Angiotensin II produces endothelial dysfunction by simultaneously activating eNOS and NAD(P)H oxidase

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TABLE OF CONTENTS

Figures and Legends

Appendices

Abstract

Blockade of the renin-angiotensin system lowers the rate of cardiovascular events in patients at risk for vascular disease and also improves endothelial function but the mechanism remains unclear. HUVECs were stimulated with Ang II (100 nM). Ang II produced a 2-fold increase in O_2^- production, which was measured by lucigenin-enhanced chemiluminescence. This increase was blocked by NAD(P)H oxidase inhibitor DPI, but not by eNOS inhibitor L-NAME. Ang II increased monocyte adhesion to ECs by 4.5-fold, and this increase was blocked by candesartan (AT1 receptor antagonist), DPI, L-NAME, wortmannin (PI3K inhibitor), dominant negative-AKT, and p22phox siRNA. Dominant active-AKT increased adhesion by 1.5-fold. Our findings indicate that the simultaneous activation by Ang II of eNOS and NAD(P)H oxidase leads to endothelial activation. This process can partially explain the therapeutic benefits of reducing the action of Ang II.

Résumé

Le blocage du système Rénin-Angiotensine abaisse le niveau d'évènements cardiovasculaires chez les patients à risque pour les maladies vasculaires et améliore aussi la fonction endothéliale, mais ce mécanisme demeure non élucidé. Les cellules endothéliales (HUVECs) sont stimulées avec Ang II (100 nM). Ang II produit une augmentation double en production d'O_2^-, qui est mesuré par la chemiluminescence induite par la lucigenine. Cette augmentation est bloquée par l'inhibiteur de la NAD(P)H oxydase DPI, mais pas par l'inhibiteur de eNOS, le L-NAME. Ang II augmente l'adhésion aux monocytes des CEs de 4.5 fois, et cette augmentation est bloquée par le candesartan (le récepteur antagoniste de AT1), le DPI, le L-NAME, la wortmannin (l'inhibiteur de la PI3K), le dominant négatif-AKT, et le p22phox si ARN. Le dominant actif-AKT augmente l'adhésion de 1.5 fois. Nos résultats indiquent que l'activation simultanée par Ang II de eNOS et la NAD(P)H oxydase mène à une activation endothéliale. Ce processus peut expliquer partiellement les bénéfices thérapeutiques de la réduction de l'action de Ang II.

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Abbreviations

Ang II: angiotensin II

RAS: renin-angiotensin system

ARB: angiotensin II receptor blocker

ACEi: angiotensin converting enzyme inhibitor

AKT/PKB: serine/threonine kinase-Akt/ protein kinase-B

HUVEC: human umbilical vein endothelial cell

EC: endothelial cell

SMC: smooth muscle cell

eNOS: endothelial nitric oxide synthase

NO: nitric oxide

O_2^-: superoxide

ROS: reactive oxidative species

NAD(P)H: nicotinamide adenine dinucleotide (phosphate)-oxidase

PI3K: phosphoinositide-3 kinase

DPI: diphenyleneneiodonium chloride

L-Name: N_ω-Nitro-L-arginine methyl ester hydrochloride

Wm: wortmannin

Cd: candesartan

TNF-α: tumor necrosis factor-α

1. Introduction

Cardiovascular diseases encompass malfunctions of the system that comprises the heart and the blood vessels of the entire body including the brain. Cardiovascular disease accounts for the death of more Canadians than any other disease. In 2002 cardiovascular disease accounted for 74,626 Canadian deaths, specifically, 32% of all male deaths and 34% of all female deaths (1). When broken down, 54% of all cardiovascular deaths are due to coronary artery disease; 21% to stroke; 16% to other forms of heart diseases and the remaining 9% to vascular problems such as high blood pressure and hardening of the arteries. Furthermore, cardiovascular diseases cost the Canadian economy over $18 billion a year and cause a major burden on the health care system (2).

Environmental and genetic risk factors of cardiovascular diseases have been identified with the help of epidemiological studies conducted over the past 50 years. The interaction between risk factors has also been extensively studied. Atherosclerosis is a pathological condition that is the cause of various vascular events such as stroke, peripheral arterial disease, coronary artery disease (CAD), and most of the cardiovascular morbidity and mortality in the current Western world. Epidemiological studies indicate that the prevalence of atherosclerosis is increasing world-wide due to the adoption of Western life-style and is likely to reach epidemic proportions in the coming decades (3). Defining the cellular and molecular mechanisms of diseases such as atherosclerosis has proven to be a bigger challenge due to the complex and involved nature of such pathologies (4).

One of the components involved in cardiovascular diseases such as atherosclerosis is the endothelium, for it is the barrier between blood and vascular tissue. Endothelial dysfunction is an initial event in atherosclerosis and contributes to diseased states such as hypertension, hyperlipidemia and diabetes (5). Endothelial dysfunction is characterized by altered anticoagulant and anti-inflammatory properties, and impaired modulation of vascular growth. Also, there

is a lack of physiological release of nitric oxide (NO), which hinders maintenance of the vessel wall. Amongst the regulators of the vascular endothelium function is angiotensin II (Ang II), a component of the renin-angiotensin system (RAS) (6). The RAS has long been identified as an important regulator of blood pressure (renal salt and water regulation) and cardiovascular hemodynamics.

The main effector of the RAS, Ang II, has been linked to vascular cell growth, differentiation, gene expression. Ang II raises blood pressure directly via vascular receptors and indirectly via facilitation of the vasoconstrictor actions of the sympathetic nervous system. Ang II acts through two receptors: AT1R and AT2R, but AT1R is dominant (7). Early research on Ang II concerned its role in the development of hypertension. More recent investigation of Ang II has focused on its role in atherosclerosis, myocardial infarction, vascular and myocardial remodeling and congestive heart failure (8). Ang II has major effects on cardiovascular structure, and reducing its effects has potential clinical benefits. Angiotensin II receptor blockers (ARBs, i.e. candesartan, losartan, valsartan) or angiotensin converting enzyme inhibitors (ACEi's i.e. quinapril, captopril, ramipril, perindopril) have been commonly prescribed to patients for decades to reduce excessive vasoconstriction and also have protective effects on the endothelium by large scale clinical trials (9), (10), (11).

Given that in the field of Ang II signaling, pathways have been studied more in vascular smooth muscle cells than in endothelial cells, and that the mechanism behind the endothelial protective effect resulting from Ang II inhibition remains unclear, we chose to investigate Ang II signaling in ECs. Our research undertaking focused on the oxidative signaling mechanisms of Ang II. More specifically, our studies focused on explaining the paradox of why Ang II-induced Akt activation and NO production have a harmful rather than a beneficial effect on the endothelium, as NO is known to improve endothelial function.

Ang II promotes the formation of reactive oxygen species (ROS) mainly by three systems: xanthine oxidase, nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide (phosphate)-oxidase (NAD(P)H oxidase); the later system is the one of particular interest. ROS have a physiological role in the vasculature such as promoting growth in the case of SMCs; however, when they are present in excess, they contribute to cardiovascular pathologies (8).

Oxidative stress has been linked to endothelial dysfunction, inflammation and monocyte infiltration. Redox sensitive genes play a role in the expression of pro-inflammatory adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), which are involved in the initiation and progression of atherosclerosis (12). NAD(P)H oxidases have been shown to be the predominant source of ROS production in the vasculature, especially in pathological conditions (13). Oxidative stress has also been linked to endothelial dysfunction and increasing pro-inflammatory gene expression (14). In ECs, superoxide production increases adhesion molecule expression which results in monocyte infiltration. Many functions of the endothelium are altered by the presence of excessive ROS. The most well known is endothelium-dependent vasorelaxation, which is impaired by a loss of NO bioactivity in the vessel wall (15).

In particular, oxygen radical production has been shown to be an important component of Ang II-mediated cardiovascular disease (16). Thus, inhibiting Ang II-induced ROS production in ECs could be of therapeutic value. Interestingly, groups such as Harrison *et al.* are working on possibilities of developing drugs that directly inhibit Nox (NAD(P)H oxidases) activation and in turn inhibit oxidative stress (17). The NAD(P)H oxidase enzyme complex is a major source of O_2^- and it is found in both ECs and SMCs. Furthermore, Ang II has been shown to stimulate NAD(P)H oxidase activation in the vascular endothelium and

VSMCs (18), (16), (19). H_2O_2 produced by NAD(P)H oxidase activates a number of downstream targets such as c-src, p38 MAPK, JNK and Akt (15).

Akt is a serine/threonine kinase that regulates cell survival and protein synthesis and is key to several signaling cascades including those of VEGF, insulin and estrogen. It is activated by a number of growth factors and cytokines in a PI3K–dependent manner. In SMCs, Ang II-induced O_2^-, and consequently H_2O_2 production, leads to the phosphorylation and activation of Akt. This Akt activation is inhibited by diphenyleneneiodonium chloride (DPI) suggesting a role for NAD(P)H oxidase (19). This pathway contributes to vascular hypertrophy and is thus pro-atherogenic. In response to shear stress, one of Akt's targets is eNOS: Akt has been shown to phosphorylate and activate eNOS (at residue Ser-1177) which then leads to NO production (20), (21).

NO and eNOS are of interest in vascular signaling as they play important roles in maintaining normal status of the vessel wall. NO alone leads to improved endothelial function, beneficial anti-inflammatory and thus anti-atherosclerotic response. Growing evidence indicates that unlike smooth muscle cells, EC's contain eNOS as well as NAD(P)H oxidase; thus, there is the potential for stimulants such as Ang II (as proposed by our studies) to trigger the simultaneous production of superoxide anion, O_2^- (from NAD(P)H oxidase) and NO (from eNOS). Together, O_2^- and NO can produce the potent oxidant peroxynitrite, eliminating NO's beneficial anti-inflammatory effects and resulting in endothelial activation and dysfunction.

2. Literature Review

2.1. The Renin-Angiotensin system (RAS)

The RAS, a system composed of enzymes and effector proteins, functions as a regulator of blood pressure, water, salt homeostasis and can affect the CNS, kidneys, heart and vasculature. Renin, an aspartyl protease, is released from the juxtaglomerular cells of the kidney, under conditions of salt/volume loss or by sympathetic activation. Once released, it cleaves angiotensinogen, a liver-derived precursor molecule to yield the inactive decapeptide Ang I. Angiotensin converting enzyme (ACE), released from the pulmonary system, converts Ang I by cleavage to the active octapeptide hormone Ang II (22). ACE, by acting as kininase II, also affects the kallikrein-kinin systems by inactivating kinins, including bradykinin. A recently identified carboxypeptidase, ACE2, cleaves one amino acid from either Ang I or Ang II, decreasing Ang II levels and increasing the metabolite Ang 1–7. Thus, the balance between ACE and ACE2 is a factor controlling Ang II levels (22).

The ACE pathway is not the only mechanism for generating Ang II, although it is the primary Ang II generating enzyme. Non-ACE pathways including cathepsin G, chymase, tonin, and tissue plasminogen activator (t-PA) have been reported (23) and play important functional roles in human blood vessels (24). In the heart, the majority of Ang I is converted by chymase. Bader M. *et al.* showed that tissues such as the brain, kidneys, adrenals, heart and vasculature contain RAS components and are thus capable of producing Ang II locally (25). Furthermore, local Ang II is more closely related to the vascular inflammatory response (26).

Ang II, whether produced by the ACE or the non-ACE mechanisms, raises blood pressure directly via vascular receptors and indirectly via facilitation of the vasoconstrictor actions of the sympathetic nervous system. Aldosterone secretion or renal tubule site regulation regulates the osmo- and volume- effects of Ang II,

such as, water and salt retention. Interestingly, central mechanisms have been identified as inducing natriuretic effects (27).

The RAS is of particular clinical interest as it is associated with the pathophysiology of hypertension, alterations in the vasculature, kidney and heart, including nephrosclerosis and post-infarction remodeling. Extensive clinical evidence shows that reducing RAS activity in turn reduces cardiovascular events in patients at risk for vascular disease (28), (29). Examining the individual molecules of the RAS and their related pathways helped initiate a new method of treating cardiovascular ailments. For example, inhibiting Ang II via ACEi's and ARB's has been shown to effectively treat hypertension, congestive heart failure, and coronary artery disease (27). In a large scale clinical study, Trial on Reversing Endothelial Dysfunction (TREND), treating patients with coronary artery disease using the ACEi quinapril significantly improved their vasodilatory response to acetylcholine in their coronary arteries (9). The findings from this trial suggest that Ang II strongly affects the endothelium *in vivo* and that inhibiting Ang II improves cardiovascular health partly by improving the state of the endothelium (9).

Similarly, the large scale randomized Heart Outcomes Prevention Evaluation (HOPE) study, evaluated the effects of the ACEi ramipril on cardiovascular events in patients with risk factors such as atherosclerosis, diabetes, hypertension, hypercholesterolemia, vascular disease, or congestive heart failure (11). Investigators observed improvements among all subgroups treated with ramipril measured by a reduction in the occurrence of myocardial infarction (20%), stroke (32%), cardiac arrest (37%), and cardiovascular death (16%) (11). Interestingly, patients treated with ramipril had a reduced incidence of new onset type II diabetes (34%) (11).

2.1.1. Angiotensin III, IV and (1-7)

The other angiotensin metabolites, Ang III (2-8), Ang IV (3-8) and the Ang (1-7) fragment are generated from Ang I and II. Aminopeptidase A cleaves Ang II to yield Ang III. Ang IV is formed when aminopeptidase N cleaves Ang III (30). Ang III and IV have been shown to increase Ang II-induced inflammation in the vasculature; however their effect is thought to be minor compared to Ang II's (31). Ang III is an active metabolite which acts through AT1 and AT2 receptors, and causes similar effects to Ang II, such as increasing blood pressure, releasing vasopressin and water consumption (27). The physiological role of Ang IV and its receptor AT4 remain elusive. The heptapeptide Ang (1-7) is also an active RAS product with its own specific receptor, recently identified in mouse kidney cells (32). It has the ability to stimulate the release of vasopressin, prostaglandins and NO; however, it does not affect the thirst response nor does it change aldosterone levels. Ang (1-7) has emerged as an angiotensin of interest as it has been shown to have cardiovascular and baroreflex actions that oppose those of Ang II in that it has vasodilator properties (33).

2.1.2. Angiotensin II receptors

It is likely that reducing Ang II results in vasculo-protective effects that benefit the endothelium. Angiotensin II acts via two types of cell surface receptors: Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors. Both AT1 and AT2 are polypeptides that contain approximately 360 amino acids. However, they are functionally distinct with a sequence homology of only 30% (29). Most of the studies that have been conducted pertain to the AT1 receptor. In the human vascular system, the most predominantly expressed receptor is the AT1 in vascular smooth muscle cells. AT1 receptors are also expressed in heart, lung, brain, liver, kidney tissue and adrenal glands (7). Both forms of Ang II receptors are present in ECs in culture (34). The large majority of cardiovascular Ang II-triggered effects are mediated through AT1 receptors (35).

The AT1 receptor is a seven transmembrane G-protein coupled receptor (GPCR). Ang II binds the AT1 receptor in a mechanism that is characteristic of cell surface hormone receptors due to the following characteristics: they have a high structural specificity, they have a limited binding capacity, they display high affinity towards Ang II (10^{-10} M), they induce signal transduction within the cell, and they are up or down-regulated via biosynthesis and recycling. These characteristics made AT1 receptors strong candidates for drug development (35).

The AT2 receptors are highly expressed during the fetal stages of life. In adult tissue, AT2 receptors are expressed in brain, adrenals and in relatively small amounts in the cardiovascular system (36). The AT2 receptor, like the AT1 receptor is a seven transmembrane GPCR receptor; however, it belongs to a unique class of receptors that differs from "classical" G protein-coupled receptors.

2.1.3. Angiotensin II

Ang II was discovered as a circulating hormone with a pivotal role in regulating blood pressure and electrolyte homeostasis. It has since been identified as a vasopressor and endothelium regulator. Vasoconstriction and the release of aldosterone occur within minutes of Ang II stimulation. Ang II is well studied in vascular smooth muscle; however, less is known about its signaling in endothelial cells (ECs). Ang II's well known effects include supporting/increasing arterial blood pressure, maintaining glomerular filtration, increasing the heart's contractility and ventricular hypertrophy (37).

Early Ang II research pertained to its role in the regulation of blood pressure and body fluid homeostasis. Over the years, numerous studies emerged suggesting a link between RAS over activity and human atherosclerosis (38). Consequently, later studies on Ang II focused on its role in atherosclerosis, myocardial infarction, SMC proliferation, vascular and myocardial remodeling and congestive heart failure (8). Ang II's effects on atherosclerotic vascular wall changes following vascular injury also became of particular interest (39).

Ang II mediates vascular wall constriction by acting directly on smooth muscle cells via AT1 receptors, and may modulate its effect by acting on the endothelium. These receptors are also present in ECs; however, their function is poorly understood. When Ang II binds the AT1 receptor, the subunits of a guanine-nucleotide-binding protein ($G_{q/11}$) dissociate. This in turn activates phospholipase C, which generates diacylglycerol and inositol triphosphate (40). Inositol triphosphate mediates the release of intracellular calcium stores. Cellular calcium levels also rise due to Ang II-induced calcium entry through cell membrane channels (41). Calcium and diacylglycerol activation signal through kinases such as PKC and calcium-calmodulin kinases which alter downstream proteins that regulate cell functions (42).

Ang II activates phospholipase A2 and C in ECs (43). Once phospholipase A2 is activated, prostaglandin follows, since metabolizing arachadonic acid yields leukotrienes or prostaglandins. The increase in calcium triggered by phospholipase C can in turn stimulate NOS. Since prostaglandins and NO act as vasodilators, Ang II's effect on ECs may modulate Ang II-induced smooth muscle constriction. Ang II signals through the AT1 receptor to activate eNOS, and releases NO from the endothelium (43). This NO production may affect the direct Ang II-triggered vasoconstriction in SMCs as it is observed to activate soluble guanylate cyclase and cGMP production in SMCs. The mechanism of NO production in this case involves inositol phosphate production and an increase in intracellular calcium. However, the production of peroxynitrite has the potential to cancel out the beneficial effects of NO, as it contributes to pathological processes in the vascular wall (43). Studies such as one by Boulanger CM *et al.* illustrate that the NO released by the endothelium decreases Ang II-induced vasoconstriction (34).

Ang II has also been shown to raise levels of endothelin-1, a vasoactive peptide. On one hand, endothelin-1 (ET-1) induces endothelium-dependent vasodilation

via ET-B receptor and NO release, but on the other hand, it mediates vasoconstriction when acting on smooth muscle cells (34). The difference in the response of the vascular wall to Ang II likely depends on the relative amounts of relaxing factors released, endothelin-1 secretion or the resulting action of endothelin-1 (44).

Additionally, Ang II contributes to growth and hypertrophy of smooth muscle cells while inhibiting endothelial cell proliferation. The predominance of different forms of Ang II receptors plays a role in the final action of Ang II: AT1 receptors are implicated in growth whereas AT2 receptors inhibit vascular smooth muscle cell proliferation (34).

2.1.4. Angiotensin II and inflammation

Atherosclerosis is a chronic disease that acts on the arterial wall and involves both innate and adaptive immunoinflammatory mechanisms. Inflammation plays a key role at all stages of atherosclerosis. Early on, it is implicated in the formation of fatty streaks, when the endothelium is activated and expresses chemokines and adhesion molecules leading to monocyte/lymphocyte recruitment and infiltration into the subendothelium (45). Inflammation also acts at the onset of adverse clinical vascular events when activated cells within the plaque secrete matrix proteases that degrade extracellular matrix proteins and weaken the fibrous cap, leading to rupture and thrombus formation (45).

Inflammation is a process that occurs in blood vessels and results in the accumulation of fluid and leukocytes. Acute inflammation is the response to all forms of injury, whereas prolonged inflammation is a response to persistent stimuli such as repeated infection or immunological responses (46). Ang II plays a role in all three major stages of the inflammatory response: 1) increase in vascular permeability, 2) leukocyte infiltration, and 3) tissue remodeling (46).

Ang II increases the permeability of the vasculature by causing pressure-mediated mechanical injury to the endothelium. Eicosanoids and vascular permeability factor have been identified as mediators of increased vascular permeability. Increased tyrosine phosphorylation of proteins at the endothelial cell junctions may also contribute to the increase in vascular permeability (47). Ang II increases protein tyrosine phosphorylation in smooth muscle cells, glomerular mesangial cells, and endothelial cells. Tyrosine phosphorylation is of considerable importance in Ang II signaling and Ang II's action on these cells (34). Furthermore, increased permeability appears to occur by Ang II signaling through the AT1 receptors, and stimulating the AT2 receptors triggers the opposite effect (48).

2.1.5. Angiotensin II and oxidative stress

Stimulation of the AT1 receptor is linked to activating NAD(P)H oxidase and thus producing ROS in vascular cells (16). ECs contain all of the system's components including gp91phox, p47phox, p67phox, p22phox and the small GTPase rac1 (49). Although it is not quite clear how the various subunits interact and how they produce superoxide, it has been established that rac1's translocation to the cellular membrane is necessary for NAD(P)H oxidase activation. Furthermore, Ang II induces this translocation in vascular smooth muscle cells and fibroblasts (50).

2.1.6. Angiotensin II, nitric oxide and atherogenesis

By signaling through the AT1 receptor Ang II promotes the atherosclerotic process at virtually all stages of the disease (8). As a result of increased Ang II-induced superoxide production, NO inactivation is observed. The two radicals superoxide and NO react with each other to form peroxynitrite at a rate of 6.7×10^9 mol/L/s. Under physiological conditions superoxide dismutases and other superoxide scavengers work to minimize peroxynitrite formation. However, when superoxide levels are increased, as in the case of Ang II stimulation, a greater amount of peroxynitrite is formed, more NO is lost and endothelial dysfunction, an early step in atherosclerosis, occurs (51). The loss of NO is detrimental since

NO acts as a vasodilator which triggers beneficial anti-inflammatory and thus anti-atherosclerotic effects. Furthermore NO inhibits the attraction and adhesion of monocytes and inflammatory molecules such as MCP-1 and VCAM-1 to the endothelium (51). Ang II is also involved in the fatty streak formation phase of atherosclerosis as well as stimulating growth and migration of vascular smooth muscle cells. Ang II contributes to plaque rupture by enhancing lipid deposition, interleukin-6 production and MMP activity (51).

2.2. The endothelium and endothelial dysfunction

2.2.1. Endothelium-physiological

The vascular endothelium, composed of a cell layer lining the cardiovascular system separates blood from tissue and plays an important role in maintaining normal vascular homeostasis. It helps maintain the delicate balance between vasodilation and vasoconstriction as well as regulating proliferation and migration of smooth muscle cells, platelet adhesion, thrombogenesis and fibrinolysis (6).

Endothelial cells are responsive to substances released by neurons, hormones, cytokines, drugs, chemical and physical stimuli (such as pH). Endothelial cells, in response to their various stimuli have the ability to regulate angiogenesis, inflammation, hemostasis, vascular tone and permeability by synthesizing and releasing multiple factors (6). Vasoactive factors released by endothelial cells include relaxing factors: adenosine, prostacyclin (PGI2), nitric oxide (NO), hydrogen peroxide (H_2O_2), epoxyeicosatrienoic acids (EETs), Cnatriuretic peptide (CNP) as well as contracting factors: thromboxane A2, isoprostanes, 20-hydoxyeicosatetraenoic acid, superoxide anion (O_2^-), H_2O_2, endothelin-1, angiotensin II, and uridine adenosine tetraphosphate (6).

Aggregating platelets, amongst other factors, stimulate the release of endothelial-derived relaxing factor production (52). Endothelial cells affect vascular smooth muscle by forming and releasing a hyperpolarizing factor which regulates the

opening of its potassium-channels (52). Endothelial cells are also capable of using myoendothelial gap junctions to communicate directly with smooth muscle cells to spread electrotonic tone, small ions and molecules such as calcium (6).

2.2.2. Endothelium-pathophysiological

The endothelium also participates in the pathogenesis of vascular diseases such as atherosclerosis. Endothelial dysfunction is identified as one of the earliest events in atherosclerosis and plays a role in the progression and clinical complications of atherosclerotic vascular disease (53). Since as early as 1856, Virchow made the link between the endothelium and the atherosclerotic process. As a result of the investigative work of Furchgott and Zawadski in the 1980's, a great deal of attention has been paid to examining the mechanisms underlying endothelium's role in maintaining vessel wall homeostasis and its pathophysiological role (54). From their experiments, they found that acetylcholine requires endothelial cells in order to relax underlying vascular smooth muscle tissue (54). On the other hand, blood vessels not fully lined with an endothelium undergo vasoconstriction in response to acetylcholine. It was later discovered that endothelium-derived prostacyclin and nitric-oxide were responsible for physiological endothelial vasodilation (54). Thereafter investigators such as Ross R. further studied these endothelial mechanisms (55), (56).

The concept of endothelial dysfunction refers to the altered functional properties of the vascular lining preceding atherosclerosis. Since the emergence of this concept, endothelial dysfunction has been associated with physiological and pathophysiological processes including aging, type I and II diabetes, inflammation, sepsis and rheumatoid arthritis (54). Moreover, because the endothelium has protective functions, endothelial damage affects its protective role and alters its response to serotonin of G-protein coupled receptors (52).

High cholesterol levels and age-related physiological changes have been shown to contribute to increased consequences of impaired endothelial function (52). A

reduction in endothelial integrity results in vascular remodeling, thrombus formation, impaired tissue perfusion and vasoconstriction (5). Cholesterol-engorged macrophages, or 'foam cells' are known to accumulate in subendothelial regions and form early atherosclerotic lesions. These lesions are seen to change locations within the vasculature depending on the age of the patients; in the aorta during the first decade of life, in the coronary arteries during the second decade, and then in cerebral arteries during the third and fourth decades (4). Studies based on animal models illustrate that following the feeding of a high-fat, high-cholesterol diet the first observable change in the artery wall is the accumulation of lipoprotein particles and their aggregates in the intima. Days or weeks later, monocytes are seen to adhere to the endothelium (4). After adhesion, they migrate across the endothelium into the intima where they proliferate, differentiate into macrophages and engulf lipoproteins to form foam cells. The remainder of the lesion-forming process involves smooth muscle cells and fibrous plaques and eventually results in the reduction in lumen diameter, cell proliferation, extracellular matrix production and the accumulation of extracellular lipid (4). Tissue culture studies on the pathological changes that take place during atherogenesis have shown that the endothelium plays a central role in mediating inflammation and that the accumulation of oxidized LDL in the intima contributes to monocyte recruitment and foam-cell formation (4). LDL is taken up efficiently by macrophages if it is in an oxidized form, and this modification is thought to involve ROS produced by ECs, macrophages and enzymes such as myeloperoxidase (4).

The endothelium, which contains tight junctional complexes, acts as a selectively permeable barrier between blood and tissues. It plays multiple roles in regulating processes such as thrombosis, inflammation, vascular tone, vascular remodeling and smooth muscle cell migration and proliferation. Endothelial cell morphology is affected by fluid sheer stress (4). Cells in tubular arterial areas, where blood flow is uniform and laminar, have an ellipsoid shape and are aligned with the flow direction. Contrastingly, cells in branched regions, where blood flow is disturbed,

have a polygonal shape, follow no particular direction and are more permeable to macromolecules such as LDL; thus, they are more susceptible to lesion formation. Moreover, minimally oxidized LDL has been shown to stimulate the endothelium to produce numerous pro-inflammatory molecules which in turn trigger the accumulation of monocytes and lymphocytes seen in atherosclerosis (4). Oxidized LDL also has the ability to inhibit NO production. NO, which will be discussed in the later sections, is a known mediator that exhibits anti-atherosclerotic properties including vasorelaxation. Other factors such as homocysteine and hormones modulate inflammation. For example, diabetes partly triggers inflammation by forming glycation end-products that interact with endothelial receptors (4).

2.2.3. Endothelial dysfunction and Angiotensin II

Numerous studies have shown that hypercholesterolemia, specifically the increased LDL plasma levels affects the pathogenesis of atherosclerosis in human and animal subjects. This effect has been hypothesized to be related to the marked increase in AT1 receptors that results from hypercholesterolemia (57). These findings may explain why lipid lowering may have antihypertensive effects. In hypercholesterolemia and atherosclerosis, components of the RAS such as ACE are up regulated. Additionally, Ang II is present in concentrated amounts in atherosclerotic plaques. Due to the high concentrations of ACE in ECs' plasma membrane, ECs are able to synthesize Ang II. In addition to their role in the formation of Ang II, circulating Ang II can reach the endothelium and potentially influence cellular functions (39). Furthermore, inhibiting the AT1 receptor using ARB's or ACEi's alleviates endothelial dysfunction (58).

2.2.4. Leukocyte adhesion to the endothelium

The formation of atherosclerotic lesions is followed by monocyte recruitment and transendothelial migration, VSMC proliferation, amongst other events. ACE inhibitor treatment *in vivo* decreases endothelial dysfunction and the subendothelial infiltration of mononuclear cells in the aorta and carotid artery of hypertensive rats (59). Furthermore, Ang II increases monocyte adhesion to ECs

(60). The increase in monocyte adhesion triggered by Ang II occurs through a mechanism that remains unclear.

Intracellular Adhesion Molecule-1 (ICAM-1) plays a role in modulating stable polymorphonuclear leukocyte (PMN) adhesion to the vascular endothelium, and its upregulation is involved in atherogenesis (61). ICAM-1 upregulation occurs in ECs covering human atheromas and correlated with plaque intimal T-lymphocyte density (62). ICAM-1 mediates firm adhesion of PMN to the endothelium by acting as a counter-receptor for leukocyte β_2-integrins, CD11a/CD18 and CD11b/CD18. Under normal conditions, the endothelium expresses ICAM-1; however, its expression increases dramatically when stimulated by inflammatory cytokines such as TNF-α, a pro-inflammatory cytokine released during sepsis, through activation of nuclear transcription factor-κB (NF-κB) (63) and Ang II (62). In ECs, NF-κB is the key regulator of ICAM-1 gene expression following stimulation with TNF-α (64) and Ang II (65).

Several studies, such as the one conducted by Pastore *et. al* showed that Ang II regulated ICAM-1 expression in the living endothelium (62). Their study established a link between Ang II and vascular damage in humans. Ang II upregulated ICAM-1 gene expression and stimulates soluble ICAM-1 release by HUVECs. In addition, increased ICAM-1 expression and secretion by Ang II was blocked by an AT1 receptor but not an AT2 receptor antagonist (62). They also confirmed an increase in leukocyte count following Ang II treatment. Ang II also increased plasma soluble ICAM-1 levels in healthy human volunteers and essential hypertensive patients. Four-week treatment with losartan but not atenolol or placebo reduced baseline and Ang II–stimulated plasma ICAM-1 concentrations (62).

Although a clear mechanism involving Ang II has not yet been found, PKC-ζ, the a PKC isozyme present in ECs, appears to play a critical role in regulating TNF-α-induced oxidant generation and in activating NF- κB and ICAM-1 transcription

in ECs (66). Studies conducted by the same group of investigators showed that when activated, TNF-α induces early-onset endothelial adhesivity towards polymorphonuclear leukocytes (PMN) by PKC-ζ-dependent phosphorylation of ICAM-1 that precedes the de novo ICAM-1 synthesis (67). Furthermore, oxidant signaling by neutrophil NAD(P)H oxidase is an important determinant in activating TNF-α in this particular pathway in ECs (68).

2.3. Reactive oxidative species

Several reactive oxygen species such as superoxide (O_2^-), hydroxyl radical ($HO^\bullet$), hypochlorous acid (HOCl), lipid radicals, and hydrogen peroxide (H_2O_2) are found within the cell under physiological conditions as a result of oxygen reduction by various enzyme systems such as NAD(P)H oxidase, xanthine oxidase, NOSs, lipoxygenase, cyclooxygenase, cytochrome P450's, peroxidases, and mitochondrial respiratory electron-transport chain enzymes (69). Superoxide, is produced when oxygen is reduced by one electron. Hydrogen peroxide is the result of two electron donations to oxygen. Superoxide is a common progenitor of other ROSs, for example, O_2^- can be converted to H_2O_2 spontaneously or through the action of superoxide dismutase. O_2^-, H_2O_2, and their reaction products affect vascular cell function. ROS are important for maintaining normal cell functioning, such as growth in the case of vascular smooth muscle cells (69).

2.3.1. Oxidative stress

Maintaining a specific level of intracellular ROS is crucial for ensuring that the pathological state of oxidant stress does not occur. Oxidant stress results following excessive production of ROS or malfunctions in the antioxidant defense systems and has been linked to cardiovascular conditions such as atherosclerosis, cardiac hypertrophy, hypercholesterolemia, diabetes and hypertension (13), (70). At the cellular level, oxidant stress alters DNA, lipid oxidation, proteins, tyrosine kinases and phosphatases, and activates redox sensitive genes. In particular, redox sensitive genes play a role in the expression of pro-inflammatory adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular

adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), which are involved in the initiation and progression of atherosclerosis (12). NAD(P)H oxidases have been shown to be the predominant source of ROS production in the vasculature, especially in pathological conditions (13). Oxidative stress has also been linked to endothelial dysfunction and increasing pro-inflammatory gene expression (14).

2.3.2. Reactive oxidative species signaling

Production of O_2^- in the vessel wall has been shown to inactivate NO, leading to the production of peroxynitrite and impaired endothelium-dependent vasodilation (71). Production of O_2^- also oxidizes LDL and activates matrix metalloproteinases, leading to vascular remodeling (71). In ECs, superoxide production increases adhesion molecule expression which results in monocyte infiltration (72). This increase in adhesion is linked to EC redox sensitive regulatory mechanisms involving VCAM-1 gene transcription and expression (73). Furthermore, ROS have the ability to alter enzyme function, affect cell growth/migration (increase VSMC proliferation) and ultimately affect vascular, myocardial and extra-cellular matrix remodeling (8). ROS such as H_2O_2 are signaling intermediates in many pathophysiological responses.

ROS can trigger signaling events both within the cell and in adjacent cells. Most ROS such as superoxide and peroxynitrite have short half lives and do not migrate between cells. However, superoxide has been shown to have a paracrine effect by inactivating NO produced by neighbouring cells (17). More stable ROS such as H_2O_2 are able to diffuse between cells. Recent evidence suggests that endothelium-derived H_2O_2 can cause vascular smooth muscle cell vasodilation (74). The effect of ROS varies between cell types; in ECs, it stimulates angiogenesis whereas in VSMCs it triggers hypertrophy (17).

ROS have emerged as playing an important role in signaling; some of the ROS sensitive signaling molecules include p38MAPK (dependent on H_2O_2), AKT, Src,

EGFR, and transcription factors such as NF-kB, AP-1 and Nrf2 (37). The presence of ROS causes vessel inflammation by release of cytokines and leukocyte adhesion molecules that result in the increased recruitment of monocytes to the area of endothelial damage (73). Amongst its many effects, ROS formation inactivates NO in vascular cells (ECs and VSMCs) (75). This consumption of NO can occur through reactions involving superoxide, lipid peroxy radicals and alkoxy radicals, and peroxidase-based reactions (17). NO reduction is of clinical importance, as it is known to inhibit vascular disease development. NO derived from vascular ECs functions as an important regulator of vascular tone and thrombus formation. Its loss is associated with the pathology of atherosclerosis, hypertension, angina, congestive heart failure and diabetic vascular disease (13), (76). Reactive molecular species such as O_2^- can reduce NO's signaling abilities by reacting with it to form the damaging product peroxynitrite (77).

Interactions between Ang II signaling, NAD(P)H oxidase activation and ROS production lead to changes in structural and functional characteristics of the vasculature and are critical in vascular pathology (37).

2.4. NAD(P)H oxidase and superoxide production

2.4.1. NAD(P)H oxidase characteristics

NAD(P)H oxidases were first characterized in neutrophils where two membrane components, p22phox and gp91phox, comprise the cytochrome b558. Other important components include the cytoplasmic subunits p47phox, p40phox, p67phox and the small GTP-binding protein rac1. Upon activation, the cytoplasmic subunits translocate to cytochrome b558 at the membrane, resulting in oxidase activation, and an oxidative burst (69). Crystal structure analysis shows that the phosphorylation of p47phox, which disturbs an auto-inhibitory interaction between p47phox and p22phox, is an important initiating event in oxidase activation (78). Serine phosphorylation of p47phox, its translocation and binding

to the p22phox subunit is part of the mechanism involved in Ang II activation of endothelial NAD(P)H oxidase.

Although neutrophil and vascular (in ECs and SMCs) NAD(P)H oxidases share similar characteristics, such as being equally inhibited by DPI and being stimulated by agonists and arachidonic acid (71), they differ in several important aspects. For instance, neutrophil oxidase release superoxide in bursts, whereas vascular NAD(P)H oxidases release it in lower levels at a continuous rate. The oxidases in non-phagocytic vascular cells are constitutively active at a low level but can be acutely stimulated by agonists such as Ang II and cytokines (79). Furthermore, the superoxide production from phagocytic NAD(P)H oxidases is approximately triple that of vascular cells (71). All the major phagocyte-type NAD(P)H oxidase subunits, including p47phox, p67phox and rac1, are expressed in ECs. Furthermore, O_2^- production by ECs is predominantly NADPH- rather than NADH-dependent. ROS generated by a phagocyte-type EC oxidase is implicated in endothelial dysfunction associated with hypercholesterolemia hypertension and hypoxia-reoxygenation injury (80).

Recently, a family of gp91phox-like proteins, the non-phagocytic NAD(P)H oxidase (NOX) proteins, has been discovered (81). In addition to the constitutively active phagocyte-type NAD(P)H oxidases expressed in vascular cells, ECs contain NOX1, NOX2, NOX4 and NOX5, and vascular smooth muscle cells express NOX1, NOX4 and NOX5 proteins (82). NOX1 and 4 were shown to be responsible for ROS generation in aortic SMCs (83).Other proteins including DUOX1 and 2, NOX organizer 1 and activator 1 are partly homologous to NOX, p47phox and p67phox respectively. These proteins are hypothesized to regulate NAD(P)H oxidase enzymatic activity (17).

2.4.2. NAD(P)H oxidase and Angiotensin II

Ang II triggers oxidative stress in the vasculature. Ang II in pathophysiologically high amounts was first shown to activate NAD(P)H oxidase in rat VSMCs (16).

This activation was linked to superoxide and hydrogen peroxide production through a mechanism that remains not fully understood in endothelial cells (37). Ang II (100 nM) increases superoxide production in a NAD(P)H-dependent manner in HUVECs (80). In addition to ECs and SMCs, Ang II can activate NAD(P)H oxidases in fibroblast, cardiomyocytes and mesangial cells (79). Other vascular NAD(P)H oxidase agonists include TNF-α, PDGF, and thrombin (71). Moreover, in ECs mechanical forces such as unidirectional and oscillatory shear stress stimulate NAD(P)H oxidase activity.

In VSMCs, Ang II signals through the AT1R, acts through the intermediate c-Src and leads to the phosphorylation and translocation of p47phox to the membrane (84). It has also been established that Ang II activation of NAD(P)H oxidase involves upstream mediators Src/EGFR/PI3K/Rac-1 and PLD/PKC/p47phox phosphorylation (15), (85). PLD and PKC activation likely precede p47phox phosphorylation. C-Src activation also stimulates epidermal growth factor receptor (EGFR), which signals through phosphatidylinositol 3-kinase (PI3K) to produce phosphatidylinositol (3,4,5)-trisphosphate and consequently activates Rac-1. Rac-1 then moves to join the membrane components, which leads to the second part of oxidase activation (15). Furthermore, c-Src is redox sensitive and stimulated by H_2O_2, thus, its activation is increased perhaps through a positive feedback mechanism with NAD(P)H stimulation and H_2O_2 production.

The increase in ROS resulting from NAD(P)H activation triggers various events within the cell. Amongst these events is the Ang II/H_2O_2-induced hypertrophy of VSMCs (71) and endothelial dysfunction. Ang II has also been linked to inducing cellular inflammatory responses such as increasing the expression of monocyte chemoattractant 1 (MCP-1), vascular cell-adhesion molecule 1 (VCAM-1), and interleukin 6 via pathways involving extracellular signal-regulated kinase 1,2 (ERK1,2), p38 MAPK, protein tyrosine kinases, and JAK2 (Janus-activated kinase 2)–STAT (signal transducers and activators of transcription). ROS production also appears to activate matrix metalloproteinases (MMPs) in addition

to regulating angiogenesis via PKC and hypoxia-induced factor 1α (HIF1α) activation (17).

In particular, the Ang II-triggered activation of Akt has been shown in VSMCs. Furthermore, Akt phosphorylation is inhibited by DPI, indicating a role for NAD(P)H oxidase in Akt activation (19). It has also been shown that Ang II, when administered over extended periods of time (hours-days) increases the expression of NAD(P)H oxidase subunits. The particular subunits that are upregulated depend on if the stimulation is *in vitro* or *in vivo*, and the cell type (17).

Ang II has been shown to increase levels of H_2O_2-derived from NAD(P)H oxidase, which then activates eNOS (86). Thus, Ang II has the ability to simultaneously produce superoxide and nitric oxide in ECs and thus there exists the potential to form the damaging product peroxynitrite. The formation of peroxynitrite through this mechanism has been shown to cause endothelial damage *in vivo* (87). The production of peroxynitrite consequently inactivates nitric oxide, diminishing its beneficial effects on the vascular wall. Ang II-induced H_2O_2 production stimulates Akt activation (19).

Activation of NAD(P)H oxidases not only induces *in vitro* effects but also triggers changes in certain clinically relevant animal models. Rats with Ang II-induced hypertension exhibit elevated superoxide levels and NAD(P)H oxidase activity. Moreover, administering SOD effectively lowers blood pressure in these rats (88). Vascular NAD(P)H oxidases contribute to elevated O_2^- production in rabbits with atherosclerosis and in conditions where Ang II tissue concentrations are elevated (71). Furthermore, AT1 receptor inhibitors reduce atherosclerotic lesion formation and restore endothelial function. Vascular NAD(P)H oxidases are associated with diabetic vascular diseases, cardiac hypertrophy, heart failure, and nitrate tolerance (17).

The presence of ROS has an effect on the vascular wall. O_2^- and H_2O_2 are important intracellular signaling molecules and act as second messengers to activate multiple intracellular signaling pathways. H_2O_2 induces eNOS gene expression via a Ca^{2+}/calmodulin-dependent protein kinase II/JAK2 signaling pathway, in endothelial cells (18). ROS also activate EGFR, STATs, PKC and trigger changes in Ca^{2+} levels. Downstream of Ang II-induced ROS production, several intracellular targets have been identified such as the mitogen activated protein kinase (MAPK) family members (ERK 1/2, p38 MAPK, ERK 5 and JNK), tyrosine phosphatases, ras/rac, c-src and Akt (8). The MAPKs ERK1/2 and p38, as well as p70S6 and Akt/PKB play pivotal roles in Ang II-induced VSMC protein synthesis and cellular hypertrophy.

2.5. Akt

2.5.1. Akt structure and activation

Akt is a serine/threonine kinase activated by a number of growth factors and cytokines in a PI3K–dependent manner. Akt is implicated in numerous intracellular signaling mechanisms including insulin, estrogen and VEGF pathways (89). Akt is a critical regulator of PI3K-mediated cell survival and protein synthesis and a large number of studies have demonstrated in various cell types that constitutive activation of Akt signaling is sufficient to block cell death induced by a variety of apoptotic stimuli. Although Akt plays a crucial role in cell survival, it is classified as a multifunctional protein kinase rather than a simple regulator of cell survival (90). The PI3K–Akt signaling axis in endothelial cells is activated by many angiogenic growth factors and regulates downstream target molecules that are potentially involved in blood vessel growth and homeostasis. Mammalian genomes contain three Akt genes, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. All three genes are widely expressed in various tissues but Akt1 is the most abundant in brain, heart, and lung tissue, whereas Akt2 is predominantly expressed in skeletal muscle and embryonic brown fat, and Akt3 is predominantly expressed in brain, kidney, and embryonic heart tissue (91).

Akt contains an A-terminus pleckstrin homology (PH) domain in the amino terminus, a central kinase domain and a carboxy terminal regulatory domain (89). In unstimulated cells, Akt exists in the cytoplasm and its two regulatory phosphorylation sites, threonine (Thr) 308 and serine (Ser) 473 are unphosphorylated. Upon PI(3)K activation, Akt's PH domain binds to the lipid product of PI(3)K. Akt then moves from the cytoplasm to the plasma membrane (92). There, the upstream kinases 3-phosphoinositide–dependent protein kinase (PDK) 1 and 2 phosphorylate Thr 308 and Ser 473 respectively, thus activating the kinase activity of Akt (92). Potential Ser473-kinases include integrin-linked kinase (ILK), MAP kinase–activated protein kinase 2 (MK2), PDK1 (conversion of substrate specificity in association with protein kinase C–related kinase-2 [PRK2]) and Akt itself (auto-phosphorylation). Fully activated Akt is able to phosphorylate its downstream substrates and some of these molecules detach from the plasma membrane and translocate to various subcellular locations including the nucleus. Akt is inactivated by being dephosphorylated by protein phosphatases such as protein phosphatase 2A (PP2A) (89).

2.5.2. Akt stimulants, targets and cellular pathways

Several EC stimuli are able to enhance cell survival by activating PI3K-Akt signaling. These stimuli include VEGF, angiopoietin-1 (Ang-1), insulin, estrogen and reactive oxygen species. Growth factor activation of angiogenesis depends on EC–extracellular matrix attachment, for without this attachment ECs undergo apoptosis (89). These findings suggest that matrix attachment, involving integrin and molecules, is required for growth factors to activate Akt and maintain endothelial cell viability. Cell attachment is mostly mediated through interactions between the extracellular matrix (i.e. actin filaments), integrin and adaptor/signaling molecules (89).

Several downstream targets of Akt are recognized as apoptosis regulatory molecules including Bad, FKHR family of forkhead transcription factors, and

IKKα and these findings are consistent with the notion that Akt acts as a survival kinase (89). However, other downstream effectors of Akt are involved in different aspects of cellular regulation such as glucose transport (transporter GLUT4), glycogen synthesis (glycogen synthase kinase-3 (GSK-3)), cell cycle regulation (E2F, p21, MDM2) and protein synthesis (mTOR, 4E-BP1, S6K1). Thus, considering its variety in effects, Akt is considered to be a multifunctional protein kinase rather than a simple regulator of cell survival (90).

H_2O_2 is able to stimulate Akt phosphorylation and activation and its elevation ultimately leads to VSMC hypertrophy and inflammation. This inflammation largely contributes to the onset of pathophysiological conditions such as hypertension and atherosclerosis (19). Ang II is also able to activate Akt in a PI3-K-dependent manner by increasing intracellular H_2O_2, indicating that Akt is part of a redox-sensitive signaling pathway–in SMCs. Furthermore, Ang II signaling through the AT1 receptor triggers an increase in superoxide production in vascular smooth muscle cells in a NAD(P)H oxidase-dependent manner resulting in PI3K activation and Akt phosphorylation and activation (19). This Ang II-induced Akt activation ultimately results in VSMC hypertrophy (19).

VEGF induces hypotension in the intact organism, NO–dependent vasodilation in isolated coronary arteries, and NO release in isolated vessels and in cultured endothelial cells. Early studies demonstrated that VEGF-induced increase in NO release from endothelial cells is attenuated by PI3K inhibitors (93), and subsequently, it was demonstrated that VEGF stimulates Akt-mediated eNOS phosphorylation at Ser1177 (in human eNOS, equivalent to Ser1179 in bovine eNOS), leading to an increase in eNOS activity (20). In another study VEGF stimulation in BAEC's (bovine aortic ECs) was shown to activate the PI3K/Akt pathway which leads to eNOS phosphorylation (at ser 1177) and activation (21). Amongst its various targets, Akt, under certain stimulus conditions such as shear stress or flow-stimulation, leads to the phosphorylation of eNOS at Ser-1177, which leads to increased NO production in ECs (20), (94). TNF-α stimulation

triggers a similar pathway where eNOS activation by occurs through sequential activation of neutral sphingomyelinase and of the PI3K/Akt pathway (95). S1P stimulation of eNOS phosphorylation also involves Gi protein, PI3K and Akt.

2.6. Nitric oxide and nitric oxide synthase

NO is formed by the enzyme nitric oxide synthase (NOS) via a reaction involving the guanidino nitrogen atom of L-arginine in various cells including VSMCs, ECs, macrophages, and neuronal cells (96). NOSs exist in three distinct isoforms: inducible NOS (iNOS), endothelial constitutive NOS (ecNOS) and neuronal cNOS. The iNOS enzyme is expressed in VSMCs, macrophages, hepatocytes, amongst others and is activated by bacterial lipopolysaccharides and several cytokines in a Ca^{2+}/calmodulin-independent manner. cNOSs, on the other hand, are Ca^{2+}/ calmodulin-dependent enzymes (51).

NO produced by ecNOS activates endothelium-derived relaxing factor (EDRF), and in turn stimulates soluble guanylate cyclase in VSMC and increases cyclic GMP (cGMP), which act as an intracellular mediator in vasorelaxation and regulator of vascular tone (97). The interaction between NO and Ang II has been an active area of investigation. NO is implicated in the regulation of Ang II receptors in VSMCs (98) and studies indicate that endothelial NO is responsible for the endothelium-dependent inhibition of constriction induced by Ang II. Conversely, Ang II stimulates NO release by activation of Ca^{2+}/calmodulin-dependent cNOS via AT1 receptors in ECs (97). Additionally, activation of PKC by diacylglycerol resulting from the Ang II-stimulated phosphoinositide breakdown may be partly involved in the mechanism of Ang II-induced cNOS activation (97).

The delicate interplay and the balance between the mediators Ang II and NO affect vascular remodeling. Vascular remodeling is characterized by cell hypertrophy and extracellular matrix synthesis, which leads to medial thickening

and luminal narrowing. Ang II and NO often have antagonistic effects on vascular remodeling and they both have beneficial and harmful effects on the vasculature. The final result in the cell depends on the balance of antioxidants and NO distribution (96). For example, in diabetes there is an increase in basement membrane; thus, there is an increased diffusion distance for NO to reach SMCs. Consequently there is more NO-O_2^- interaction and more peroxynitrite formation (96). EC's generate both Ang II and NO that are targeted to SMCs. In terms of benefits, NO is a vasodilator that also down-regulates AT1 receptors in SMCs and has a beneficial anti-inflammatory, anti-proliferative and anti-atherosclerotic response (99). Correspondingly, NOS inhibition (using L-NAME) has been shown to contribute to high blood pressure. Interestingly, Ang II increases NO synthesis, which helps control vessel integrity. Ang II is also beneficial to the endothelium since it activates the Akt signaling pathway, which leads to endothelial cell survival and proliferation (89). On the other hand, Ang II when acting through the AT1 receptor also promotes vascular SM hypertrophy and EC proliferation which contributes to atherosclerosis (8).

Ang II induces injury and vascular constriction in addition to playing a role in all stages of inflammation (increase in vascular permeability, leukocyte infiltration, and tissue remodeling) (46). Similarly, clinical trials indicate that excessive amounts of Ang II triggers diseased states; and, inhibiting Ang II reverses endothelial dysfunction and reduces cardiovascular events (9), (10), (11). Furthermore, NO can be harmful if it combines with O_2^- to form peroxynitrite, which is a potent oxidant with damaging effects on the endothelium.

2.7. Peroxynitrite formation

Nitric oxide (NO) is a product of the enzymatic conversion of arginine to citrulline by nitric oxide synthase. NO reacts rapidly with superoxide (6.7×10^9 $M^{-1}sec^{-1}$) to form peroxynitrite. When O_2^- is present in the cell in excess the reaction proceeds and shifts the equilibrium from NO being a beneficial signaling molecule to a reagent that forms peroxynitrite and redox-related toxic products

(100). At physiological pH and in the presence of transition metals, peroxynitrite undergoes heterolytic cleavage to form hydroxyl anion and nitronium ion, the latter of which nitrates protein tyrosine residues.

The presence of nitrotyrosine on proteins is widely used as a marker for peroxynitrite formation *in vivo* (77). 3-nitrotyrosine (3-NT) is accepted stable biomarker of tissue peroxynitrite formation. In a study conducted by Wattanapitayakul SK *et al.* showed increased amounts of 3-NT in Ang II-treated vascular tissues, and moreover that the 3-NT was concentrated in the endothelium (100). They also found that there was an inverse correlation between Ach relaxant response and 3-NT immunoreactivity; thus, the efficacy of NO-mediated vascular relaxation is reduced. They showed that endothelial dysfunction may be mediated by peroxynitrite formation *in vivo* and that it precedes other Ang II-induced vascular pathologies (100).

Nitration of protein tyrosine residues has the effect of inhibiting various cellular pathways including mitochondrial respiration, high-energy phosphate utilization, prostaglandin synthesis, and superoxide dismutase activity. Peroxynitrite can also induce DNA strand breakage in HUVECs (100). Peroxynitrite is a potent oxidant that functions by directly oxidizing various biological molecules. It is mostly described as a mediator of NO toxicity, but has been associated with physiological activities (43). Its action strongly depends on its cellular environment. For example, it has been shown to cause apoptosis in transformed cell lines (101) and generate NO in the presence of thiols (102).

3. Rationale

Endothelial dysfunction is an early stage in atherosclerosis and blocking Ang II is cardio-protective and preserves the endothelium (28). Clinical trials such as TREND (9) and BANFF (10) studies have demonstrated that excess Ang II is harmful for the endothelium and that ACEi's improve endothelial-dependent dilation. The milestone HOPE trials demonstrated that reducing Ang II using the ACEi ramipril was important for maintaining cardiovascular health and for reducing cardiovascular events (11). Recently, it has been shown that the AT1 antagonist losartan induces similar effects to the ACEi enalapril suggesting that inhibiting the RAS is essential for the protective effect (28), (103), (104).

However, amongst its other effects, Ang II increases NO, and NO exerts a beneficial effect on the endothelium; thus presenting us with an interesting paradox. Another important consideration is that unlike SMCs, ECs contain both ecNOS and NAD(P)H oxidase. Thus, in ECs, there is the potential for the simultaneous production of O_2^- and NO and generation of the potent oxidant peroxynitrite. Peroxynitrite formation causes endothelial dysfunction, which results in increased leukocyte infiltration and adhesion.

4. Research Objective

We examined the cellular mechanism involved in Ang II-induced endothelial cell activation. We sought out to determine the interacting effects of Ang II-induced NO and superoxide production on endothelial activation and dysfunction. Part of the signaling mechanism studied focused on the activation of Akt and its downstream pathways. Akt is a central signaling molecule (involved in insulin, estrogen, VEGF pathways); thus, the presence of other factors could affect the *in vivo* response to Ang II. This study was also designed to make the molecular link between NAD(P)H oxidase-induced Ang II activation and peroxynitrite's damaging effects on the endothelium. We also studied the role of Akt activation by Ang II in the production of peroxynitrite.

Identifying effector molecules involved in Ang II signaling will provide possible paths for developing more precisely targeted therapies for Ang II-related ailments. Preventing Ang II-mediated endothelial activation/injury may be of therapeutic value for preventing atherosclerosis and hypertension.

5. Hypothesis

Ang II increases O_2^- production by NAD(P)H oxidase which leads to activation of Akt and increases intracellular NO by eNOS activation. Simultaneous production of O_2^- and NO results in the formation of peroxynitrite, and consequently endothelial dysfunction, activation and cell injury (**Figure 1**).

6. Materials and Methods

6.1. Materials

HUVECs were extracted from donors from the Royal Victoria Hospital birthing centre. Water-soluble Angiotensin II and the following inhibitors were purchased from Sigma-Aldrich: DMSO-soluble Diphenyleneneiodonium chloride (DPI), PBS-soluble N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), DMSO-soluble wortmannin and candesartan. Bis (*N*-methylacridinium) nitrate (Lucigenin) soluble in 1% in acetic acid, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (ß-NAD(P)H) dissolved in sodium hydroxide and water-soluble Tumor Necrosis Factor-α (TNF-α) were also purchased from Sigma-Aldrich.

3,3'-dioctadecylindocarbocyanin-iodide (DiI) dye for fluorescent labeling of U937 monocytes was purchased from Biotium Inc. and 10% neutral buffered formalin from SurgiPath. Gfp virus, Ad5-CMV-eGFP as well as a dominant negative-Akt virus (Ad5-CMV-Akt-AAA) and a catalytically (constitutively) active-Akt virus (Ad5-CMV-myrAkt) were kind gifts from Dr. K. Walsh. Scrambled siRNA (19 base pairs) 'siCONTROL non-targeting siRNA #1' was purchased from Dharmacon RNA technologies and the p22 siRNA (21 base pairs) from Qiagen. Qiagen's HiPerfect reagent was used for siRNA transfections.

6.2. Cell culture

6.2.1. HUVECs

HUVECs were harvested from umbilical cords, isolated and pooled from 6 different donors using a previously developed protocol (72). Experiments were carried out on primary cells that were cultured up to 3 passages.

HUVECs were plated (approximately 1,500,000 cells/vial) onto 100 mm tissue culture plates and T-75 mm culture flasks. Cells were cultured in a 37°C

incubator of 5% (v/v) CO_2. As cells approached confluency (approximately 70-80%), trypsin-EDTA (0.25%, Gibco-invitrogen) was used to detach cells from plate surface and cells were passaged. Cells were seeded on 100 mm plates, 6 well or 48 well dishes, depending on the experiment. They were used at a pre-confluency of approximately 95%.

Cells were cultured in endothelial cell growth medium: MCDB 131 media (Gibco-invitrogen) supplemented with 20% heat inactivated fetal bovine serum (FBS), 1% L-Glutamine (Gibco-invitrogen), 1% P/S/F (antibiotic-antimycotic, prepared with 10,000 units/mL penicillin G sodium, 10,000 μg/mL streptomyocin sulfate and 25 μg/mL amphotericin B as Fungizone in 0.85 % saline, Gibco-invitrogen,), 3 mg/100 mL endothelial mitogen growth factor (Biomedical Technologies Inc.), 135 μL Hepalean/100 mL media (heparin sodium injection 10,000 U.S.P. units/mL, Oragon Canada). The cell culture media was changed every two days. Prior to, during and following experiments, cells were frequently monitored using a microscope to check for growth, changes in appearance and to rule out contamination.

6.2.2. U937 monocytes

U937 monocytes were seeded on 150 mm culture dishes and grown in 20-25 mL media per dish. These cells grow in suspension, thus do not adhere to the base of the dish. Cells were cultured in a 37°C incubator of 5% (v/v) CO_2. Cells were cultured in RPMI media 1640 (Invitrogen) + 1% L-Glutamine and 1% P/S/F and 10% FBS. Media was changed every 4 days. As cells approached approximately 70-80% confluency, they were passaged. Prior to, during and following experiments, cells were frequently monitored using a microscope to check for growth, changes in appearance and to rule out contamination.

6.3. Superoxide detection with lucigenin-enhanced chemiluminescence

HUVECs were grown to approximately 95% confluency and plated onto 100 mm culture dishes in endothelial cell growth medium (see above). Cells were then stimulated with Ang II (100 nM) for 1 hour in growing media; no serum deprivation was used. Control cells and stimulated cells were scraped thoroughly using 200 μL of Hepes Buffer (see above) per plate, collected in sterilized eppendorf tubes, repeatedly vortexed and placed on ice for the remainder of the experiment. The following conditions were run: blank, control, Ang II, Ang II + DPI (5 μM) and Ang II + L-Name (3 mM). The inhibitors were added following cell scraping into suspensions of Ang II stimulated cells. Hepes buffer was used to equalize the volume of all wells (250 μL/well). A 96 well assay plate was used.

LMax II 384 luminometer (Molecular Devices) using SoftMax Pro (version 4.7.1) software were used to measure superoxide levels in each well. Lucigenin (10 μM) and NAD(P)H (250 uM) were injected into each well. The superoxide level was measured every 31 seconds for a period of 30 minutes. The background signal was subtracted from the total output and the area under the curve of output was calculated (by the SoftMax Pro software). Following superoxide measurements, cells were counted (a sample from the control pool and one from the Ang II stimulation). Results were normalized to the number of cells (we used a conversion factor to obtain the effective results for 1,000,000 cells per 1 mL).

6.4. Adhesion assay

The monocyte adhesion assay was modeled after Fiedler U *et. al.* (105). Passage 3 HUVECs were seeded in 48 well plates in 200 μL endothelial growth medium per well (see above), with the only exception being that 10% FBS was used. HUVECs were grown to approximately 95% confluency. Saturating concentrations of TNF-α (above 0.04 ng/mL) have been shown to induce intense U937 monocyte adhesion to the cultured HUVEC monolayer and was therefore chosen as a positive control (105). In our experiments, TNF-α was used at a concentration of 10 ng/mL.

DiI labeling of U937 monocytic cells

Monocytes were diluted and counted using a hemocytometer. 500,000 monocytes per well were centrifuged and resuspended in 5 mL of D-PBS. 5 μL of 1 mM stock DiI was then added to the cell suspension, to make a final labeling concentration of 1 μM. Cells and added dye were gently pipetted to ensure proper mixing. The cell-dye mixture was incubated in 37°C water bath for 20 min with occasional mixing by inversion of the tube. Following incubation, cells were centrifuged, washed twice with PBS and finally resuspended in stimulation medium (250 μL of stimulation medium plus monocyte solution per well). The cell suspension was then added to each well of confluent HUVECs at the end of the 6 hour Ang II stimulation.

6.4.1. Initial control conditions and inhibitors

A triplicate of the following conditions was used: Control-Stimulation media, Control-Ang II solvent (ddH_20), positive control-TNF-α (10 ng/mL), Ang II (100 nM), Ang II + DPI (5 μM), Ang II + Wortmannin (100 nM), Ang II + L-Name (3 mM), Ang II + Candesartan (10 μM).

Cells were incubated with inhibitors for 1 hour prior to 6 hour stimulation with Ang II or TNF-α. The only difference between the stimulation media and the growing media was that the stimulation media contained 5% FBS. Stimulation volume was 200 μL per well. Following stimulation, media was removed and to each well 200 μL of stimulation medium containing 500,000 3,3'-DiI-labeled U937 monocytic cells were added (see above for DiI labeling technique). Monocytes were allowed to adhere to plated HUVECs for 30 min in 37°C on a rocking platform. After the initial 15 min of shaking, the plate was rotated 90° for the remainder of the shaking time. After removing the media and suspended monocytes, the wells were then washed three times, at room temperature, with PBS. Washing was important for ensuring only tightly adhering monocytes remained. Following the washes, cells were fixed for 30 min using 10% neutral

buffered formalin, at room temperature. The formalin was then removed and cells were visualized. U937 adhesion was quantified in four fields per well (each condition was a triplicate) viewed under 10X magnification, with an automated image analysis system using an Olympus 1X70 inverted microscope (under the red fluorescence filter) and Image-Pro Plus software (version 4.5.0.29).

6.4.2. Use of Akt viruses

In order to study the specificity of the response, Akt viruses were used as a tool to examine the role of Akt in monocyte adhesion. HUVECs were plated at passage 3 and grown on 48 well plates using the same growing media as for the initial adhesion assay (see above). At approximately 70% confluency, cells were transfected with viruses for 4 hours using serum deprived media (MCDB 131 + 1% L-Glutamine). Gfp virus, Ad5-CMV-eGFP ($1.3x10^{12}$ particles/mL, $4x10^{10}$ titer pfu/mL) was used as a positive control to indicate transfection efficiency. Dominant negative-Akt virus (Ad5-CMV-Akt-AAA) ($1.3x10^{12}$ particles/mL, $1x10^{10}$ titer pfu/mL) and a catalytically (constitutively) active Akt virus (Ad5-CMV-myrAkt) ($1.1x10^{12}$ particles/mL, $2x10^{10}$ titer pfu/mL). The Akt viruses were used at a dose of 50 molecule of infection (MOI=PFU/mL), 200 μL per well was used. Following the transfection time course, cells were washed three times with PBS to remove any residual virus media and supplied with growing media (250 μL per well was used-slightly higher than the usual 200 μL cells are grown in to compensate for serum deprivation and stress caused by virus transfection). Visualization of GFP was used to ensure successful virus transfection. Cells were then grown to full confluency and the monocyte adhesion assay was carried out following the same protocol described above.

6.4.3. Use of p22phox siRNA

In order to examine the effect of NAD(P)H oxidase on monocyte adhesion to HUVECs following Ang II stimulation, siRNA against the p22phox subunit was used. HUVECs were grown to approximately 70% confluency (the protocol is optimized to 50-70% confluency) on 48 well plates. Cells were washed with PBS

before growth medium was replaced by starving media (MCDB 131 + 1% L-Glutamine) of 200 μL/well. siRNA was mixed with HiPerfect reagent and starving media and allowed to form complexes for 10 min at room temperature. Scrambled siRNA was used at a concentration of 10 nM and p22 siRNA at 1.4 μg. 0.78 μL/well of HiPerfect solution was used and mixed with starving media. Following the room temperature incubation, the mixture of siRNA and HiPerfect was added drop wise to each well (50 μL/well). Plates were tilted to ensure even mixing and incubated at 37°C for 5 hours. Following incubation, complexes were removed, cells were washed twice with PBS and EC growth media was added back to HUVECs. Cells were then grown to full confluency and 3 days later, the monocyte adhesion assay was carried out following the same protocol described above.

6.5. Statistical analysis

Data are expressed as ±SEM from 3-5 separate experiments for each set of experiments. Statistical analysis between the groups was performed with one-way or two-way ANOVA and using pair wise multiple comparison procedure (Student-Newmann-Keuls method). P values below 0.05 were considered statistically significant.

7. Results

7.1. Ang II increases superoxide production

In order to determine if Ang II enhanced O_2^- production, the levels between control cells and Ang II-stimulated cells were compared over 30 minutes by measuring lucigenin-enhanced chemiluminescence. **Figure 2** illustrates data averaged over 3 separate experiments. Following 1 hour of Ang II stimulation, there appeared to be just over a 4-fold increase in the O_2^- level compared with the control cells. The data passed the normality and equal variance tests. A one-way analysis of variance (pair wise multiple comparison procedure-using the Student-Newman-Keuls Method) was performed on the data and indicated that the differences in the mean values among the treatment groups were greater than would be expected by chance; thus, there was a statistically significant difference ($P < 0.05$).

7.2. Pharmacological blocker DPI inhibits the Ang II-triggered increase in superoxide production

In order to examine the particular role of NAD(P)H oxidase and eNOS in Ang II-enhanced O_2^- production, pharmacological inhibitors L-Name (eNOS inhibitor) and DPI (NAD(P)H oxidase inhibitor) were used in the assay. The O_2^- levels between control cells, Ang II-stimulated cells and Ang II-stimulated cells incubated with inhibitors were compared over 30 minutes of measuring lucigenin-enhanced chemiluminescence. **Figure 3** illustrates data averaged over 4 separate experiments. Following 1 hour of Ang II stimulation, there appeared to be approximately a 1.9-fold increase in the O_2^- level compared with the control cells. Cells pre-treated with L-Name displayed approximately a 2.24-fold increase in O_2^- level compared with the control cells, a value very close to Ang II stimulated cells. Cells pre-treated with DPI produced O_2^- levels slightly less than the control cells (approximately 0.89 fold of the control cell level). DPI treated Ang II-stimulated cells when compared to Ang II stimulated cells produced approximately half the amount of O_2^-. The data passed the normality and equal

variance tests. A two-way analysis of variance (pair wise multiple comparison procedure-using the Student-Newman-Keuls Method) was performed on the data and indicated that the differences in the mean values between the control group and the Ang-II treated group were greater than would be expected by chance; thus, there was a statistically significant difference ($P < 0.05$). A pair wise comparison between Ang-II treated groups (with and without DPI) indicated a statistically significant difference ($P < 0.05$). Similarly, when Ang-II treated groups (pre-treated with L-Name vs. DPI) were compared, a statistically significant difference ($P < 0.05$) was also reported.

7.3. Ang II enhances monocyte adhesion to HUVECs

The extent of U937 monocyte adhesion to HUVEC monolayers was used to as an indicator of initial stages of endothelial dysfunction. After taking pictures of each well for each condition using the Olympus 1X70 inverted microscope, the fluorescently labeled monocytes were manually counted. The data presented in **Figure 4** is a representative experiment of 3 separately conducted experiments. The positive control TNF-α increases monocyte adhesion to HUVECs (105). TNF-α-stimulated HUVECs had 5.57 and 8.44 times as many monocytes adhering as control conditions (ddH_2O and stimulation media respectively). The data passed the normality and equal variance tests. Pair wise comparison tests between TNF-α-stimulated HUVECs and both control groups (ddH_2O and stimulation media) indicated statistically significant differences ($P < 0.001$ for both control groups). There was also a reported statistically significant difference ($P < 0.05$) between TNF-α-stimulated HUVECs and Ang II-stimulated cells. Ang II was also shown to increase the number of monocytes that attached to HUVECs by 3.29 and 4.98 times as many monocytes adhering as control conditions (ddH_2O and stimulation media respectively). Ang II's effect on adhesion was 58.97% of that induced by TNF-α. Pair wise comparison tests between Ang II-stimulated HUVECs and both control groups (ddH_2O and stimulation media) indicated statistically significant differences ($P < 0.05$ for both control groups).

7.4. Pharmacological blockers Cd, DPI, Wm and L-Name inhibit the Ang II-triggered increase in monocyte adhesion to HUVECs

The next stage in examining monocyte adhesion to HUVECs involved pharmacological inhibitors. Specific inhibitors were chosen to target key players in the proposed pathway in order to assess how they affect monocyte adhesion. The adhesion assay was conducted in the same way as the initial set, and the fluorescent monocytes were also counted in the same manner. The data shown in **Figure 5** is a representative experiment of 3 separately conducted experiments. TNF-α-stimulated HUVECs had 14.35 and 5.59 times as many monocytes adhering as control conditions (ddH_2O and stimulation media respectively). The data passed the normality and equal variance tests. Pair wise comparison tests between TNF-α-stimulated HUVECs and both control groups (ddH_2O and stimulation media) indicated statistically significant differences ($P < 0.001$ for both control groups). There was also a reported statistically significant difference ($P < 0.05$) between TNF-α-stimulated HUVECs and Ang II-stimulated cells. Ang II was also shown to increase the number of monocytes that attached to HUVECs by 11.5 and 4.48 times as many monocytes adhering as control conditions (ddH_2O and stimulation media respectively). This increase in monocyte adhesion was 80.17% of that induced by TNF-α. Pair wise comparison tests between Ang II-stimulated HUVECs and both control groups (ddH_2O and stimulation media) indicated statistically significant differences ($P < 0.001$ for both control groups).

Monocyte adhesion in response to Ang II was decreased by candesartan (by 0.37- and 0.14-fold compared to ddH_2O and stimulation media control groups respectively), DPI (by 2.83-and 1.10-fold compared to ddH_2O and stimulation media control groups respectively), L-NAME (by 0.26-and 0.10-fold compared to ddH_2O and stimulation media control groups respectively), wortmannin (by 0.32- and 0.13-fold compared to ddH_2O and stimulation media control groups respectively). Pair wise comparison tests were performed between Ang II-stimulated HUVECs and Ang II-stimulated HUVECs pre-treated with each inhibitor. Comparing Ang II stimulated cells (no inhibitor vs. wortmannin)

indicated a statistically significant difference ($P < 0.001$). Similarly, comparing Ang II stimulated cells (no inhibitor vs. DPI) indicated a statistically significant difference ($P < 0.001$).When Ang II stimulated cells (no inhibitor vs. L-Name) were compared, the test indicated a statistically significant difference ($P < 0.001$). And finally, comparing Ang II stimulated cells (no inhibitor vs. candesartan) indicated a statistically significant difference ($P < 0.001$).

7.5. Dominant negative-Akt virus inhibits the Ang II-triggered increase in monocyte adhesion to HUVECs and constitutively active-Akt virus elevates the Ang II-triggered increase in monocyte adhesion to HUVECs

The next stage in examining monocyte adhesion to HUVECs involved Akt viruses. Specific inhibitors were chosen to target Akt in the proposed pathway in order to assess their effects on monocyte adhesion. The adhesion assay was conducted in the same way as the initial set except for transfecting the HUVECs with viruses before running the Ang II stimulation and adding monocytes; the fluorescent monocytes were also counted in the same manner. In this set of experiments a GFP virus was used as a positive control, to indicate successful transfection. Three hours following HUVEC transfection, GFP was visualized using the Olympus 1X70 inverted fluorescent microscope. The following day, cells transfected with GFP viruses strongly expressed the protein throughout the wells. The data shown in **Figure 6** is a representative experiment of 5 separate experiments. The data passed the normality and equal variance tests.

The dominant negative-AKT virus decreased monocyte adhesion by 0.39 fold, while the constitutively active-AKT virus increased adhesion by 1.54 fold, compared to Ang II-stimulated GFP-transfected cells. Pair wise comparison tests between all Ang II-stimulated groups and all control groups indicated statistically significant differences ($P < 0.001$). Pair wise comparison tests were performed on cells within each virus. Control cells transfected with the dn-Akt virus vs. ca-Akt virus showed a statistically significant difference ($P < 0.05$). Comparison of Ang

II stimulated cells (GFP virus vs. dn-Akt virus) indicated a statistically significant difference ($P < 0.001$). Comparing Ang II stimulated cells (GFP virus vs. ca-Akt virus) indicated a statistically significant difference ($P < 0.001$). Comparing Ang II stimulated cells (dn-Akt virus vs. ca-Akt virus) indicated a statistically significant difference ($P < 0.001$).

Furthermore, comparing the effect of the dn-Akt virus (control cells vs. Ang II-stimulated cells), a statistically significant difference was noted ($P < 0.05$). Similarly, when comparing the effect of the ca-Akt virus (control cells vs. Ang II-stimulated cells), a statistically significant difference was noted ($P < 0.001$).

7.6. p22phox siRNA inhibits the Ang II-triggered increase in monocyte adhesion to HUVECs

In order to further examine the specific role of NAD(P)H oxidase on Ang II-induced monocyte adhesion to HUVECs, siRNA specific to the p22phox component was used, along with a scrambled siRNA as a control. As shown in **Figure 7**, the p22phox siRNA decreased Ang II-induced monocyte adhesion by 1.94-fold, compared to the Ang II-stimulated HUVECs transfected with scrambled siRNA. The data passed the normality and equal variance tests. Pair wise comparison tests between all Ang II-stimulated groups and all control groups indicated statistically significant differences ($P < 0.001$). Pair wise comparison tests were performed on cells within each siRNA group. Control cells transfected with the scrambled siRNA vs. p22 siRNA did not show a statistically significant difference ($P > 0.05$). However, comparing Ang II stimulated cells (scrambled siRNA vs. p22 siRNA) did in fact indicate a statistically significant difference ($P < 0.001$). Furthermore, comparing the effect of the p22 siRNA (control cells vs. Ang II-stimulated cells), a statistically significant difference was noted ($P < 0.05$).

8. Discussion

The main finding of this study was that that Ang II stimulation in ECs led to the activation of NAD(P)H oxidase and consequently Akt, eNOS and EC activation. In our proposed mechanism it is the simultaneous production of NO from eNOS and superoxide from NAD(P)H oxidase that leads to endothelial activation. Interestingly, blocking either the source of superoxide, NAD(P)H oxidase, or the source of NO, PI3K-Akt-eNOS, resulted in a decrease of endothelial activation and consequently a decrease in monocyte adhesion to HUVECs. Our findings shed light on the paradox of why Ang II-induced Akt activation and NO production have a harmful rather than a beneficial effect on the endothelium, as NO is known to improve endothelial function. Using multiple approaches (pharmacological inhibitors, viruses and siRNA) in order to inhibit the AT1 receptor, NAD(P)H oxidase, PI3K, Akt and eNOS we observed that in order to block Ang II-induced endothelial activation, one, but not all of the aforementioned molecular players can be inhibited.

Although parts of the signaling mechanism of Ang II have been identified, the interactions between the molecular players examined in this study have not been considered. Moreover, establishing the pathway in ECs is of importance, as Ang II signaling is more clearly understood in SMCs. The precise mechanism by which Ang II triggers endothelial activation remains uncertain. Although SMCs and ECs share similarities, their differences affect how they respond to stimuli. Our rationale was based on what is known in SMCs, the characteristics of ECs and established pathways in ECs triggered by stimulants such as TNF-α.

Understanding this mechanism is of physiological and clinical significance for several reasons. First of all, Akt is a central signaling player in several cellular pathways including insulin, estrogen, VEGF, shear stress and growth factors, and its involvement and potential alteration (by inhibition or over activation) in Ang II signaling may affect other pathways. Secondly, understanding why ACEi's and

ARBs have protective effects on the vascular endothelium could have pharmaceutical applications for the design of more specifically targeted drugs. Thirdly, examining Ang II's effect on increasing both O_2^- and NO using an *in vitro* model will contribute to understanding the physiology *in vivo*, where the result depends on the overall balance of these and other oxidants, antioxidants and NO distribution. And finally, the mechanism behind Ang II-induced endothelial activation may help clarify the process of focal leukocyte recruitment by the endothelium, an important aspect of atherosclerosis.

8.1. Cell cultures

The two cell lines that were used were Human Umbilical Vein Endothelial Cells (HUVECs) and U937 monocytes.

8.1.1. HUVECs

The HUVECs used were from a primary cell line, freshly isolated from umbilical cords. These cells were chosen for our experiments partly because of their availability and their ease in culturing. HUVECs are widely used in Ang II studies since they express Ang II receptors (34). We confirmed the involvement of the AT1 receptor using Ang II stimulations and the AT1 receptor inhibitor candesartan. Since the expression of AT1 receptors in HUVECs is strongest within the first 5 passages, all experiments were conducted within 3 passages. ECs are able to produce NO in response to vasoactive pro-inflammatory cytokines (106), and more specifically, HUVECs are able to produce NO; thus, they were appropriate choice for our study (107). Since HUVECs are sensitive to environmental changes, extra care had to be taken as they were a challenge to work with during stimulations involving transfections, temperature changes, and serum deprivation. If cells were disturbed or stressed they rounded up and became unattached to the plate surface. For example, during virus transfection, the initial MOI used was 100 for 6 hours; however, HUVECs were unable to withstand the stress. Thus, the MOI was reduced to 50 and time of transfection was changed to 4 hours. Furthermore, the health and degree of confluency of the cells was critical

for successful transfections. 100 nM was the chosen dosage of Ang II for EC stimulations based on previously performed studies (43), (62), (79), (97).

8.1.2. U937 monocytes

The adhesion assay we used was modeled after Fiedler *et al.*, in which they used U937 monocytes (105). These monocytes are an immortalized cell line and are a homogenous population of cells that are grown in suspension, simple to culture and are widely used in endothelial adhesion studies (108), (109). Monocytes express various cell surface adhesion molecules and receptors that mediate rolling, activation, and firm arrest on vascular endothelial cells (110). Some of these adhesion molecules include ICAM-1, 2, 3, fibrinogen, fibronectin, VLA-4 and the leukocyte ligand for VCAM-1 (111). An important issue encountered was the tendency of U937 monocytes to clump together, especially during the adhesion assay. For this reason, mixing by rocking as well as the washing steps prior to fixation with formalin were particularly important.

8.1.3. Serum deprivation of cells

HUVECs were serum starved during the Ang II stimulation experiments for the adhesion assays. Similarly, for the adhesion assay, inhibitor incubations, virus infections, siRNA transfections and Ang II stimulations cells were in starvation media that only contained 5% FBS and 1% L-Glutamine. This period of starvation minimized the proliferative signaling of the culture medium. It is important to note that the transition from full growth to starvation media triggered EC detachment. For this reason, the starvation period was not extended beyond 6 hours and the FBS concentration was not lowered below 1%. Cells were not serum deprived for superoxide measurements using chemiluminescence, because starving HUVECs increase ROS (112).

8.2. Experimental techniques

8.2.1. Lucigenin-enhanced chemiluminescence

The measurement of superoxide levels in intact endothelial cell was done using lucigenin (10 μM)-enhanced chemiluminescence. Lucigenin reacts with ROS to emit light and the superoxide produced is measured as light units/min. This method is considered to be a reliable and sound assay for detecting superoxide (113). Lucigenin (10 μM)-enhanced chemiluminescence has been used in an Ang II study assessing superoxide production by NAD(P)H oxidase in HUVECs (80).

The use of lucigenin-enhanced chemiluminescence for detecting O_2^- production by biological tissues was under scrutiny in the past because of the potential for redox cycling and artefactual O_2^- generation when high doses (substantially higher than 5 μM) of lucigenin are used (114). Li *et al.* showed that such redox cycling only occurred at lucigenin concentrations above 5 μM, and that the potential for redox cycling was influenced by the precise system under study (114). Since we used lucigenin at 10 μM in our studies, our results were a reliable representation of superoxide produced in the cell.

Because this method is highly sensitive, there was a large variability between individual experiments. Although the pattern seen in the results was consistent (i.e. higher superoxide levels in Ang II-stimulated cells), the superoxide levels measured varied between experiment sets. To account for this variability, results were shown in terms of fold changes with respect to control samples, as seen in **Figures 2 and 3**. Statistically significant differences were reported between control and Ang II-stimulated cells and Ang II-stimulated cells incubated with inhibitors DPI and L-Name.

8.2.2. Adhesion assay

Atherosclerosis affects the arterial wall and involves both innate and adaptive inflammation. Inflammation is involved in all stages of atherosclerosis, including

endothelium activation and increased expression of chemokines/adhesion molecules leading to leukocyte recruitment and infiltration into the subendothelium (45). Ang II plays a role in the inflammatory response by increasing vascular permeability, leukocyte infiltration, and tissue remodeling (46). More specifically, Ang II increases monocyte adhesion to ECs (60) through a mechanism that remains unclear. We studied monocyte adhesion to ECs using pharmacological inhibitors, viruses and siRNA to block specific points of our proposed pathway. The adhesion assay we ran was modeled after Fiedler U *et. al.*'s study on Angiopoietin-2's effect on endothelial response to TNF-α (105). The monocyte adhesion assay is a widely used method to study endothelial cell activation (108), (109).

8.3. Experimental approach: inhibiting various points in the pathway

We examined the specific molecular players in our proposed Ang II signaling mechanism using pharmacological inhibitors, viruses and siRNA in chemiluminescence and adhesion assays. The inhibitors used in this study were AT1 receptor blocker candesartan, PI3K inhibitor wortmannin, NAD(P)H oxidase inhibitor DPI, and eNOS inhibitor L-Name. These inhibitors were used to assess their affect on Ang II-induced changes in Akt/eNOS/nitrotyrosine phosphorylation, superoxide production and monocyte adhesion to HUVECs. In order to examine Akt's role in Ang II-induced endothelial dysfunction, dominant negative-Akt virus and a constitutively active-Akt virus were used. And finally, we also used siRNA targeted at the p22phox NAD(P)H oxidase subunit, to determine the specific role of NAD(P)H oxidase in Ang II-induced endothelial dysfunction. The following sections discuss the tools used in examining our proposed pathway.

8.3.1. Inhibition of the AT1 receptor using candesartan

Candesartan (pharmaceutical drug name Atacand) is commonly prescribed to patients to treat hypertension. High blood pressure reduction helps prevent

strokes, heart attacks, and kidney problems. Clinical applications also include treatment of congestive heart failure, and to help protect the kidneys from damage due to diabetes. Candesartan blocks the vasoconstrictor and aldosterone secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor in many tissues, such as vascular smooth muscle and the adrenal gland. Its action is therefore independent of the pathways for angiotensin II synthesis. The result is blocking the action of angiotensin II thereby relaxing vascular smooth muscle, causing blood vessels to dilate. Candesartan does not inhibit angiotensin converting enzyme (ACE), nor does it bind to or block other hormone receptors or ion channels known to be important in cardiovascular regulation (115).

Cd has a high affinity for AT1 receptors (in the low nanomolar range) and a practically negligible affinity for AT2 receptors. Studies have convincingly demonstrated that candesartan, like all AT1 receptor antagonists, is a competitive inhibitor, with a very slow dissociation from the receptor. Candesartan shares the same mechanism of action as other Angiotensin II receptor antagonists but has a different pharmacokinetic profile (115). Candesartan is widely used to study Ang II signaling in HUVECs. It has been used to inhibit HUVEC migration (116) and abolish the stimulatory action of Ang II on *in vitro* and *in vivo* soluble ICAM-1 release (62). In another study, candesartan was used to decrease the expression of NAD(P)H oxidase subunit gp91-phox, reduce oxidative stress in the vessel wall and thus have an anti-atherosclerotic and anti-oxidative effect (117).

In the monocyte adhesion assay, as seen in **Figure 5**, Candesartan effectively blocked the increase in monocyte adhesion to HUVEC monolayers in both Ang II and TNF-α-stimulated HUVECs, bringing the level of adhering monocytes down to below baseline control levels. Thus, Cd acted as an effective AT1 receptor antagonist blocking Ang II's signaling cascades in ECs.

8.3.2. Inhibition of NAD(P)H oxidase using DPI

Diphenyleneneiodonium chloride (DPI) is a pharmacological inhibitor that binds strongly to flavoproteins. It acts as a powerful and specific inhibitor of several important enzymes, including nitric oxide synthase (NOS), NAD(P)H-ubiquinone oxidoreductase, NAD(P)H oxidases and NAD(P)H cytochrome P450 oxidoreductase. The inhibition of neutrophil NAD(P)H oxidase by DPI results in the loss of recoverable photoreduced flavin (FAD). The radical mechanism of action involves abstraction (by DPI) of an electron from the reduced redox centre to form a radical, which then adds back directly on to the prosthetic group or adjacent protein groups in or near the active site to form adducts (118). The NAD(P)H oxidase complex is present in various cell types, including SMCs and ECs, and increases superoxide production upon stimulation by Ang II, TNF-α, PDGF, and thrombin (71), (79). DPI is a widely used inhibitor in oxidative stress and NAD(P)H oxidase signaling studies (119), (120).

DPI was used at a concentration of 5 μM in our experiments. Although it has been used at concentrations as high as 100 μM (119) and 30 μM (120), DPI acted effectively at 5 μM in our studies. In the superoxide measurements using lucigenin-enhanced chemiluminescence, shown in **Figure 3**, DPI successfully blocked the Ang II-induced increase in superoxide production, and brought the level of superoxide to slightly below baseline levels. Also, in the monocyte adhesion assay, as seen in **Figure 5**, DPI effectively blocked the increase in monocyte adhesion in both Ang II and TNF-α-stimulated HUVECs, and brought the level of adhering monocytes down to approximately baseline control levels. Thus, DPI effectively blocked the action of NAD(P)H oxidase in producing superoxide suggesting that NAD(P)H oxidase plays a role in Ang II-induced endothelial activation.

8.3.3. Inhibition of NAD(P)H oxidase using p22phox siRNA

siRNA against the p22phox subunit of NAD(P)H oxidase was used to study the specific role of NAD(P)H oxidase on monocyte adhesion. This particular enzyme

subunit was chosen because it plays a crucial role in oxidase activation (78). In a previous study, antisense p22phox stably transfected into cultured VSMCs resulted in a 50% decrease in angiotensin II–stimulated NAD(P)H oxidase activity (121). A scrambled form of siRNA was used as a control, and HiPerfect reagent was used for transfections. The siRNA transfection protocol used for this study was optimized for HUVECs by Al Gouleh I *et al.* (122). **Figure 7** illustrates the effect of the siRNA on monocyte adhesion to endothelial cells. The p22phox siRNA successfully blocked the Ang II-induced increase in monocyte adhesion whereas the scrambled siRNA did not have an effect on control or Ang II-stimulated HUVECs.

8.3.4. Inhibition of PI3K using wortmannin

The potent and specific phosphatidylinositol 3-kinase inhibitor wortmannin, used in our study was isolated from penicillium funiculosum. Inhibition of PI3K/Akt signal transduction cascade enhances the apoptotic effects of radiation or serum withdrawal and blocks the antiapoptotic effect of cytokines. Inhibition of PI3K by wortmannin also blocks many of the short-term metabolic effects induced by insulin receptor activation. The inhibition induced by Wm is irreversible (123). Wm has been used in previous signaling studies on the role of Akt on mitogen activated protein kinase kinase/extracellular signal-regulated kinase 1/2 (MEK/ERK1/2) and eNOS activation (86), (21). Wm has also been used to study Akt's response to shear stress (20), (124) and VEGF (21) amongst other stimulants.

Wm was used at a concentration of 100 nM in our studies, as this concentration was determined to be optimal for the HUVECs used. Wm has a non-cytotoxic effect of cells at the concentration used, yet it remains effective. In fact, concentrations as low as 10 nM have been shown to effectively block the PI3K/Akt survival signaling pathway in HUVECs (112). In the monocyte adhesion assay, as seen in **Figure 5**, Wm effectively blocked the increase in monocyte adhesion to HUVEC monolayers in both Ang II and TNF-α-stimulated

HUVECs, bringing the level of adhering monocytes down to below baseline control levels. Thus, the use of Wm in our studies helped illustrate the involvement of the PI3K-Akt signaling cascade in Ang II-induced endothelial activation.

8.3.5. Inhibition of Akt using viruses

We used, a dominant negative (dn)-Akt virus (Ad5-CMV-Akt-AAA) and a catalytically (constitutively) active (ca)-Akt virus (Ad5-CMV-myrAkt) in order to study the role of Akt on monocyte adhesion to HUVEC monolayers. The dn-Akt virus is an HA-tagged, inactive phosphorylation AKT mutant that contains an alanine substitution at the active site (residue 179) in addition to two alanine substitutions at the regulatory phosphorylation sites (125). The dominant-negative mutant Akt protein cannot be activated by phosphorylation and it functions in a dominant-negative fashion. The constitutively active Akt construct has the c-*src* myristoylation sequence fused in-frame to the N terminus of the HA-Akt (wild-type) coding sequence. The mutant Akt and constitutively active Akt adenoviral vectors were constructed by the same protocol (125).

This dn-Akt virus was previously used by Hussain SN and coworkers and was shown to successfully inhibit Akt signaling pathways in HUVECs (126); thus, the same virus infection protocol was used. The ca-Akt virus used in our study was used in a previous study to confer survival to ECs in suspension and under serum-deprived conditions (125). The green fluorescent protein (Gfp) virus, Ad5-CMV-eGFP was used as a control virus to indicate successful endothelial cell infection. This virus was also used as a control by Harfouche R *et al*, (126). After 3 hours of infection, several Gfp's were visualized per view using an Olympus 1X70 inverted microscope. The day following cell infection, nearly all endothelial cell in view expressed gfp.

Although in the Harfouche *et al.* virus transfection protocol 100 molecule of infection (MOI) were used for gfp and dn-Akt viruses, the amount was too high

for our particular strain of HUVECs and this concentration resulted in cell death. The cells in the Harfouche *et al.* study were purchased from GlycoTech Corporation, whereas our cells were freshly isolated. Since the cells were from different sources, perhaps their response to viruses also differed. In an earlier study, an MOI of 50 was used for Akt and Gfp viruses (127). In the Fujio Y *et al.* study, the ca-Akt virus was used at a MOI of 50 as well. In our experiments, the MOI for all 3 viruses was subsequently reduced to 50 MOI, and with this concentrations, the health of the cells was not compromised. **Figure 6** illustrates the effect of the viruses on monocyte adhesion to endothelial cells. The dn-Akt virus successfully blocked the Ang II-induced increase in monocyte adhesion whereas the ca-Akt virus successfully increased monocyte adhesion, compared to cells incubated with Ang II alone.

8.3.6. Inhibition of eNOS using L-Name

N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-Name) is an analog of arginine that inhibits nitric oxide synthase. It affects the vascular system in various ways; it blocks relaxation induced by acetylcholine and induces an increase in arterial blood pressure. L-Name has been used as an eNOS inhibitor in various studies involving ECs (43), (93). L-Name was used at a concentration of 3 mM in our experiments and was shown to be effective. A range of L-Name concentrations is used in *in vitro* studies depending on the cell type and the nature of the experiment. The concentration used in our studies was approximately the same as that of other HUVEC studies (93), (128).

In the superoxide measurements using lucigenin-enhanced chemiluminescence, shown in **Figure 3** With L-Name there was still over a 2-fold increase in superoxide levels compared with the control cells, a value very close to Ang II stimulated cells. This result is consistent with previous studies (80), (129), (130). Because L-Name blocks eNOS, which produces NO, less NO is available within the cell. Thus, less NO is able to react with superoxide and the result is an increase in superoxide levels. In the monocyte adhesion assay, as seen in **Figure**

5, L-Name effectively blocked the increase in monocyte adhesion to HUVEC monolayers compared to both Ang II and TNF-α-stimulated HUVECs, bringing the level of adhering monocytes down to below baseline control levels. Thus, L-Name's ability to block monocyte adhesion to HUVECs suggests the involvement of eNOS in Ang II-induced endothelial activation.

8.4. Significance

Ang II has been shown to activate the endothelium and increase monocyte adhesion to endothelial cells, but a mechanism has not been established (39). The objective of this study was to determine the cellular mechanism involved in Ang II-induced endothelial cell activation (measured as monocyte adhesion) and moreover, to determine the roles of NAD(P)H oxidase-produced superoxide, Akt and eNOS in this mechanism. We found that Ang II signals through the AT1 receptor to stimulate superoxide production by NAD(P)H oxidase. As a result of this cascade, Akt and subsequently eNOS are activated. This leads to the production of NO. NO is proposed to react with the superoxide and produce the potent oxidant peroxynitrite which goes on to damage and activate the endothelium, making it more responsive to leukocyte adhesion.

A likely cause of increased monocyte adhesion is Ang II-induced up regulation of ICAM-1. Increased expression of ICAM-1 has been observed as a result of endothelial cell stimulation (62), (66), (108). Furthermore, numerous studies have linked increased ICAM-1 expression to Ang II stimulation in ECs (62). Although this link has been established, the specific pathways remain unclear.

We examined the specific molecular players in our proposed Ang II signaling mechanism using pharmacological inhibitors, viruses and siRNA in chemiluminescence and adhesion assays. In our adhesion assay, Cd illustrated the role of the AT1 receptor in Ang II signaling cascade in ECs. Previously conducted studies have shown that hydrogen peroxide (86), shear stress (20), and TNF-α (95), amongst other stimulants stimulate the Akt pathway which leads to eNOS

activation. These findings concur with our finding that Ang II is able to activate Akt and eNOS. We demonstrated the involvement of the PI3K-Akt cascade in Ang II-induced endothelial activation and dysfunction using Wm and Akt viruses in the adhesion assay. We also demonstrated the involvement of eNOS in Ang II-induced endothelial activation and dysfunction using L-Name in chemiluminescence and adhesion assays. Moreover, we also showed that elevated superoxide levels resulting from Ang II stimulation were likely attributed to NAD(P)H oxidase activation by using the pharmacological inhibitor DPI in chemiluminescence assays and adhesion assay in addition to p22phox siRNA in the adhesion assay.

Together, our findings indicate that the simultaneous activation by Ang II of eNOS and NAD(P)H oxidase in endothelial cells leads to HUVEC activation. Our results help to explain the paradox of why Ang II-induced Akt activation and NO production harms rather than helps the endothelium. In our proposed mechanism it is the simultaneous production of NO from eNOS and superoxide from NAD(P)H oxidase that react to form peroxynitrite and ultimately lead to endothelial dysfunction and activation. Our findings suggest that blocking any one part of the pathway effectively inhibits Ang II-induced endothelial activation.

9. Final conclusions and summary

- Ang II (100 nM) produced a 1.9-fold increase in O_2^- production above control levels
  - increase was blocked by DPI
  - increase was not blocked by L-NAME

- Ang II increased monocyte adhesion to HUVEC by 4.5-fold
  - this increase corresponded to 80% of the response induced by TNF-α

- Monocyte adhesion in response to Ang II was blocked by candesartan, DPI, L-Name, and wortmannin

- Adhesion after Ang II treatment was decreased by dominant negative-AKT and increased by 1.5-fold by the constitutively active-AKT compared to Ang II-stimulated GFP-transfected cells.

- Ang II-induced monocyte adhesion was inhibited a p22phox siRNA

Together, these findings indicate that the simultaneous activation by Ang II of eNOS and NAD(P)H oxidase in endothelial cells leads to increased HUVEC activation. This process can partly explain the beneficial effects of therapies that reduce the action of Ang II.

Figures and Legends

Figure 1. Proposed Ang II signaling pathway leading to endothelial cell activation

In endothelial cells, Angiotensin II binds and activates the AT1 receptor (34), (35). When Ang II binds the AT1 receptor, the subunits of a guanine-nucleotide-binding protein ($G_{q/11}$) dissociate. This in turn activates phospholipase C, which generates diacylglycerol and inositol triphosphate (40). Inositol triphosphate mediates the release of intracellular calcium stores. Cellular calcium levels also rise due to Ang II-induced calcium entry through cell membrane channels (41). Calcium and diacylglycerol activation signal through kinases such as PKC and calcium-calmodulin kinases which go on to alter downstream proteins (42). Stimulation of the AT1 receptor by Ang II has been recently linked to activating NAD(P)H oxidase and thus producing ROS in vascular cells (16). In the scheme shown NAD(P)H oxidase is hypothesized to be the source of superoxide, which is then converted to H_2O_2. In VSMCs, Akt phosphorylation is inhibited by DPI, indicating a role for NAD(P)H oxidase in Akt activation (19). Our scheme proposes a similar mechanism in ECs. Ang II has been shown to increase levels of H_2O_2-derived from NAD(P)H oxidase, which then activates eNOS (86). Thus, as shown in the scheme, Ang II has the ability to simultaneously produce superoxide and nitric oxide and consequently the damaging product peroxynitrite. The link between Akt and eNOS activation has been established in studies involving VEGF (93), (21), shear stress (20), (94), and TNF-α (95) stimulation. We propose that Ang II triggers a similar Akt-eNOS signaling cascade in ECs. Ang II increases monocyte adhesion to ECs (60), and ACE inhibitor treatment *in vivo* decreases endothelial dysfunction and the subendothelial infiltration of mononuclear cells (59). The proposed mechanism attributes the increased monocyte adhesion to endothelial activation caused by peroxynitrite formation.

Figure 1

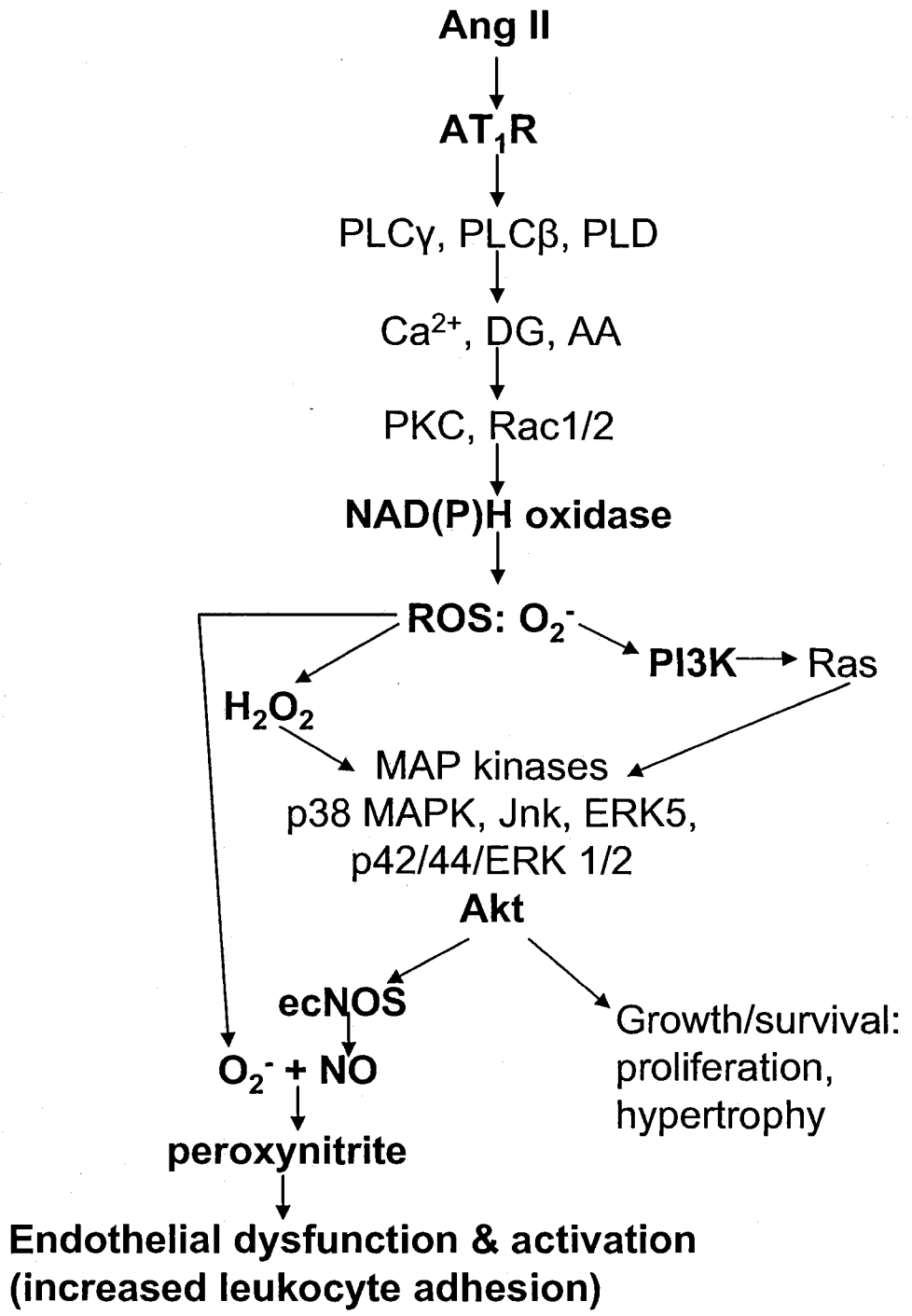

Ang II
AT1R
PLCγ, PLCβ, PLD
Ca2+, DG, AA
PKC, Rac1/2
NAD(P)H oxidase
ROS: O2-
PI3K
Ras
H2O2
MAP kinases
p38 MAPK, Jnk, ERK5,
p42/44/ERK 1/2
Akt
ecNOS
O2- + NO
peroxynitrite
Growth/survival:
proliferation,
hypertrophy
Endothelial dysfunction & activation
(increased leukocyte adhesion)

Figure 2. Superoxide production measured using lucigenin-enhanced chemiluminescence: control vs. Angiotensin II-stimulated HUVECs

HUVECs grown to approximately 95% confluency in endothelial cell growth medium were stimulated with Ang II (100 nM) for 1 hour. Control cells and Ang II-stimulated cells were scraped thoroughly using Hepes Buffer. Hepes buffer was used to equalize the volume of all wells prior to superoxide readings. LMax II 384 luminometer using SoftMax Pro software was used to measure superoxide levels of each well. Lucigenin (10 μM) and NAD(P)H (250 μM) were injected into each well. The superoxide level was measured every 31 seconds for a period of 30 min. The background signal was subtracted from the total output and the area under the curve of output was calculated. Following superoxide measurements, cells from each sample were counted. Results were normalized to 1,000,000 cells/mL. Data represents 3 individual experiments. Data represents ± SEM.

* statistically significant difference ($p<0.05$) using the Student-Newman-Keuls comparison Method.

Figure 2

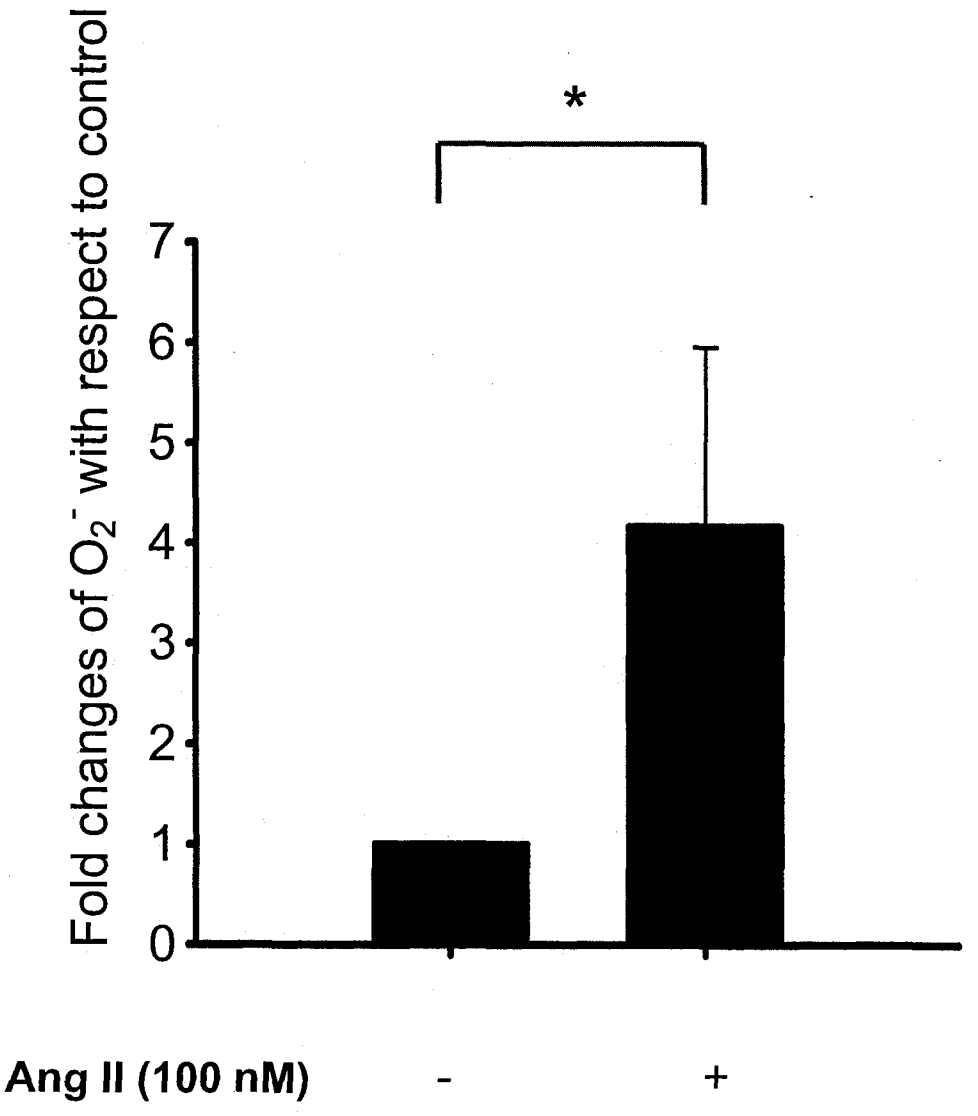

Fold changes of O_2^- with respect to control
*
7
6
5
4
3
2
1
0
Ang II (100 nM)
-
+

Figure 3. Superoxide production measured using lucigenin-enhanced chemiluminescence: effect of L-Name and DPI on Angiotensin II-stimulated HUVECs

HUVECs grown to approximately 95% confluency in endothelial cell growth medium were stimulated with Ang II (100 nM) for 1 hour. The following conditions were run: blank, control, Ang II, Ang II + DPI (5 μM) and Ang II + L-Name (3 mM). The inhibitors were added following cell scraping into suspensions of Ang II stimulated cells. Cells were scraped thoroughly using Hepes Buffer. Hepes buffer was used to equalize the volume of all wells prior to superoxide readings. LMax II 384 luminometer using SoftMax Pro software was used to measure superoxide levels of each well. Lucigenin (10 μM) and NAD(P)H (250 μM) were injected into each well. The superoxide level was measured every 31 seconds for a period of 30 min. The background signal was subtracted from the total output and the area under the curve of output was calculated. Following superoxide measurements, cells from each sample were counted. Results were normalized to 1,000,000 cells/mL.

Data represents 3 individual experiments. Data represents ± SEM.

* statistically significant difference ($p<0.05$) using the Student-Newman-Keuls comparison Method.

Figure 3

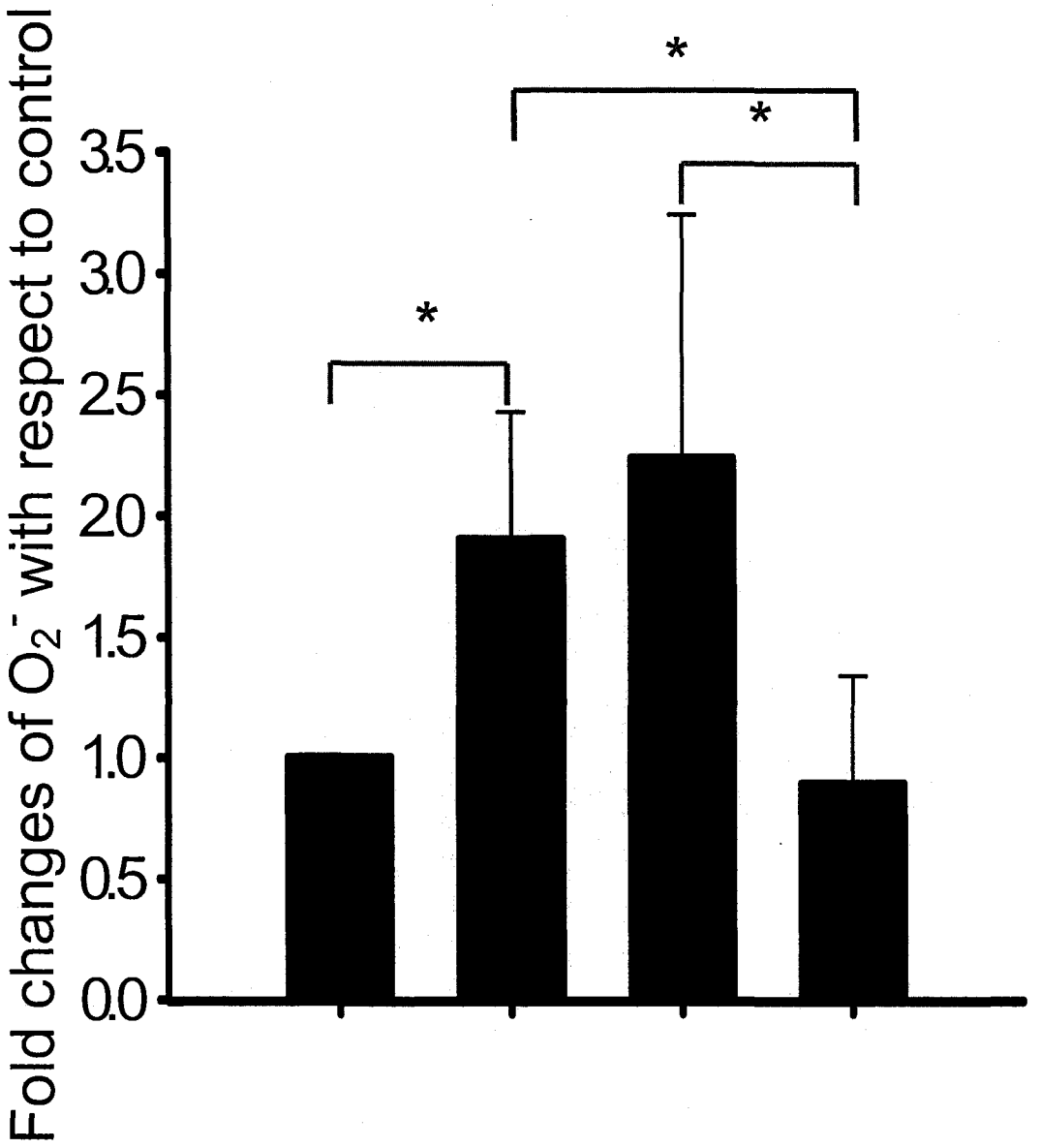

Fold changes of O_2^- with respect to control
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Ang II (100 nM) - + + +
L-Name (3 mM) - - + -
DPI (5 µM) - - - +

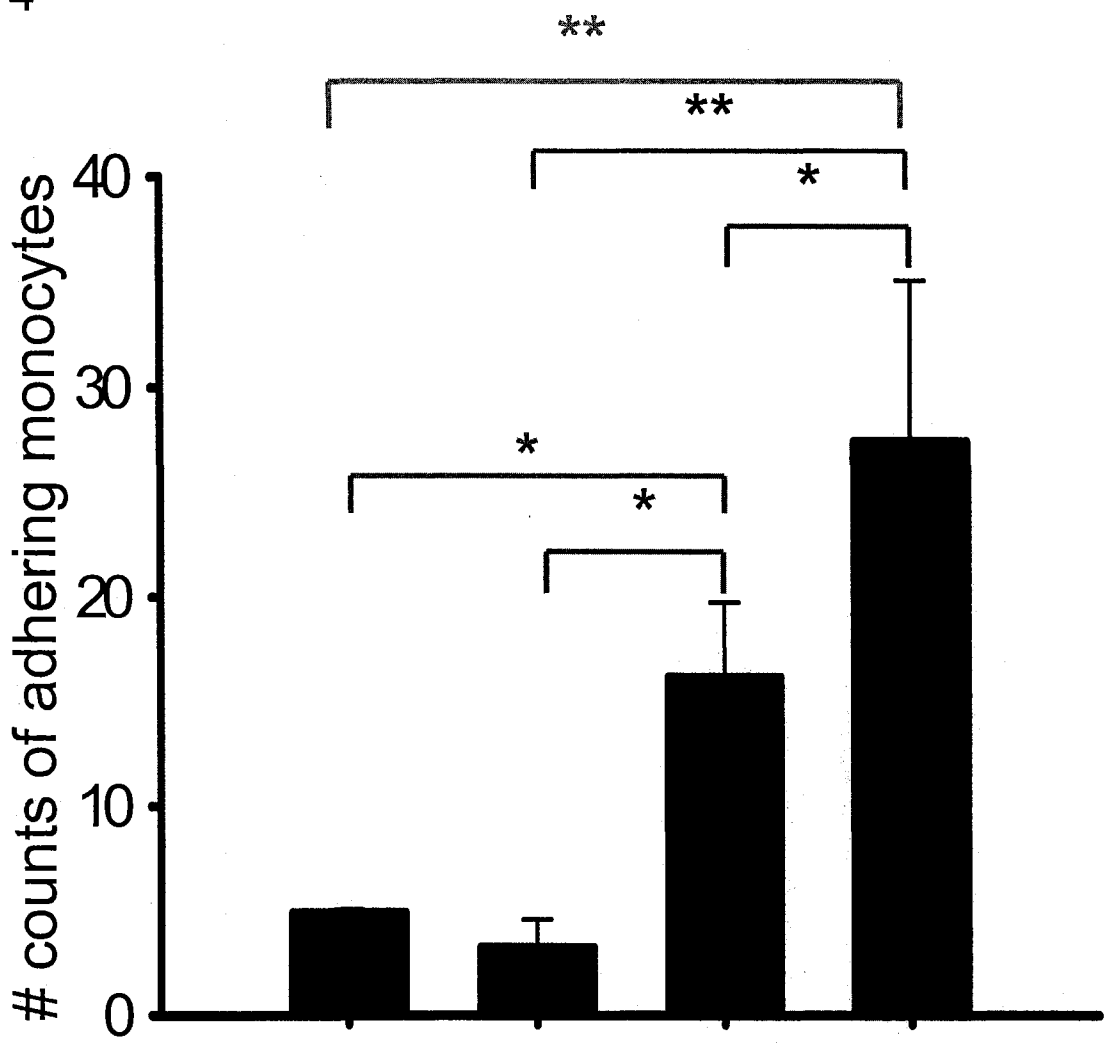
Figure 4. Monocyte adhesion to HUVECs: control conditions

The monocyte adhesion assay was performed on passage 3 HUVECs grown using endothelial growth medium to approximately 95% confluency. The following conditions were used: Control (Stimulation media), Control (Ang II solvent, ddH_20), positive control-TNF-α (10 ng/mL) and Ang II (100 nM).

Cells were incubated with Ang II or TNF-α for 6 hours using stimulation media. Following stimulation, 500,000 DiI-labeled U937 monocytic cells were added to each well. Monocytes were allowed to adhere to plated HUVECs for 30 min in 37°C on a rocking platform. After the initial 15 min of shaking, the plate was rotated 90° for the remainder of the shaking time. After removing the media and suspended monocytes, the wells were then washed three times, at room temperature, with PBS. Following the washes, cells were fixed for 30 min using 10% neutral buffered formalin, at room temperature. The formalin was then removed and cells were visualized. U937 adhesion was quantified in four fields per well (each condition was a triplicate) viewed under 10X magnification, with an automated image analysis system using an Olympus 1X70 inverted microscope and Image-Pro Plus software (version 4.5.0.29).
Data represents 3 individual experiments. Data represents ± SEM.

* statistically significant difference ($p<0.05$) using the Student-Newman-Keuls comparison Method.
** $p<0.001$

Figure 4



ddH_2O (Ang II solvent)	+	-	-	-
Media	-	+	-	-
Ang II (100 nM)	-	-	+	-
TNFα (10 ng/mL)	-	-	-	+

Figure 5. Monocyte adhesion to HUVECs: effect of candesartan, DPI wortmannin, and L-Name

The monocyte adhesion assay was performed on passage 3 HUVECs grown using endothelial growth medium to approximately 95% confluency. The following conditions were used: Control (Stimulation media), Control (Ang II solvent, ddH_20), positive control-TNF-α (10 ng/mL), Ang II (100 nM), Ang II + DPI (5 μM), Ang II + Wortmannin (100 nM), Ang II + L-Name (3 mM), Ang II + Candesartan (10 μM).

Cells were incubated with inhibitors for 1 hour prior to 6 hour stimulation with Ang II or TNF-α in stimulation media. Following stimulation, 500,000 DiI-labeled U937 monocytic cells were added to each well. Monocytes were allowed to adhere to plated HUVECs for 30 min in 37°C on a rocking platform. After the initial 15 min of shaking, the plate was rotated 90° for the remainder of the shaking time. After removing the media and suspended monocytes, the wells were then washed three times, at room temperature, with PBS. Following the washes, cells were fixed for 30 min using 10% neutral buffered formalin, at room temperature. The formalin was then removed and cells were visualized. U937 adhesion was quantified in four fields per well (triplicate per condition) viewed under 10X magnification, with an automated image analysis system using an Olympus 1X70 inverted microscope and Image-Pro Plus software (version 4.5.0.29).

Data represents 3 individual experiments. Data represents ± SEM.

* statistically significant difference ($p<0.001$) using the Student-Newman-Keuls comparison Method.

** $p<0.05$

Figure 5

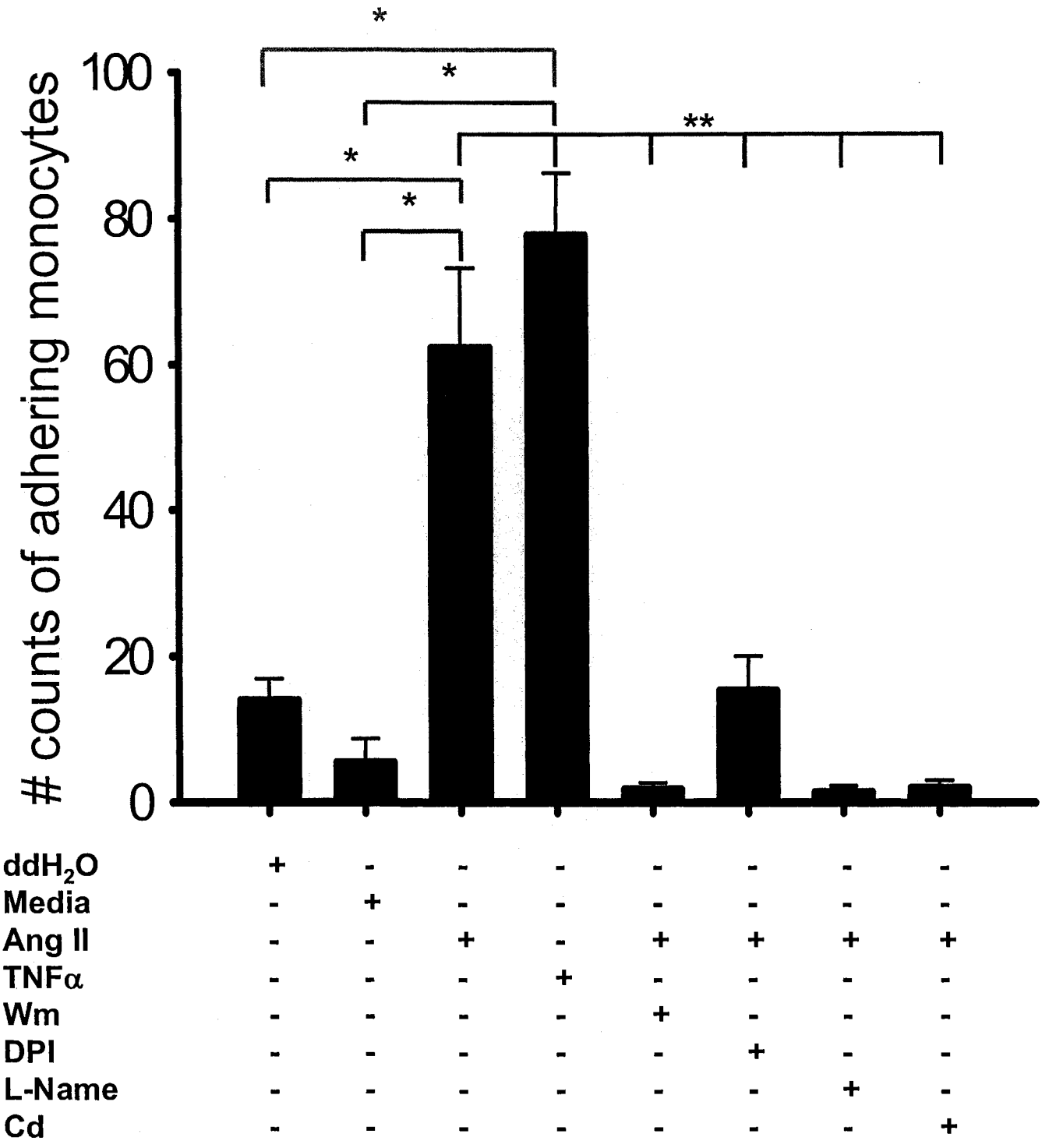

counts of adhering monocytes
100
80
60
40
20
0
*
*
*
*
**
ddH2O + - - - - - - -
Media - + - - - - - -
Ang II - - + - + + + +
TNFα - - - + - - - -
Wm - - - - + - - -
DPI - - - - - + - -
L-Name - - - - - - + -
Cd - - - - - - - +

Figure 6. Monocyte adhesion to HUVECs: effect of Akt viruses

Akt viruses were used to examine the role of Akt in monocyte adhesion. At approximately 70% confluency, cells were transfected with viruses for 4 hours using serum deprived media (MCDB 131 + 1% L-Glutamine). Gfp virus, Ad5-CMV-eGFP ($1.3x10^{12}$ particles/mL, $4x10^{10}$ titer pfu/mL) was used as a positive control to indicate transfection efficiency. Dominant negative (dn) Akt virus (Ad5-CMV-Akt-AAA) ($1.3x10^{12}$ particles/mL, $1x10^{10}$ titer pfu/mL) and a constitutively active (ca) Akt virus (Ad5-CMV-myrAkt) ($1.1x10^{12}$ particles/mL, $2x10^{10}$ titer pfu/mL). The viruses were used at a dose of 50 molecule of infection (MOI=PFU/mL). Following the transfection, cells were washed three times with PBS, supplied with growth media and grown to confluency before performing the adhesion assay.

The following conditions were used: GFP, GFP + Ang II (100 nM), dn-Akt, dn-Akt + Ang II, ca-Akt, ca-Akt + Ang II. Cells were stimulated with Ang II for 6 hours using stimulation media. Following stimulation, 500,000 DiI-labeled U937 monocytic cells were added to each well. Monocytes were allowed to adhere to plated HUVECs for 30 min in 37°C on a rocking platform. After the initial 15 min of shaking, the plate was rotated 90° for the remainder of the shaking time. After removing the media and monocytes, the wells were washed three times with PBS. Following the washes, cells were fixed for 30 min using 10% neutral buffered formalin. The formalin was then removed and cells were visualized. U937 adhesion was quantified in four fields per well (triplicate per condition) viewed under 10X magnification, with an automated image analysis system using an Olympus 1X70 inverted microscope and Image-Pro Plus software (version 4.5.0.29). Data represents 3 individual experiments. Data represents ± SEM.

* statistically significant difference ($p<0.001$) using the Student-Newman-Keuls comparison Method.

** $p<0.05$

Figure 6

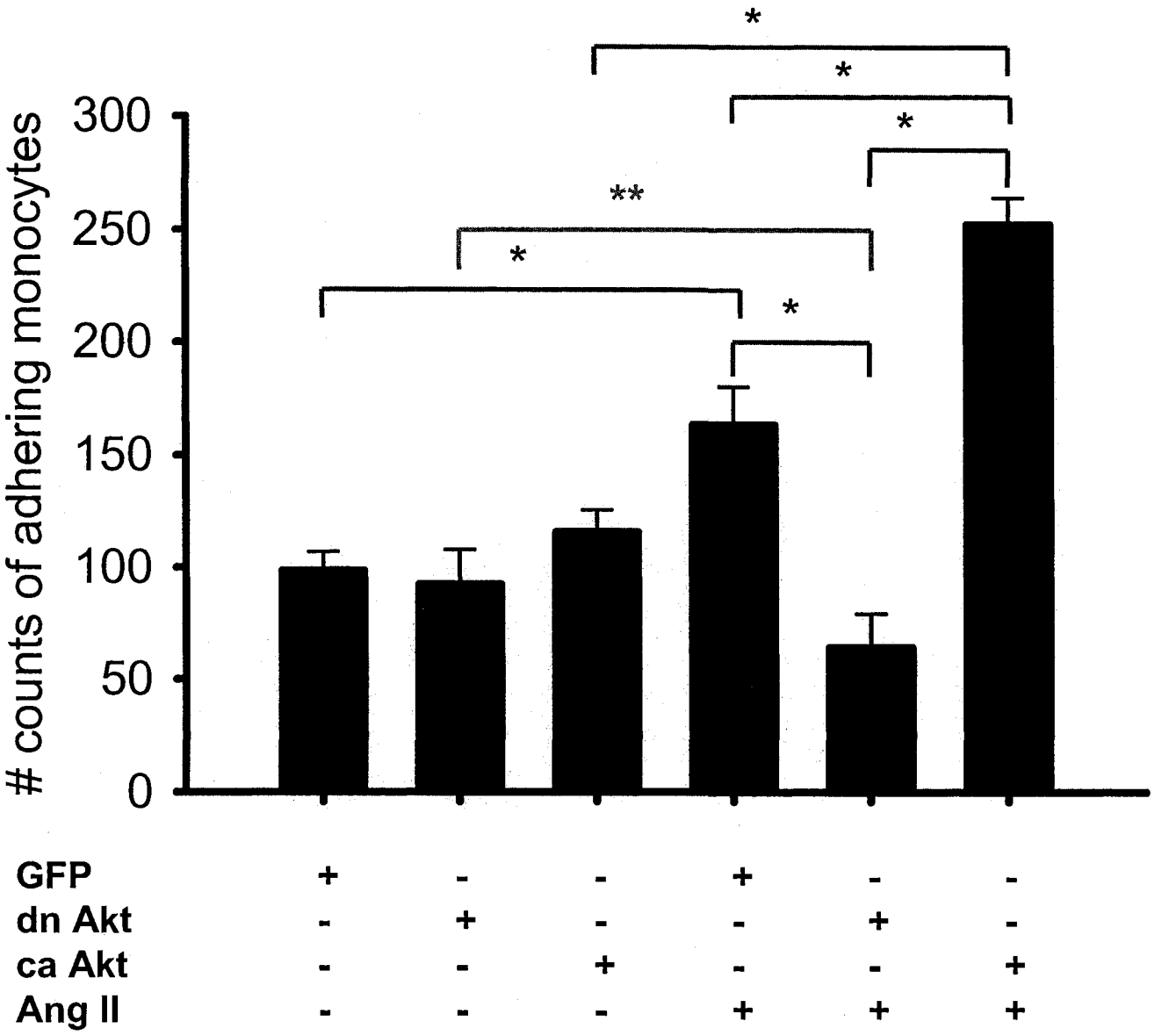
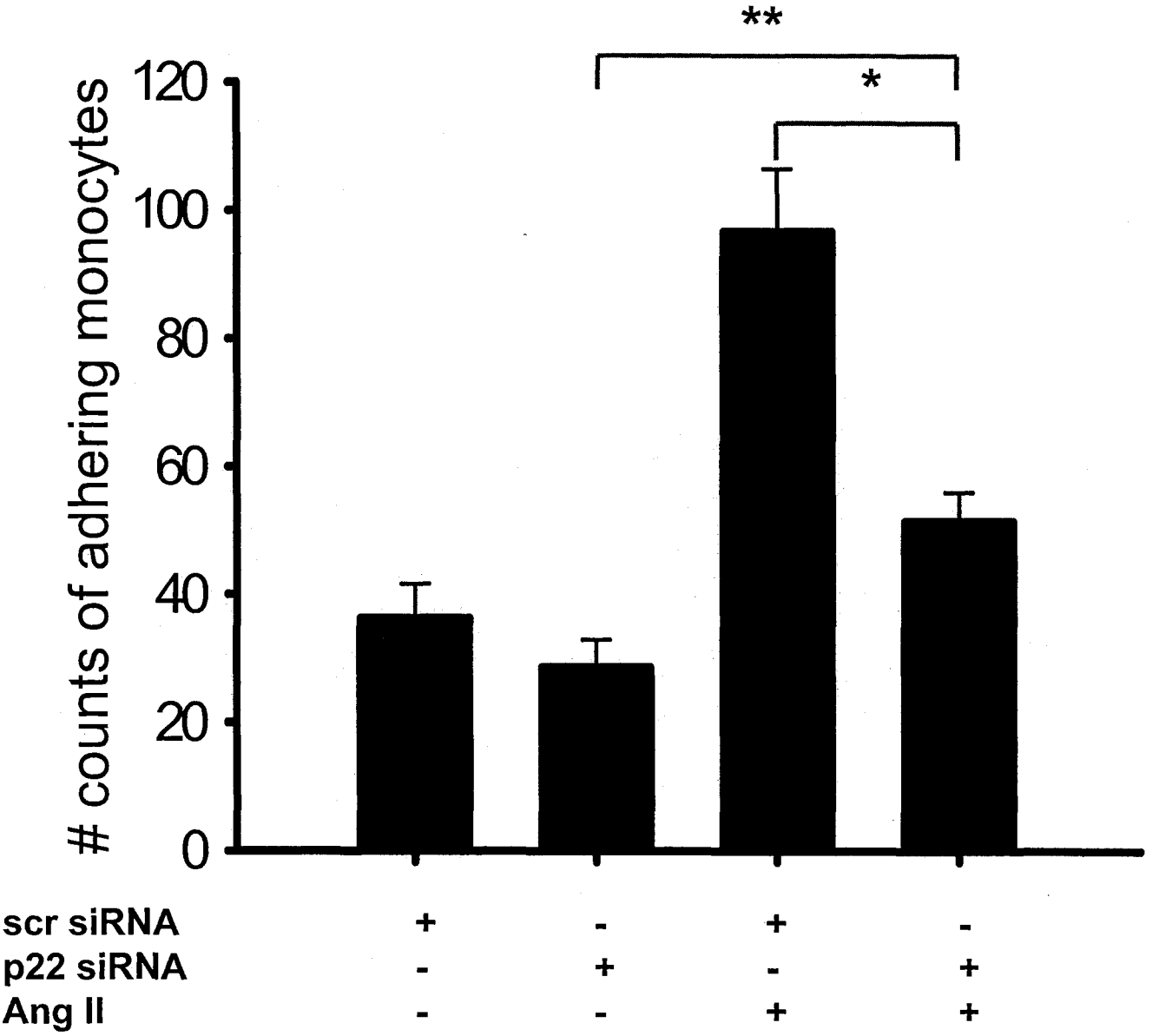

counts of adhering monocytes
300
250
200
150
100
50
0
*
*
*
**
*
*
GFP + - - + - -
dn Akt - + - - + -
ca Akt - - + - - +
Ang II - - - + + +

Figure 7. Monocyte adhesion to HUVECs: effect of p22phox siRNA

In order to examine the effect of NAD(P)H oxidase on monocyte adhesion to HUVECs following Ang II stimulation, siRNA against the p22phox subunit was used. HUVECs were grown to approximately 70% confluency and transfected with scrambled and p22phox siRNA. siRNA was mixed with HiPerfect reagent and serum-free media and allowed to form complexes for 10 min at room temperature. Scrambled siRNA was used at a concentration of 10 nM and p22 siRNA at 1.4 μ, and 0.78 μL/well of HiPerfect solution was used. Following the room temperature incubation, the mix of each siRNA was added drop wise to each well (50 μL/well). Plates were tilted to ensure even mixing and incubated at 37°C for 5 hours. Following incubation, cells were washed twice with PBS and EC growth media was added back to HUVECs. Cells were then grown to full confluency and 3 days later, the monocyte adhesion assay was performed.

The following conditions were used: GFP, GFP + Ang II (100 nM), dn-Akt, dn-Akt + Ang II, ca-Akt, ca-Akt + Ang II. Cells were stimulated with Ang II for 6 hours using stimulation media. Following stimulation, 500,000 DiI-labeled U937 monocytic cells were added to each well. Monocytes were allowed to adhere to plated HUVECs for 30 min in 37°C on a rocking platform. After the initial 15 min of shaking, the plate was rotated 90° for the remainder of the shaking time. After removing the media and monocytes, the wells were washed three times with PBS. Following the washes, cells were fixed for 30 min using 10% neutral buffered formalin. The formalin was then removed and cells were visualized. U937 adhesion was quantified in four fields per well (triplicate per condition) viewed under 10X magnification, with an automated image analysis system using an Olympus 1X70 inverted microscope and Image-Pro Plus software (version 4.5.0.29). Data represents 3 individual experiments. Data represents ± SEM.
* statistically significant difference ($p<0.001$) using the Student-Newman-Keuls comparison Method. ** $p<0.05$

Figure 7


**
*
120
100
80
60
40
20
0
counts of adhering monocytes
scr siRNA + - + -
p22 siRNA - + - +
Ang II - - + +

Appendix A

Future directions

Although our findings contributed to the understanding of Ang II-induced endothelial activation, there is the possibility of the involvement of other molecular players such as ICAM-1 that, due to time constraints were not assessed. There are various possibilities for future studies, including ICAM-1 and superoxide studies. Confirming the result of previous studies of an increase in protein expression of ICAM-1 following Ang II exposure, for the time period studied would be useful. Another possible useful variation of the monocyte adhesion assay is to study the effect of blocking ICAM-1 (via antibodies) on adhesion. Studying the interaction between superoxide produced by NAD(P)H oxidase and ICAM-1 could provide further evidence for the proposed mechanism. Studying the role of NAD(P)H oxidase on adhesion may also be done using p47phox -/- mice, as p47phox is a crucial NAD(P)H oxidase subunit. Understanding signaling pathways of other stimulants such as TNF-α in endothelial cells is helpful in trying to piece together Ang II's signaling mechanism. Another possible study that focuses on understanding the role of PKCζ activation in Ang II-induced ICAM-1 clustering and endothelial adhesivity towards leukocytes, as this kinase and ICAM-1 are involved in TNF-α signaling (67), (66). In order to confirm our findings, NO production in response to Ang II stimulation may be measured using a Greiss reaction and luminal-enhanced chemiluminescence may be used to measure peroxynitrite formation.

Appendix B

Claims to original research

I hereby declare that this submission is my own work and that to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to substantial extent has been accepted for the award of any other degree or diploma of McGill university or any other institute of higher learning, except where acknowledgements have been made in the text.

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