle (data not shown).

F. Statistical Analysis

All data are presented as mean ± SD. Data points were compared by nonparametric ANOVA one-way analysis of variance points and with Bonferroni’s correction. \(P\) values <0.05 are considered significant, and \(p\) values <0.01 and <0.001 are considered highly significant.
III. RESULTS

A. Angiotensin II-induced Proliferation in SV and IMA VSMC.

1. Angiotensin II-induced SV VSMC Proliferation: Time and Dose-dependent Responses.

To assess the functional role of proliferation, SV VSMCs between passages four and seven were grown to approximately 70-90% confluency, serum starved for 24 hours, and stimulated with three various concentrations of angiotensin II for various time periods. SV VMSC proliferation was detected via BrdU incorporation with an ELISA immunoassay (previously described in the Methods section.) The results for a dose and time-dependent response to angiotensin II-induced proliferation are shown (Figure 5, a—e).

Stimulation of SV VSMCs for 4 hours did not induce significant proliferation in comparison to the control (untreated) group (Figure 5, a). Additionally, angiotensin II stimulation for 8, 12, and 24 hours also resulted with no significant induction of proliferation in comparison to the control (untreated) groups (Figure 5, b-d). Angiotensin II, therefore, did not induce significant proliferation in SV VSMCs with 4, 8, 12, and 24 hours of angiotensin II treatment at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations.
Figure 5. Angiotensin II-induced SV VSMC Proliferation. SV VSMCs were stimulated with Angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations. Proliferation was measured using ELISA to detect the incorporation of BrdU. 

A. No significant differences in SV VSMC proliferation between control (untreated) and angiotensin II-induced groups were observed after 4 hours of stimulation.

B. Similar to 4 hours of stimulation, no significant differences in SV VSMC proliferation between the control (untreated) and angiotensin II-induced groups were detected after 8 hours of stimulation.
Figure 5 (continued). Angiotensin II-induced SV VSMC Proliferation. SV VSMCs were stimulated with Angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations. Proliferation was measured using ELISA to detect the incorporation of BrdU. 

A. No significant differences in SV VSMC proliferation between control (untreated) and angiotensin II-induced groups were observed after 12 hours of stimulation. 

B. As detected with 4, 8, and 12 hours of stimulation, no significant differences in SV VSMC proliferation between the control (untreated) and angiotensin II-induced groups were detected after 24 hours of stimulation.

To assess the functional role of proliferation, IMA VSMCs between passages four and seven were grown to approximately 70-90% confluency, serum starved for 24 hours, and stimulated with three various concentrations of angiotensin II for various time periods. IMA VMSC proliferation was detected via BrdU incorporation with an ELISA immunoassay (previously described in the Methods section.) The results for a dose and time-dependent response to angiotensin II-induced proliferation are shown (Figure 6, a—e).

Stimulation of IMA VSMCs for 4 hours did not induce significant proliferation in comparison to the control (untreated) group (Figure 6, a). Additionally, angiotensin II stimulation for 8, 12, and 24 hours also resulted with no significant induction of proliferation in comparison to the control (untreated) groups (Figure 6, b-d). Angiotensin II, therefore, did not induce significant proliferation in IMA VSMCs with 4, 8, 12, and 24 hours of angiotensin II treatment at 1 x 10^{-10}, 1 x 10^{-8}, and 1 x 10^{-6} M concentrations.
Angiotensin II-induced IMA VSMC Proliferation, 4 hrs. Stimulation

<table>
<thead>
<tr>
<th>Angiotensin II Concentration (M)</th>
<th>BrdU Incorporation (O.D.)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<tr>
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<tr>
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<td>$1 \times 10^{6}$</td>
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Angiotensin II-induced IMA VSMC Proliferation, 8 hrs. Stimulation

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<tr>
<th>Angiotensin II Concentration (M)</th>
<th>BrdU Incorporation (O.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.74</td>
</tr>
<tr>
<td>$1 \times 10^{10}$</td>
<td>0.72</td>
</tr>
<tr>
<td>$1 \times 10^{8}$</td>
<td>0.73</td>
</tr>
<tr>
<td>$1 \times 10^{6}$</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Figure 6. Angiotensin II-induced IMA VSMC Proliferation. IMA VSMCs were stimulated with Angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations. Proliferation was measured using ELISA to detect the incorporation of BrdU. A, No significant differences in IMA VSMC proliferation between control (untreated) and angiotensin II-induced groups were observed after 4 hours of stimulation. B, Similar to 4 hours of stimulation, no significant differences in IMA VSMC proliferation between the control (untreated) and angiotensin II-induced groups were detected after 8 hours of stimulation.
Figure 6 (continued). Angiotensin II-induced IMA VSMC Proliferation. IMA VSMCs were stimulated with Angiotensin II at 1 x 10^{-10}, 1 x 10^{-8}, and 1 x 10^{-6} M concentrations. Proliferation was measured using ELISA to detect the incorporation of BrdU. A. No significant differences in IMA VSMC proliferation between control (untreated) and angiotensin II-induced groups were observed after 12 hours of stimulation. B. Furthermore, no significant differences in IMA VSMC proliferation between the control (untreated) and angiotensin II-induced groups were detected after 24 hours of stimulation.
Angiotensin II-induced Apoptosis in SV VSMC.

1. Dose and Time-dependent Response to Angiotensin II-induced Apoptosis.

To assess the functional role of apoptosis, SV VSMCs between passages four and seven were grown to approximately 70-90% confluency, serum starved for 24 hours, and stimulated with various concentrations of angiotensin II for various time periods. SV VSMCs were assessed for apoptosis using annexin V, propidium iodide, and analyzed using flow cytometry (previously described in the Methods section.) The results for a dose and time-dependent response to angiotensin II-induced apoptosis are shown (Figure 7, a—e).

Stimulation of SV VSMCs for 4 hours induced apoptosis in comparison to the control (untreated) group (Figure 7, a). However, there was not a significant difference between the control group and the angiotensin II-stimulated groups. Angiotensin II, therefore, did not induce significant apoptosis in SV VSMCs with 4 hours of angiotensin II treatment at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations.

SV VSMCs were next untreated (control group) or treated for 8 hours with angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations (Figure 7, b). In comparison to the control (untreated) group, angiotensin II-induced apoptosis was observed with significance at $1 \times 10^{-8}$ M (** $p < 0.01$) and $1 \times 10^{-6}$ M (** $p < 0.01$) concentrations.
With 12 and 24 hours of stimulation, SV VSMCs treated with angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations were compared with control (untreated) SV VSMCs, and apoptosis was again assessed (Figure 7, c-d). By 12 hours of stimulation, the SV VSMCs from three variable concentrations did not produce significantly different percentages of apoptosis in comparison to the control group (Figure 7, c). Similarly, 24 hours of angiotensin II stimulation also at the same variable concentrations did not produce significantly different percentages of apoptosis between angiotensin II-treated groups and control groups (Figure 7, d). Although not significantly different than the control groups, increases in apoptosis were observed between the $1 \times 10^{-8}$ and $1 \times 10^{-6}$ M concentration groups and the control group (Figure 7, d). Thus, significant apoptosis was not produced with 12 or 24 hours of angiotensin II treatment at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations.

A significant difference between the control (untreated) and angiotensin II-induced SV VSMCs apoptosis was observed with 8 hours of $1 \times 10^{-6}$ M angiotensin II treatment. Therefore, a time-course assessment of SV VSMC apoptosis was next examined to compare the control (untreated) group with the angiotensin II-stimulated group at 4, 8, 12, and 24 hours of angiotensin II treatment with $1 \times 10^{-6}$ M concentration. Consequently, angiotensin II-induced apoptosis in SV VSMCs was significantly increased (** $p < 0.01$) only with 8 hours of treatment at $1 \times 10^{-6}$ M concentration (Figure 7, e).
Figure 7. Angiotensin II-induced SV VSMC Apoptosis. SV VSMCs were stimulated with Angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations. Apoptosis was measured using annexin V and propidium iodide labeling, and analyzed with flow cytometry. 

A, Angiotensin II-induced SV VSMC apoptosis was observed after 4 hours of stimulation, but significant differences were not found between the control (untreated) group and the three variable angiotensin II-stimulated groups. 

B, Significant angiotensin II-induced SV VSMC apoptosis was detected with 8 hours of stimulation at $1 \times 10^{-8}$ and $1 \times 10^{-6}$ M concentrations (** $p < 0.01$).
Angiotensin II Induced SV VSMC Apoptosis, 12 hrs. Stimulation

Angiotensin II concentration (M)

- Control
- $1 \times 10^{-10}$
- $1 \times 10^{-8}$
- $1 \times 10^{-6}$

% Apoptotic Cells

Angiotensin II Induced SV VSMC Apoptosis, 24 hrs. Stimulation

Angiotensin II concentration (M)

- Control
- $1 \times 10^{-10}$
- $1 \times 10^{-8}$
- $1 \times 10^{-6}$

% Apoptotic Cells

**Figure 7 (continued).** SV VSMCs were stimulated with angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations. VSMC apoptosis was measured using annexin V and propidium iodide, and analyzed with flow cytometry. **C-D,** Angiotensin II-induced SV VSMC apoptosis was observed at 12 and 24 hours of stimulation, but significant differences were not found between the control (untreated) group and the three variable angiotensin II-stimulated groups.
Figure 7 (continued). E. SV VSMCs were stimulated with angiotensin II at 1 x $10^{-6}$ M concentrations. VSMC apoptosis was measured using annexin V and propidium iodide, and analyzed with flow cytometry. The time-course stimulation of SV VSMCs with 1 x $10^{-6}$ M concentration angiotensin II induced the up regulation of apoptosis, peaking with significant apoptotic activity at 8 hours of stimulation in comparison to the control (untreated) group. Following 8 hours of stimulation, no significance was observed in angiotensin II-induced apoptosis at 12 and 24 hours (** $p < 0.01$).
SV VSMCs were treated with $1 \times 10^{-6}$ M concentration angiotensin II for 4, 8, 12, and 24 hours for comparison against control (untreated) group to assess the expression of the pro-apoptotic Bax protein.

Using Western Blot analysis (Figure 8, a), Bax protein expression appeared to be up regulated with 4 and 8 hours of stimulation, while down regulation occurred at 12 and 24 hours of stimulation to conclude with near-control levels of Bax protein expression. Thus, angiotensin II at $1 \times 10^{-6}$ M concentration appeared to up regulate Bax protein expression to climax at 8 hours of stimulation, while thereafter Bax protein expression declined to near-control levels at 24 hours of stimulation. Also using Western Blot analysis, GAPDH expression was detected to demonstrate equal quantities of protein were compared between all the control (untreated) and variable angiotensin II-stimulated groups (Figure 8, b).

With the Western Blot detection of increasing levels of Bax protein in response to $1 \times 10^{-6}$ M concentration angiotensin II (Figure 8, a-b), the previously collected data was analyzed using densitometry analysis to quantify Bax protein expression (Figure 8, c). Bax was up regulated with significance (** $p < 0.01$) after 8 hours of treatment, followed by a decrease in Bax protein expression at 12 and 24 hours. Thus, angiotensin II significantly induced the up regulation of the Bax protein at 8 hours.
**Figure 8. Angiotensin II-induced Up Regulation of Bax Expression.** SV VSMCs were stimulated with angiotensin II at $1 \times 10^{-6}$ M concentration, and the pro-apoptotic Bax protein was assessed at 4, 8, 12, and 24 hours for comparison against the control (untreated) group. *A,* Western Blot analysis demonstrated an up regulation of Bax at 4 and 8 hours of stimulation, while down regulation occurred at 12 and 24 hours of stimulation to conclude with near-control levels of Bax expression. *B,* GAPDH expression was detected for the SV VSMCs analyzed in Figure 8, *a* to demonstrate equal quantities of protein between all the control (untreated) and angiotensin II-stimulated groups. *C,* Densitometry analysis was applied to the SV VSMCs previously stimulated with angiotensin II (*Figure 8, *a-b*), quantifying the up regulation of the Bax protein at 8 hours as significantly different from the control (untreated) group ($** p < 0.01$).
3. **Down Regulation of Bcl-2 Expression in Response to Angiotensin II.**

SV VSMCs were treated with $1 \times 10^6$ M concentration angiotensin II for 4, 8, 12, and 24 hours for comparison against the control (untreated) group to assess Bcl-2 protein expression.

Western Blot analysis detected an overall decrease in Bcl-2 protein expression, beginning at 4 hours through 24 hours of stimulation with $1 \times 10^6$ M concentration of angiotensin II (*Figure 9, a*). Additionally, Western Blot analysis was further used to assess GAPDH expression for the SV VSMCs analyzed in *Figure 9, a*, and demonstrated equal quantities of protein between the control and angiotensin II-stimulated groups (*Figure 9, b*). 

Following Western Blot analysis, which suggested decreasing levels of Bcl-2 in response angiotensin II at $1 \times 10^6$ M concentration (*Figure 9, a*), the previously collected data was next analyzed using densitometry analysis to quantify Bcl-2 protein expression (*Figure 9, c*). Bcl-2 was down regulated with significance at 8 hours ($p < 0.01$), 12 hours ($p < 0.001$), and 24 hours ($p < 0.001$) hours of stimulation with angiotensin II (*Figure 9, c*).
Figure 9. Angiotensin II-induced Down Regulation of Bcl-2 Expression. A, At 1 x 10^{-6} M concentration, angiotensin II induced the down regulation of anti-apoptotic Bcl-2 protein expression in SV VSMCs at 4, 8, 12, and 24 hours of stimulation in comparison to the control (untreated) group. B, GAPDH expression for the SV VSMC analyzed in Figure 9, A was assessed to demonstrate equal quantities of protein between the control and angiotensin II-stimulated groups. C, Densitometry analysis was used on the SV VSMC data collected in Figure 9, A to quantify a significant reduction in Bcl-2 protein expression between the control (untreated) and angiotensin II-stimulated groups at 4 hours, 8 hours, 12 hours, and 24 hours (* p < 0.05, ** p < 0.01, *** p < 0.001).
C. Effect of Angiotensin II on the Apoptosis of IMA VSMCs.


IMA VSMCs between passages three and seven were grown to approximately 70-90% confluency, serum starved for 24 hours, and stimulated with 1 $\times$ 10$^{-10}$, 1 $\times$ 10$^{-8}$, and 1 $\times$ 10$^{-6}$ M concentrations of angiotensin II for 4, 8, 12, and 24 hours. To assess the responsiveness of IMA VSMCs to angiotensin II-induced apoptosis, IMA VSMC apoptosis was detected using annexin V, propidium iodide, and analyzed using flow cytometry (previously described in Methods section.)

In contrast to the angiotensin II-induced apoptosis detected in SV VSMCs, an angiotensin II-induced apoptotic response was not detected with 1 $\times$ 10$^{-10}$, 1 $\times$ 10$^{-8}$, and 1 $\times$ 10$^{-6}$ M concentrations of angiotensin II during any of the time-course intervals (Figure 10, a-d). These data, therefore, suggest that angiotensin II-induced apoptosis does not occur in IMA VSMCs. Additionally, these data demonstrate a differential response to angiotensin II-induced apoptosis between VSMCs of the SV and IMA.
Figure 10. IMA VSMC Resistance to Angiotensin II-induced Apoptosis. IMA VSMCs were stimulated with angiotensin II at $1 \times 10^{-10}$, $1 \times 10^{-8}$, and $1 \times 10^{-6}$ M concentrations. Apoptosis was measured using annexin V and propidium iodide, and analyzed with flow cytometry. A, No significant difference in IMA VSMC apoptosis was detected between the angiotensin II-stimulated and control (untreated) groups at 4 hours of stimulation. B. Similar to the data collected in Figure 10, a, no significant difference between the angiotensin II-stimulated and control (untreated) IMA VSMC groups was detected at 8 hours of stimulation.
Figure 10 (continued). IMA VSMCs were stimulated with angiotensin II at 1 x 10^{-10}, 1 x 10^{-8}, and 1 x 10^{-6} M concentrations. Apoptosis was measured using annexin V and propidium iodide, and analyzed with flow cytometry. C, Consistent with data collected in Figure 10, a-b, significant differences in apoptosis were not detected between the angiotensin II-stimulated and control (untreated) IMA VSMC groups at 12 hours of stimulation D. During 24 hours, no significance differences in apoptosis between the angiotensin II-stimulated and control (untreated) groups was not detected in IMA VSMCs.
As another indication for pro-apoptotic activity, IMA VSMCs were assessed for Bax protein expression at 4, 8, 12, and 24 hours of stimulation for a comparison between the control (untreated) and angiotensin II-stimulated groups at $1 \times 10^{-6}$ M concentration (previously described in Methods section).

Western Blot analysis suggested that Bax protein expression was not effected in response to angiotensin II treatment at $1 \times 10^{-6}$ M (Figure 11, a). Specifically, angiotensin II did not appear to up regulate Bax protein expression during 4, 8, 12, and 24 hours of stimulation. Also using Western Blot analysis, GAPDH expression was detected and demonstrated equal quantities of protein between all the control and angiotensin II-stimulated groups (Figure 11, b).

Following Western Blot analysis, densitometry analysis was used to quantify Bax protein expression in response to angiotensin II (Figure 9, c). Densitometry analysis demonstrated that Bax protein expression was not significantly different between the control (untreated) and angiotensin II-stimulated groups at 4, 8, 12, and 24 hours of stimulation.
IMA VSMC Resistance to Angiotensin II-induced Up Regulation of Bax. IMA VSMCs were stimulated with 1 x 10^{-6} M concentration of angiotensin II for 4, 8, 12, and 24 hours. **A**, To assess apoptotic activity in response to angiotensin II, Bax protein expression was detected using Western Blot analysis to compare the control (untreated) and angiotensin II-treated groups. **B**, GAPDH expression was detected using Western Blot analysis to demonstrate equal quantities of protein between the control (untreated) and angiotensin II-treated groups of IMA VSMCs. **C**, Densitometry analysis was applied to the data collected in Figure 11, **A**, which quantified Bax expression. Densitometry analysis demonstrated that no significant difference between the control (untreated) and angiotensin II-treated IMA VSMC groups.
3. Up Regulation of Bcl-2 Expression in Response to Angiotensin II

To assess that anti-apoptotic activity was antagonistic to Bax protein activity in IMA VSMC, the anti-apoptotic Bcl-2 protein expression was next detected. Bcl-2 protein expression was detected 4, 8, 12, and 24 hours of angiotensin II stimulation for comparison against the control (untreated) group (previously described in Methods section).

Through the use of Western blot analysis, Bcl-2 protein expression was suggested to be up regulated with angiotensin II treatment at $1 \times 10^{-6}$ M concentration. Bcl-2 protein expression appeared to increase in a time-dependent manner beginning at 4 hours of stimulation through 24 hours of stimulation (Figure 12, a). Additionally, GAPDH expression demonstrated equal quantities of protein were analyzed between the control (untreated) and angiotensin II-treated groups (Figure 12, b).

The Western Blot data revealing an up regulation of Bcl-2 protein in response to angiotensin II in IMA VSMCs (Figure 12, a) was next analyzed for statistical differences between the control (untreated) and angiotensin II-treated groups using densitometry (Figure 12, c). In comparison to the control group, significant up regulation of the Bcl-2 protein was demonstrated at 12 (p < 0.05) and 24 (p < 0.01) hours of stimulation in a time-dependent manner.
Figure 12. Angiotensin II-induced Up Regulation of Bcl-2 in IMA VSMCs. IMA VSMCs were untreated (control) and stimulated with 1 x 10^{-6} M concentration of angiotensin II for 4, 8, 12, and 24 hours. 

A, to assess anti-apoptotic activity in response to angiotensin II, Bcl-2 expression was detected using Western Blot analysis to compare the control (untreated) and angiotensin II-treated groups. 

B, GAPDH expression was detected using Western Blot analysis to demonstrate equal quantities of protein between the control and angiotensin II-treated groups of IMA VSMCs. 

C, Densitometry analysis was next applied to the data collected in Figure 12, a, to quantify Bcl-2 expression. Densitometry analysis demonstrated that significant differences between the control (untreated) and angiotensin II-treated IMA VSMC groups existed at 12 and 24 hours of stimulation (*p < 0.05, **p < 0.01).
D. Angiotensin II-induced Up Regulation of Phospho-p38 MAP Kinase Activity in SV VSMC.

The p38 MAP kinase pathway has been implicated in the induction of VSMC apoptosis. Although associated with VSMC apoptosis, the affects of angiotensin II on inducing the phosphorylation of the p38 MAP kinase are unknown. Having previously demonstrated angiotensin II-induced apoptosis at $1 \times 10^6$ M concentration in SV VSMCs, the p38 MAP kinase pathway was next assessed as a possible mechanism responsible for the observed, angiotensin II-induced SV apoptosis.

Using Western Blot analysis, the response of angiotensin II on the phosphorylation of p38 MAP kinase in SV VSMC was assessed by comparing the control (untreated) and angiotensin II-treated groups using $1 \times 10^6$ M concentration of angiotensin II (Figure 13, a). Although several time-course intervals were examined (data not shown), the phosphorylation of p38 MAP kinase was most dominantly active at 5, 15, 30, 60, and 120 minutes of stimulation with $1 \times 10^6$ M concentration of angiotensin II. Western Blot analysis revealed the up regulation of phospho-p38 MAP kinase beginning at 5 minutes through 30 minutes. Following the 30-minute measurement interval, phospho-p38 MAP kinase appeared to be down regulated during the 60 and 120 minutes of angiotensin II-stimulation. Also using Western Blot analysis, GAPDH expression demonstrated equal quantities of protein were compared between the control and angiotensin II-stimulated groups (Figure 13, b).
Densitometry analysis was next performed to quantify significant differences in phospho-p38 MAP kinase activity between the control (untreated) and angiotensin II-treated groups of IMA VSMCs (Figure 13, c). Densitometry analysis demonstrated that phospho-p38 MAP kinase activity was significantly upregulated ($p < 0.05$) to a maximal kinase activity following 30 minutes of angiotensin II treatment. At 60 and 120 minutes of treatment, densitometry analysis also quantified a decrease in phospho-p38 MAP kinase activity.
Figure 13. Angiotensin II-induced Up Regulation of Phospho-p38 MAP Kinase in SV VSMCs. SV VSMCs were untreated (control) and stimulated with $1 \times 10^{-6}$ M concentration of angiotensin II for 5, 15, 30, 60, and 120 minutes. A, To assess phosphorylation of p38 MAP kinase in response to angiotensin II, phospho-p38 MAP kinase was detected using Western Blot analysis to compare the control (untreated) and angiotensin II-treated groups. The phosphorylation of p38 MAP kinase appeared to be up regulated between 5 and 30 minutes of angiotensin II stimulation, while down regulation occurred at 60 and 120 minutes. B, GAPDH expression using Western Blot analysis demonstrated equal quantities of protein had been examined between the control (untreated) and angiotensin II-treated groups of SV VSMCs. C, Densitometry analysis was next applied to the data collected in Figure 13, a, to quantify phospho-p38 MAP kinase expression. Densitometry analysis demonstrated that a significant increase in phospho-p38 MAP kinase expression existed between the angiotensin II-treated SV VSMC groups at 30 minutes of stimulation and the control (untreated) ($p < 0.05$).
E. Effect of p38 MAP Kinase Inhibitor on the Apoptosis of SV VSMCs.

1. Down Regulation of Angiotensin II-induced Apoptosis.

   To assess if the observed angiotensin II-induced phosphorylation of p38 MAP kinase in SV VSMCs lead to apoptosis, p38 MAP kinase in SV VSMCs was inhibited using SB 203580, a known p38 MAP kinase inhibiting agent, and stimulated again with $1 \times 10^{-6}$ M concentration of angiotensin II. In response to p38 MAP kinase pathway inhibition, apoptotic activity was first assessed with an annexin V-based apoptosis assay using flow cytometry.

   Following kinase inhibition for 30 minutes using SB 203580, SV VSMCs were untreated (control) or stimulated with $1 \times 10^{-6}$ M angiotensin II for 4, 8, 12, and 24 hours (as described in the Methods section). Annexin V-based apoptosis analysis suggested that the previously detected angiotensin II-induced apoptosis in SV VSMCs had been reduced such that significance no longer existed between the untreated (control) and angiotensin II-stimulated groups (Figure 14). This data suggests, therefore, that p38 MAP kinase inhibition, using SB 203580, limited angiotensin II-induced SV VSMC apoptosis.
Figure 14. Down Regulation of Angiotensin II-induced Apoptosis in p38 MAP Kinase-inhibited SV VSMCs. The p38 MAP kinase pathway was inhibited in SV VSMCs using SB 203580. Following pathway inhibition, SV VSMCs were untreated (control) or stimulated with angiotensin II at $1 \times 10^{-6}$ M concentration for 4, 8, 12, and 24 hours to detect apoptotic activity via annexin V detection. Although apoptotic activity was observed during all time intervals, significance was not detected between the time intervals.
2. Down Regulation of Angiotensin II-induced Bax Expression.

To furthermore examine the role of the p38 MAP kinase inhibition on angiotensin II-induced apoptosis, SV VSMCs were next assessed for pro-apoptotic Bax protein expression. The p38 MAP kinase was inhibited, using SB 203580, and then stimulated with angiotensin II at $1 \times 10^6$ M concentration. Using the same time intervals as assessed without p38 MAP kinase inhibition (Figure 8, a), Bax protein expression was detected during 4, 8, 12, and 24 hours of stimulation with $1 \times 10^6$ M concentration of angiotensin II.

Western Blot analysis was used to assess the protein expression of Bax in response to p38 MAP kinase inhibition (Figure 15, a). Data obtained from the Western Blot analysis suggested that p38 MAP kinase resulted with a drastically decreased expression of the Bax protein in comparison to the untreated (control) and angiotensin II-treated SV VSMC groups. Thus, p38 MAP kinase inhibition appeared to reduce angiotensin II-induced Bax protein up regulation (shown in Figure 8, a).

Next, densitometry analysis was applied to quantify the observed decrease in Bax protein expression between the control (untreated) with the p38 MAP kinase-inhibited and angiotensin II-treated groups of SV VSMCs (Figure 15, c). Densitometry analysis demonstrated that Bax protein expression was significantly ($p < 0.001$) down regulated in response to p38 MAP kinase inhibition, which opposes the previously observed up regulation of the Bax.
protein expression prior to p38 MAP kinase inhibition with SB 203580 (Figure 8, a). Thus, these data suggest the p38 MAP kinase pathway may contribute to SV VSMC apoptosis through the up regulation of the pro-apoptotic Bax protein.
Figure 15. Down Regulation of Angiotensin II-induced Bax Expression in p38 MAP kinase-inhibited SV VSMCs. The p38 MAP kinase was inhibited in SV VSMCs using SB 203580. Following kinase inhibition, SV VSMCs were stimulated with angiotensin II at $1 \times 10^{-6}$ M concentration for 4, 8, 12, and 24 hours to assess Bax protein expression. A, Western Blot analysis demonstrated a decrease in Bax protein expression between the control (untreated) group in comparison to the p38 MAP kinase-inhibited and angiotensin II-treated groups. B, GAPDH expression was next detected using Western Blot analysis to demonstrate equal quantities of protein between the control (untreated) and angiotensin II-treated and p38 MAP kinase-inhibited groups of SV VSMCs. C, Densitometry analysis demonstrated that a significant difference in Bax expression existed between the control (untreated) groups in comparison to the p38 MAP kinase-inhibited and angiotensin II-treated groups (*** $p < 0.001$).
3. **Up Regulation of Angiotensin II-induced Bcl-2 Expression.**

   In opposition to the pro-apoptotic activity of the Bax protein, the anti-apoptotic activity of the Bcl-2 protein was next examined in response to p38 MAP kinase inhibition, using SD 203580, and angiotensin II stimulation at 1 x 10^-6 M concentration. Applying the same time intervals as previously used for Bax protein detection in response to p38 MAP kinase inhibition (*Figure 15*) as well as initially used to determine Bcl-2 protein expression without p38 MAP kinase pathway inhibition (*Figure 9, a*), Bcl-2 expression in the presence of the p38 MAP kinase inhibitor was detected during 4, 8, 12, and 24 hours of stimulation with 1 x 10^-6 M concentration of angiotensin II.

   Through Western Blot analysis, the protein expression of Bcl-2 was detected in response to p38 MAP kinase inhibition (*Figure 16, a*). Western Blot analysis demonstrated that p38 MAP kinase inhibition resulted with an increase in Bcl-2 protein expression in comparison to the untreated (control) and angiotensin II-treated SV VSMC groups. Therefore, the up regulation of Bcl-2 protein expression in response to p38 MAP kinase inhibition opposes the previously detected down regulation of Bcl-2 protein expression observed prior to p38 MAP kinase inhibition (shown in *Figure 9, a*).
Quantification of Bcl-2 protein expression detected through Western Blot analysis (*Figure* 16, *a*) was accomplished through a densitometry analysis (*Figure* 16, *c*). In response to p38 MAP kinase inhibition, Bcl-2 protein expression was up regulated following angiotensin II treatment after 8 hours, while significant (*p* < 0.001) up regulation was observed after 12 and 24 hours of treatment. In furtherance, these data oppose the previously detected down regulation of the Bcl-2 molecule without the p38 MAP kinase inhibitor, SB 203580 (*Figure* 9, *a-c*). These data, therefore, support the concept that the p38 MAP kinase may promote SV VSMC apoptosis through the down regulation of the anti-apoptotic Bcl-2 protein (*Figure* 9, *a-c*) in addition to the up regulation of the pro-apoptotic Bax protein (*Figure* 8, *a-c*).
Figure 16. Up Regulation of Angiotensin II-induced Bcl-2 in p38 MAP Kinase-inhibited SV VSMCs. The p38 MAP kinase was inhibited in SV VSMCs using SB 203580. Following kinase inhibition, SV VSMCs were stimulated with angiotensin II at $1 \times 10^{-6}$ M concentration for 4, 8, 12, and 24 hours, and Bcl-2 protein expression was measured. A, Although light in band expression, Western Blot analysis indicated an increase in Bcl-2 expression between the control (untreated) groups in comparison to the p38 MAP kinase inhibited and angiotensin II-treated groups. B, GAPDH expression was next detected using Western Blot analysis to demonstrate equal quantities of protein between the control and angiotensin II-treated groups of SV VSMCs. C, Densitometry analysis was then applied to the data collected in Figure 16, a, to quantify Bcl-2 protein expression. Densitometry analysis demonstrated that angiotensin II-induced SV VSMCs, initially blocked with the p38 MAP kinase inhibitor, up regulated Bcl-2 protein expression in a time-course dependent pattern in comparison to the control (untreated) groups. Significance was observed in comparison between the control (untreated) group with the 12 and 24 hour groups ($* p < 0.01$, $** p < 0.05$).
IV. DISCUSSION

The unique physiological characteristics of the saphenous vein (SV) in comparison to the internal mammary artery (IMA) have many implications on the development of restenosis following coronary artery bypass grafting. Consequently, understanding the mechanisms contributing to restenosis as well as intimal hyperplasia and atherosclerosis in the SV and IMA has become a significant area for cardiovascular research. The current research, therefore, aims to gain a heightened understanding about the differential properties associated with vascular smooth muscle cells (VSMCs) of the SV in comparison to the IMA in response to the widely reactive and unclear effects of angiotensin II.

In summary, this study has revealed three major findings: (1) Angiotensin II induced significant apoptosis in VSMCs of the SV following 8 hours of treatment, whereas there was no pro-apoptotic effect of angiotensin II on VSMCs of the IMA. The effect of angiotensin II to induce apoptosis in SV VSMCs was supported by the up regulation of a pro-apoptotic molecule, Bax, and decrease in the expression of a pro-survival protein, Bcl-2. Angiotensin II had no effect on Bax expression in IMA VSMC, but increased Bcl-2 expression. (2) Angiotensin II induced the phosphorylation of p38 MAP kinase in SV VSMCs, whereas this effect was not observed in IMA VSMC, and (3) inhibition of p38 MAP kinase, using SB 203580, decreased angiotensin II-induced apoptosis in SV VSMCs, thereby suggesting a linkage between p38 MAP kinase and angiotensin II-induced apoptosis. The current research, therefore, demonstrated a differential response to angiotensin II-induced apoptosis between VSMCs of SV in comparison to IMA.
Restenosis resulting from coronary artery disease is commonly treated with coronary artery bypass grafting. Although the IMA and SV are both routinely used as conduit bypass vessels, long term results vary depending on which vessels are implanted. Patency rates of IMA grafts are higher in comparison to those of the SV, indicating intrinsic differences between the vessels (Loop et al. 1986). Additionally, previous reviews from this laboratory have focused on the differences between the SV and IMA during the complex development of intimal hyperplasia, which serves as a hallmark to the development of restenosis (Mitra, Gangahar, and Agrawal 2006; Mitra and Agrawal 2006). Although VSMC proliferation is a defining process occurring during restenosis, much importance also remains in examining the functional role of apoptosis as a differential response between the SV and IMA VSMCs. Consequently, apoptosis is often overlooked as a mechanism contributing to the development of cardiovascular diseases and restenosis (Bennett and Boyle 1998; Best et al. 1999; Geng and Libby 2002; Kockx et al. 1998).

Angiotensin II has been a contributing agent to the pathogenesis of cardiovascular diseases, including restenosis, primarily as a potent proliferative agent to VSMCs. Research has identified angiotensin II as inducing both proliferative and apoptotic activities, thereby creating some controversy in the functional role of angiotensin II. For example, in the carotid artery following balloon catheter-induced injury, angiotensin II was observed to induce VSMC proliferation in the cross-sectional area of the neointima, media, (Daemen et al. 1991; van Kleef, Fingerle, and Daemen 1996), and mesenteric vessels (Su et al. 1998). Furthermore, increased VSMC DNA replication in the neointimal and medial layers of carotid artery was also observed in response to angiotensin II stimulation,
supporting its role as a proliferative agent (deBlois et al. 1996). Conversely, however, angiotensin II-induced apoptosis has been detected to occur through an AT₂ receptor-mediated mechanism in human endothelial cells, myocytes, and vascular smooth muscle cells (Ono and Ishimitsu 2002; Jugdutt and Menon 2004; Touyz and Berry 2002). Additionally, angiotensin II-induced apoptosis in human VSMCs has been reported to activate the extrinsic apoptotic signaling pathway via the up regulation of FasL expression (Li et al. 2006a). Also supportive of angiotensin II-induced apoptosis, recent findings have linked myocardial apoptosis (Foo et al. 2006) through a mechanism involving the activation of G-protein coupled receptors (Satoh et al. 2006). Therefore, the functional role of angiotensin II as a proliferative and apoptotic agent remains controversial although many recent studies are supporting angiotensin II-induced VSMC apoptosis. In agreement with many of these recent reports, this research observed angiotensin II as an agent capable of inducing apoptosis in SV VSMCs (Figure 7, b), while angiotensin II-induced VSMC proliferation was not detected in SV and IMA (Figures 5-6).

The differential responsiveness to angiotensin II-induced apoptosis in SV verses IMA VSMCs was supported through the expression of the pro-apoptotic Bax protein. Angiotensin II induced the up regulation of Bax protein expression in SV VSMCs with significance, furthermore supporting the novel concept of angiotensin II-induced apoptosis occurring in SV VSMCs. In contrast, however, IMA VSMCs lacked significant Bax protein expression when stimulated with identical concentrations of angiotensin II. Consequently, these findings continue to support differential properties existing between
SV and IMA VSMCs through the up regulation of the Bax protein in SV VSMCs (Figure 17).

In contrast to the pro-apoptotic functions of the Bax protein, the Bcl-2 protein functions to antagonize Bax with anti-apoptotic functionality. Consequently, Bcl-2 protein expression also differed in response to identical concentrations of angiotensin II. While angiotensin II induced the down regulation of Bcl-2 protein in SV VSMCs, Bcl-2 protein expression was up regulated in VSMCs of the IMA. The paramount finding through this research, therefore, suggests that SV VSMCs differ from IMA VSMCs in response to angiotensin II-induced apoptosis at $1 \times 10^{-6}$ M concentration as supported through annexin V-based apoptosis detection and Western Blot analysis of Bax and Bcl-2 protein expressions (Figure 18).

Having demonstrated the differential response to angiotensin II-induced apoptosis between the SV and IMA VSMCs, the activation of a signaling pathway with the capability of inducing SV VSMC apoptosis was next identified. The p38 MAP kinase pathway has been correlated to the pathogenesis of cardiovascular disease (Li et al. 2006b). Although pathway activation was assessed in response to angiotensin II treatment via the JAK/STAT pathway and the ERK1/2 pathway (data not shown), significant activation and up regulation was only observed to occur across the p38 MAP kinase pathway. In comparison to IMA VSMCs, however, phosphorylated p38 MAP kinase was not observed (data not shown). These data, therefore, not only continue to support differential properties existing between SV and IMA VSMCs, but this data identifies the angiotensin II-induced activation of the p38 MAP kinase pathway in SV VSMCs.
Figure 17. **SV vs. IMA VSMC Bax Expression with Angiotensin II at 1 x 10^{-6} M concentration.** Angiotensin II induced the significant up regulation of Bax protein expression in SV VSMCs at 8 hours of stimulation. In IMA VSMCs, however, angiotensin II did not induce the significant up regulation of Bax protein expression (**p < 0.001**).

Figure 18: **SV vs. IMA VSMC Bcl-2 Expression with Angiotensin II at 1 x 10^{-6} M concentration.** Angiotensin II induced the down regulation of Bcl-2 protein expression in SV VSMCs. In IMA VSMCs, conversely, angiotensin II induced the up regulation of Bcl-2 expression (*p < 0.05, **p < 0.01, ***p < 0.001).
In support of a differential response existing between SV and IMA VSMCs, apoptosis induction and p38 MAP kinase pathway activation have been identified as uniquely different events occurring between the SV and IMA VSMCs in response to angiotensin II. Possibly of most significance to this study, however, may be the correlation between SV VSMC apoptosis and p38 MAP kinase pathway phosphorylation, suggesting that activation via phosphorylation of p38 MAP kinase does, indeed, contribute to SV VSMC apoptosis as demonstrated with the p38 inhibitor, SB 203580. Following p38 MAP kinase pathway inhibition with SB 203580, the apoptotic-inducing effects of angiotensin II were not observed with significance in SV VSMCs (Figure 19). Consistent with this research, another recent study conducted by Li et al. has linked p38 MAP kinase inhibition to improved cardiac functionality through reducing myocardial apoptosis (Li et al. 2006b). Thus, our finding of a reduction of apoptosis in SV VSMCs may be supported through recent connections between p38 MAP kinase pathway inhibition and reductions to apoptosis.

Also indicative of p38 pathway inhibition leading to reduced apoptosis, Bax protein expression was significantly down regulated in a time-dependent manner, while Bcl-2 protein expression was significantly up regulated in a similar time-dependent manner in response to the p38 MAP kinase inhibitor, SD 203580 (Figure 20). Therefore, this observation of the down regulation of Bax protein expression in combination with up regulation of Bcl-2 protein expression in response to p38 MAP kinase inhibition supports the opposing data collected under similar conditions without inhibition of the p38 MAP kinase pathway.
Figure 19: Down Regulation of Angiotensin II-induced SV VSMC Apoptosis with p38 MAP Kinase Inhibitor. With p38 MAP kinase pathway inhibition, the significant angiotensin II-induced up regulation in apoptosis after 8 hours was not observed (** $p < 0.01$).
Figure 20: Down Regulation of Angiotensin II-induced SV VSMC Bax Expression with p38 MAP Kinase Inhibitor. In response to the p38 MAP kinase inhibition, Bax protein expression was significantly down regulated in a time-dependent manner, suggesting that p38 MAP kinase-induced SV VSMC apoptosis may be mediated via a Bax-dependent mechanism (**p < 0.01, ***p < 0.001).
Taken together, these data suggest that angiotensin-II induced SV VSMC apoptosis is mediated via the p38 MAP kinase pathway, and angiotensin II-induced SV VSMC apoptosis involves interaction with Bax and Bcl-2 proteins indicative of the intrinsic branch of the apoptotic pathway. In summary, reductions in apoptosis, the down regulation of the Bax protein, and the up regulation of the Bcl-2 protein in response to p38 MAP kinase inhibition have only been demonstrated in SV VSMCs, supporting the existence of differential properties between SV and IMA VSMCs in angiotensin II-induced apoptosis mediated by the p38 MAP kinase pathway. Additionally, these data support the novel concept that the p38 MAP kinase pathway contributes to SV VSMC apoptosis induced by angiotensin II.

Although this study supports the novel role of angiotensin II-induced SV VSMC apoptosis, limitations exist to the in vivo applicability of these findings. First, the homeostatic and physiologically-occurring concentrations for angiotensin II falls have been reported to be within the range of $1 \times 10^7$ and $1 \times 10^8$ (Mehta and Griendling 2006). Consequently, the angiotensin II concentration of $1 \times 10^{-6}$ M applied in this study to assess the in vitro responsiveness of angiotensin II-induced SV VSMC apoptosis would most likely not be physiologically possible. However, in the experiments protease inhibitors were not used. Since many cells including VSMCs are known to release proteases which can degrade angiotensin II, the final concentration of angiotensin II in this system is uncertain. Therefore, it is likely that this in vitro system could be similar to what may be observed under in vivo conditions.
Of additional importance, significant angiotensin II-induced SV VSMC apoptosis was also detected at $1 \times 10^{-8}$ M concentration, although SV VSMC apoptosis was most significantly different from the control (untreated) groups at $1 \times 10^{-6}$ M concentrations. These findings, therefore, also suggest that an angiotensin II-induced SV VSMC apoptotic response may be physiologically possible in vivo at $1 \times 10^{-8}$ M concentration although the $1 \times 10^{-6}$ M concentration was used to examine the most significant in vitro SV VSMC responsiveness to angiotensin II. Nonetheless, future investigations are necessary to continue defining the relationship between angiotensin II and VSMC apoptosis.
V. CONCLUSION

Since the likelihood for developing restenosis following coronary artery bypass grafting surgery remains a viable threat, assessing the physiological differences of conduit bypass vessels has become a crucial topic for vascular research. With much recognition of the challenges in addressing the differential properties of bypass conduit vessels, the current research demonstrates a differential response to *in vitro* angiotensin II-induced apoptosis between SV and IMA VSMCs.

Consequently, these novel findings not only demonstrate differential physiological properties between the SV and IMA VSMCs, but also raise question as to the functional role of angiotensin II-induced VSMC apoptosis. Traditionally being regarded as a potent mitogen with proliferative effects on nearly all VSMCs, angiotensin II has been demonstrated to affect VSMC of the SV differently than those of the IMA. The current research, therefore, demonstrated a differential response to angiotensin II-induced apoptosis between VSMCs of the SV in comparison to the IMA. The clinical significance of these findings warrants further investigations. Thus, these results support the hypothesis that differential physiological properties do, indeed, exist between SV and IMA VSMCs in response to *in vitro* angiotensin II-induced apoptosis.
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Ref Type: Internet Communication


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