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ROLE OF CYCLOOXYGENASE-2 IN ABDOMINAL AORTIC ANEURYSMS IN MICE

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ROLE OF CYCLOOXYGENASE-2 IN ABDOMINAL AORTIC ANEURYSMS IN MICE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

ROLE OF CYCLOOXYGENASE-2 IN ABDOMINAL AORTIC ANEURYSMS IN MICE

Abdominal aortic aneurysm (AAA) is a chronic inflammatory disease with no available pharmacological treatment. AAA formation reduces the structural integrity of the vessel and increases the susceptibility to rupture. The inflammatory response within human aneurysmal tissue is characterized by increased expression of cyclooxygenase-2 (COX-2). Similarly, in a mouse model of the disease induced by chronic Angiotensin II (AngII) infusion, we have shown that COX-2 expression in the abdominal aortic smooth muscle layer increases early in the development of the disease. Furthermore, genetic or pharmacological inactivation of COX-2 prior to disease initiation reduces AAA incidence.

The current study utilized nonhyperlipidemic mice to determine the effectiveness of COX-2 inhibition initiated after AAA formation. COX-2 inhibitor treatment was initiated 5 days after beginning the AngII infusion, a time-point where significant aneurysmal pathology is observed. COX-2 inhibition with celecoxib significantly reduced the incidence as well as severity of AAAs as compared to the control group. Celecoxib treatment also protected the mice from aortic rupture and death. AAA development is characterized by degradation of the aortic smooth muscle layer with loss of the contractile phenotype. We found that the effectiveness of celecoxib was associated with significantly increased mRNA expression of alpha-actin, SM22alpha and desmin, all of which are markers of a differentiated smooth muscle cell phenotype. Celecoxib treatment also decreased mRNA expression of a marker of dedifferentiated smooth muscle (hyaluronic acid synthase 2). We also examined the role of altered expression of COX-2 in the increased susceptibility of the abdominal segment to AAA formation. We found a prolonged and greater induction of COX-2 in the abdominal aortic smooth muscle layer in contrast to a transient induction of COX-2 in the other regions of the aorta throughout disease progression. Overall, these findings suggest that COX-2 plays an important role in AAA development in mice, and COX-2 inhibition with celecoxib attenuates progression of aneurysm development by maintaining a differentiated phenotype in abdominal aortic smooth muscle cells.

KEYWORDS: Abdominal aortic aneurysm (AAA), Cyclooxygenase-2 (COX-2), COX-2 inhibitor, Celecoxib, Angiotensin II (AngII)

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To Mum and Papa

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BACKGROUND

1.1. ABDOMINAL AORTIC ANEURYSMS

1.1.1. Epidemiology and natural history of abdominal aortic aneurysms

Abdominal aortic aneurysms (AAAs) have emerged to be a major health concern throughout the world, particularly in the developing countries.[1] It is known to affect mostly the ageing population, and is the 10th leading cause of death in men over the age of 55 years.[2] AAA is a chronic vascular condition of the aorta involving an increase in diameter of at least 50% as compared to the expected normal diameter, and has potentially life-threatening implications.[3] It begins as minor vessel dilation that progress over years to produce a weakened aorta with increased susceptibility for rupture.[4] Ruptured AAAs have greater than a 90% incidence of mortality, resulting in approximately 15,000 deaths per year in the United States.[5, 6] Of particular concern is that, unlike coronary heart disease, the incidence of AAAs is reported to be increasing in the western countries.[7, 8] Moreover, although AAAs are thought to be responsible for approximately 2% of all deaths, these estimates of mortality are suggested to be hampered by attributing a portion of sudden deaths to cardiac failure rather than the actual cause of ruptured AAAs, because a pre-existing AAA had not been documented.[9]

The diameter of the normal abdominal aorta in humans is approximately 2 cm. Thus, a person having an abdominal aortic diameter of ≥3 cm is considered to be diagnosed of an AAA.[10] The rate of aneurysmal growth has been found to be positively

correlated to the diameter of the AAA.[11] The smallest AAAs (3 cm – 3.5 cm in diameter) require approximately 5 years to attain a diameter of 5.5 cm, at which stage surgical intervention is suggested.[12] The average rate at which an AAA, which has a diameter of ≤ 5.5 cm, expands has been suggested to be 2.6 mm per year.[13] Moreover, the AAA diameter has been suggested to be the strongest predictor of rupture of the abdominal aorta. Lederle et al. have suggested that the rupture rates of AAAs measuring 5.5 to 5.9, 6 to 6.9, and ≥ 7 cm are approximately 8%, 10% and 20% per year, respectively.[11]

1.1.2. Risk factors for AAAs

AAAs have been associated with a number of non-specific risk factors based on data collected from epidemiological screening studies. Of these, male gender and increasing age are probably the most prominent although non-modifiable risk factors for AAAs.[9] The incidence of AAAs is known to increase with increasing age in men. Thus, AAAs have a prevalence of approximately 1.3% in men 45 to 54 years of age which increases to approximately 5% in men 65 years or older. However, in men 75 to 84 years of age, the prevalence of AAAs is reported to be as high as 12.5%. In contrast, in the same age groups as above, the prevalence of AAAs in women was reported to be 0% in the youngest group and 5.2% in the oldest age group.[5, 14, 15]

Tobacco smoking has been suggested to be the most important environmental risk factor for the development and progression of AAAs.[1] The prevalence of the disease in people smoking tobacco is known to be approximately 4 times more than those who have not smoked during their life.[16] The rate of growth of these aneurysms in current

smokers has also been reported to be 15 to 20% higher than that in non-smokers.[13] However, this risk has also been found to decrease gradually with termination of smoking.[17] Thus, the above reports suggest that smoking cessation should be one of the major priorities for patients with AAAs.

AAA has been traditionally regarded as a consequence of atherosclerosis.[18] However, recent evidences suggest that the pathobiology associated with these two diseases are significantly different from each other, at least during the initial development.[19] While macrophages in atherosclerotic lesions are usually localized in the subintimal space, macrophages in AAAs are most often identified in the medial layer.[20] Brady et al., while reporting on the risk factors associated with AAAs during the UK Small Aneurysm Trial, have also shown that patients with the greatest atherosclerotic burden exhibit the slowest growth of AAAs.[13] In contrast, a recent study by Golledge et al. suggests that a low high-density lipoprotein level is likely one of the most sensitive predictors of AAAs.[21] Thus, with different studies reporting conflicting evidence, the role of atherosclerosis in the development of AAAs is not clear.

Interestingly, diabetes mellitus, another cardiovascular risk factor, has been reported to be negatively associated with AAA growth. Patients with diabetes were found to have a 30% reduction in AAA growth, as compared to those without the disease.[13] The wall of the aorta has been shown to be thicker as the stress on the vascular wall is reduced in diabetic patients.[22] Moreover, it has been recently suggested that the glycation of extracellular matrix molecules such as collagen, that occurs during diabetes may increase the resistance of the aortic wall to proteolysis during aneurysmal degeneration and may contribute to a protective effect during AAA expansion.[23]

Family history is now widely accepted as a significant risk factor correlated with AAA development.[10, 20] At least 15% of people detected with AAAs were found to have first-degree family members with AAAs.[24] Ethnicity of the patients may also be important with evidence that the incidence of AAAs is more common in Northern Europeans than Asians or Africans.[10, 25] Finally, arterial hypertension was found to have only a minor impact on the incidence of AAAs. It should be noted however, that it is difficult to assess the correlation of blood pressure and AAA, as hypertension is usually defined on the basis of the patient receiving an anti-hypertensive medication.[9] Therefore, although the primary risk factors associated with AAA development, including advanced age, male gender and smoking show similarity to atherosclerosis, the majority of evidence suggests that the etiology of AAAs is distinct from other cardiovascular diseases.

1.1.3. Pathobiology of AAAs

AAAs are known to develop in the infrarenal region of the aorta i.e., just below the renal arteries. The major characteristic features during the pathogenesis of AAAs include chronic inflammation of the aortic wall, uncontrolled protease production leading to destructive remodeling of the vessel wall, and depletion of medial smooth muscle cells.[12]

Alteration of the connective tissue in the aortic wall is a primary feature of AAA development in humans. The connective tissue is comprised mainly of elastin and collagen, which impart mechanical properties to the aorta. While the network of elastic fibers is responsible for the elastic properties of the aorta, collagen (types I and III)

provides tensile strength, and thereby maintains the structural integrity of the vessel wall.[1] AAA formation is characterized by significant elastin fragmentation as well as a decrease in the concentration of elastin.[26, 27] Thus, loss of elastin in the medial layer has been suggested to contribute primarily to the initial dilation of the aorta during AAA formation. In the absence of sufficient medial elastin necessary for providing structural integrity, collagen which is present mostly in the adventitial tissue is thought to be providing compensatory function for maintaining the resistance of the aorta. Thus, degradation of the adventitial collagen has been suggested to be the ultimate step leading to the rupture of the aorta.[28, 29]

The proteolytic degradation within the aneurysmal wall is believed to be mediated by a variety of proteases which are produced by the medial smooth muscle cells, adventitial fibroblasts and the inflammatory infiltrate.[1] The most prominent among these are the matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9. Various studies have reported the increased expression and activity of MMPs in the aortic wall during AAA development.[30, 31] Both MMP-2 and MMP-9 have elastolytic as well as collagenolytic activities. While MMP-2 is primarily active during the early expansion of non-ruptured AAAs, MMP-9 is less prevalent in the smaller aneurysms, and is believed to play a role in the continued expansion of AAAs during the advanced stages of the disease.[20] Both MMP-8 and MMP-9 have been reported to be increased at the site of aortic rupture.[32] Thus, in addition to their proteolytic activities, MMPs are also thought to regulate the arterial neovascularization important during AAA rupture.[20, 33, 34]

Various cell types have been reported to be responsible for producing the enzymes necessary for AAA progression. These include mononuclear phagocytes,

activated tissue macrophages, vascular smooth muscle cells, adventitial fibroblasts, neutrophils and lymphocytes.[35] Moreover, the presence of T cells, B lymphocytes and dendritic cells in the cellular infiltrate also suggest the role of an immune response in AAAs.[36] More than 80% of human AAAs are reported to show histological evidence of inflammatory cell infiltration.[29] These include C-reactive protein, IL-1β, TNF-α and IL-6.[12, 37] In addition, COX-2 and resultant PGE_2 have also been shown to be expressed in human aneurysmal tissues.[4, 38] Thus, inflammation is believed to play a crucial role in the development of AAAs.

1.1.4. Diagnosis and management of AAAs

AAA development and progression are known to be asymptomatic in most patients. Minor and non-specific symptoms include vague and chronic abdominal and back pain, and the diagnosis of an AAA is usually incidental while assessing other medical complaints. With few prognostic markers available, the most commonly used method to diagnose AAAs is ultrasonography. This procedure is one of the simplest and cheapest diagnostic tools for AAAs and is currently used for population screening and surveillance, pre-operative assessment, as well as post-operative evaluation of AAAs. Ultrasonography has been reported to effectively measure the internal aortic diameter in AAAs, with an accuracy of 2- 3 mm. In cases where ultrasound has identified expansion of the internal aortic diameter sufficient to warrant surgery, CT scans may also be utilized to aid in determining the specific form of surgery that is required.[1, 6, 35] Recently, new imaging techniques using computed tomography or magnetic resonance imaging or three-

dimensional reconstruction have been suggested to provide information on wall tension or shear stress in AAAs for better prediction of the risk of aortic rupture.[39]

Surgery is the only treatment option currently available for patients with AAAs.[6] The decision as to whether or not to operate is based on criteria that include the risk of rupture of the involved AAA, the risk of the surgical procedure itself, and the condition of the patient. Data from four randomized controlled trials that observed the expansion of small AAAs with an internal diameter of less than 5.5 cm show that the rate of rupture is approximately 0.5 – 1% per year, if surgery was performed when the diameter exceeded 5.5 cm. Moreover, the risk compared between the patients who underwent an early elective surgery versus those receiving late repair, was found to be similar.[40-44] Thus, regular surveillance of smaller AAAs without elective repair is considered to be safe, whereas surgery is reserved for the most severe cases where the diameter of the abdominal aorta exceeds 5.5 cm.[1]

Two types of surgery are usually performed on AAA patients – open surgical treatment and endovascular insertion of vascular stents. The open surgery is usually associated with a high perioperative mortality rate of up to 5%, as well as an extended recovery time. Although endovascular repair may show improved perioperative morbidity as compared to open surgery, complications related to limited durability and requirements involving re-intervention and follow-up imaging for extended periods have raised questions about the value of the procedure.[40, 41, 44, 45] Thus, with significant risk and expense associated with surgery, together with the effectiveness of screening procedures that facilitate early detection of slow-growing small AAAs, there is a need to

identify effective pharmacological treatments which can attenuate or eliminate the growth of AAAs.

1.1.5. Animal models of AAAs

A number of experimental models of AAA formation have been developed which mimic the cellular and biochemical characteristics of the human disease. These models utilize a variety of methods including chemical induction or genetic manipulation to induce the disease.[46] While large animals have been used to develop novel methods for AAA surgery, small animal models, especially in rodents, have been developed to better understand the pathological characteristics of the disease and thereby discover new therapeutic treatments for AAAs.[47] The elastase perfusion and the $CaCl_2$ induced AAA models have been the commonly used chemical induction models. The elastase perfusion model was initially developed in rats where Anidjar et al. showed that a 2 hour intraluminal perfusion of porcine pancreatic elastase resulted in complete loss of elastin in the involved aortic segment during the 3 weeks following the procedure.[48] The elastase perfusion model has also been adapted for use in mice.[49] The calcium chloride ($CaCl_2$) model uses a similar approach when gauze treated with $CaCl_2$ is placed directly over the adventitial layer of the abdominal aorta for 10 minutes, which results in AAA formation within 2 to 4 weeks.[50] However, these models are not only technically challenging because they involve a major surgery and prolonged exposure to an anesthetic, but there is also concern for their ability to effectively mimic the chronic development of AAAs in humans.[47]

One of the most widely utilized animal models to study AAAs is the Angiotensin II (AngII) infusion model in mice. This model was originally developed by Daugherty et al. in apolipoprotein E (ApoE) -deficient hyperlipidemic mice, where chronic infusion of 500 to 1000 ng/kg/min of AngII provided subcutaneously via osmotic mini-pumps was found to induce AAAs in these mice.[51] However, over the 28 day infusion period, AAAs were found to develop in the suprarenal region of the aorta, as opposed to the infrarenal AAAs observed in humans. Similarly, with the same dose of AngII infusion, AAAs were also observed in low-density lipoprotein receptor (Ldlr) –deficient mice as well.[52] The AngII-induced AAA model was found to display multiple characteristics of human AAAs and has provided mechanistic insight into the formation and progression of the disease.[53] Similar to the disease in humans, AngII-induced AAA development in mice is characterized by inflammatory cell infiltration into the aortic media, followed by remodeling of the vessel wall, and eventual medial degradation.[54] Previous studies suggested that only hyperlipidemic mouse strains, ApoE-deficient or Ldlr-deficient, had significant susceptibility to AngII-induced AAA development. However, studies in our laboratory have identified a nonhyperlipidemic C57BL6J/129Ola mixed background strain of mice that are as susceptible to AngII-induced AAAs as the hyperlipidemic strains.[55, 56] Thus, the ability to produce AAAs in a reproducible manner using the AngII infusion model in nonhyperlipidemic mice is advantageous, as it allows the study of the mechanisms of AAA development independent of an atherosclerotic burden that may indirectly affect the disease formation or progression.

1.1.5. Potential therapeutic strategies for AAAs

Based on studies in animal models of AAAs, as well as human studies with AAA explants, association of AAAs with certain drugs and randomized controlled trials with AAA patients, a variety of strategies are currently being considered to treat AAAs.[9, 44] Smoking is considered the most important modifiable risk factor for AAAs.[1, 44] With the risk of AAAs shown to gradually decrease with smoking cessation, patients with AAAs are advised to stop smoking tobacco to allow for a decrease in the growth rate of the AAAs.[1, 13, 17] Studies using a rat AAA model have shown that reduced laminar flow in the abdominal aorta contributes to AAA expansion.[57] A recent study in humans using magnetic resonance imaging shows that exercise increases the abdominal aortic laminar blood flow in AAA patients, and thus could potentially limit AAA progression.[58] Thus, with exercise known to reduce other cardiovascular events, AAA patients are currently being advised to exercise regularly to improve hemodynamic laminar blood flow characteristics in the abdominal aorta.[44]

Previous reports with animal models as well a limited number of human studies have suggested the beneficial effect of β-blockers in reducing AAA growth.[59, 60] However, 2 large randomized controlled trials have failed to show a significant effect of the β-blocker, propranolol on the growth rate of small AAAs.[44, 61] With the central role of MMPs in AAA development and rupture, several animal studies were carried out examining the role of protease inhibitors in limiting AAA expansion. Doxycycline, a synthetic tetracycline derivative, known to inhibit MMPs, has been shown to prevent aneurysmal growth in experimental AAAs.[62] Studies in humans have also shown doxycycline to effectively reduce the growth of small AAAs, as well as in AAA patient

that have undergone endovascular repair.[63-65] Thus, doxycycline shows promise for treating AAAs and two large randomized controlled trials are currently being performed to determine its efficacy in limiting the progression of small AAAs.[44]

Several animal studies have suggested that the use of statins may be potentially beneficial in preventing AAA development.[66-68] However, in humans, there are conflicting reports on the efficacy of statins in reducing AAA growth.[69, 70] Angiotensin II (AngII) has also been well-known to play an important role in the development of AAAs in mice.[51] Inhibition of the Ang pathway, utilizing AngII receptor blockers or angiotensin converting enzyme inhibitors, has also been suggested to prevent AAA development in several animal studies.[71, 72] However, since no randomized controlled trials have been performed to determine the effect of statins or suppression of the AngII pathway in reducing AAA growth, the current role of these drugs as a potential therapeutic strategy for AAAs is uncertain.[44]

Although a number of potential medications are being considered for limiting AAA progression, there is no current evidence which shows convincingly that any of these drugs can be effectively used to treat AAAs. Thus, regular surveillance of small AAAs until the disease progresses to a sufficiently large size to meet the criteria required for surgical repair seems to be the only therapeutic strategy currently in place for AAAs.

1.2. ANGIOTENSIN II AND THE CARDIOVASCULAR SYSTEM

The renin-angiotensin system is well-known to play a vital role in the physiological and pathological responses of the cardiovascular system. Angiotensin II (AngII) is the key effector molecule of the renin-angiotensin system and affects the functionality of multiple organs including heart, kidney, brain and the vasculature. The classical pathway begins with the acid protease renin which is initially released from the juxtaglomerular cells of the kidney into the circulation. Renin thereafter cleaves angiotensinogen, synthesized by the liver, to produce the inactive decapeptide AngI. AngI is then cleaved by the angiotensin converting enzyme (ACE) to produce the active octapeptide AngII. All effectors of the renin-angiotensin system are also expressed locally in various tissues, and this local generation of AngII in the vasculature has been shown to be particularly important in regulating vascular inflammation.[73-75]

The primary effect of AngII is in the regulation of vascular tone, blood pressure and electrolyte homeostasis.[73] In the vasculature, AngII has been shown to elicit vasoconstriction as well as hypertrophy of vascular smooth muscle cells.[76] One of the most well-defined characteristics of AngII is its mitogenic potential, whereby AngII increases proliferation of vascular smooth muscle cells.[77-79] AngII has also been shown to be a key regulator of vascular inflammation, by increasing vascular permeability, inducing leukocyte infiltration from the circulation into the perivascular space, as well as in tissue remodeling via regulation of cell growth and matrix synthesis.[75, 80]

AngII is known to mediate its effects by binding to two G-protein coupled receptors: angiotensin receptor type 1 (AT1 receptor) and angiotensin receptor type 2 (AT2 receptor). These are typical polypeptide proteins with 7 trans-membrane domains. Most of the known pathophysiological effects of AngII, including vasoconstriction, cell growth and proliferation, vascular inflammation, stimulation of aldosterone synthesis and retention of salt and water are mediated via the AT1 receptors. The AT1 receptors are present in various tissues and organs throughout the body including blood vessels, heart, lung, liver, adrenals, kidney and the brain. While humans possess only one subtype of the AT1 receptor, rodents express 2 subtypes of the receptor, including AT1a and AT1b, which are known to share 95% sequence homology.[73-75, 81] Although similar, the AT1a receptor has been shown to be more important than the AT1b receptor in regulating blood pressure in the vasculature of mice.[82] The expression of AT1 receptors is regulated by a negative feedback control from its agonist, AngII. While an acute stimulation by AngII leads to increased AT1 receptor activation, chronic exposure to AngII is known to down-regulate the receptor expression. The intracellular cytoplasmic tail of the receptor contains multiple serine/threonine residues which are phosphorylated by G protein receptor kinases. The phosphorylated receptor is then internalized into clathrin-coated pits, mediated by β-arrestins, and is thereby desensitized to further activation by AngII.[73, 81, 83]

In contrast to the AT1 receptors, the AT2 receptors are highly expressed in the developing fetal tissue. The expression of the AT2 receptor declines after birth resulting in low levels of the receptor in adults. The AT2 receptors are known to antagonize the

effects of AT1, especially in the cardiovascular system, by inducing vasodilation, apoptosis and inhibition of cell growth.[73-75]

AngII has been widely implicated in the pathophysiology of several cardiovascular diseases including hypertension, cardiac failure, post-myocardial infarction as well as diabetic nephropathy.[74] AngII is known to lead to disease development by acting on a variety of vascular cells such as endothelial cells, smooth muscle cells, fibroblasts, monocytes and macrophages, as well as cardiac myocytes.[73] Of particular importance is the role of AngII in vascular inflammatory diseases such as atherosclerosis and abdominal aortic aneurysm.[84] AngII infusion, at a dose of 1000 ng/kg/min was found to enhance atherosclerotic lesion development in both apolipoprotein E-deficient as well as low-density lipoprotein receptor-deficient hyperlipidemic mice in 28 days.[51, 52] These lesions consisted of a significant amount of T lymphocytes and lipid-laden macrophages, both characteristic of atherosclerosis.[51, 85] This enhanced development of atherosclerosis by AngII was found to be independent of blood pressure as arterial pressure was not increased by AngII infusion in these mice.[51] In addition, Weiss et al. showed that infusion of norepinephrine in apolipoprotein E-deficient mice, which resulted in comparable increases in blood pressure to that induced by AngII, produced only a modest effect on development of atherosclerotic lesions, as compared to the effects of AngII.[86] Thus, the above reports indicate that AngII plays a major role in the pathogenesis of atherosclerosis.

Chronic infusion of AngII has been shown to result in the development of abdominal aortic aneurysms (AAAs) in mice. Daugherty et al. were the first to show that a chronic infusion of 1000 ng/kg/min AngII, delivered via subcutaneous implantation of

osmotic minipumps, resulted in development of suprarenal AAAs in hyperlipidemic mice.[51, 52] Subsequently, reports from our laboratory showed that this dose of AngII was also effective in producing a significant incidence of AAAs in mice of a nonhyperlipidemic background as well.[55, 56] The AngII infusion model has since been widely utilized to study the different factors which may potentially affect the pathophysiology of AAAs in humans.

Formation of AAAs as well as the atherosclerotic lesions by AngII infusion has been reported to be markedly reduced by antagonizing the AT1 receptor with losartan. In contrast, utilization of an AT2 receptor antagonist was found to dramatically increase AAA incidence as well as the severity of atherosclerosis.[87] Additionally, genetic deficiency of the AT1a receptor was also found to reduce the atherosclerotic lesion area in these hyperlipidemic mice, while no such effect was displayed by the AT2 receptor-deficient mice.[88] Deficiency of the AT1a receptor, particularly in the endothelial cells has also been recently reported to attenuate the incidence of AngII-induced ascending aortic aneurysms in hyperlipidemic mice.[89] Thus, the above reports suggest that the pathological effects of AngII in cardiovascular diseases such as atherosclerosis and aneurysms, is primarily mediated by the AT1a receptor.

1.3. PROSTAGLANDINS AND CYCLOOXYGENASES

1.3.1. Prostaglandins

Prostaglandins (PGs) are a group of biologically active lipid mediators, derived from arachidonic acid. The discovery of PGs dates back to the 1930s with the identification of a factor isolated from the prostate gland, later shown to be seminal vesicles, which had vasodepressor and smooth muscle contraction properties.[90, 91] Almost 30 years later, Samuelsson and Bergström elucidated the structures of the PGs and determined that they are products of the free fatty acid, arachidonic acid (AA), which is ubiquitously present in all cell membranes. Subsequently, PGs were isolated, characterized and determined to be synthesized from a variety of tissues and body fluids.[90-92] Following the identification of PGs, leukotrienes were discovered and were shown to be potent inflammatory lipid mediators associated with asthma and allergic reactions.[93] Prostaglandins, leukotrienes and other products derived from arachidonic acid are collectively known as eicosanoids.

PGs are a group of unsaturated fatty acids containing 20 carbon atoms. The basic structure of PGs is prostanoic acid which includes a 5-membered cyclopentane ring. The different PGs are classified by letters and are grouped based on substitutions in the cyclopentane ring. The thromboxanes have a 6-membered ring instead of the cyclopentane ring. The numbered subscripts at the end of the name for each PG denote the number of double bonds in that compound. The major PGs are PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2, which, together with thromboxane A_2 (TXA_2), are known as prostanoids.[91, 94]

Almost all cell types of our body, except red blood cells have the capacity to synthesize PGs from arachidonic acid (AA), and may be stimulated to produce PGs by cytokines, growth factors, immunogens, mechanical trauma, chemical irritants or other stimuli. AA is an unsaturated fatty acid which is present in large quantities in phospholipids of the cell membrane. The membrane phospholipids are acted upon by multiple isoforms of the enzyme phospholipase A_2 (PLA_2), including type IV cytosolic PLA_2 ($cPLA_2$), which shows selectivity for the release of AA. Various enzymes are known to maintain AA esterified in order to maintain its cellular levels, until mobilized by PLA_2. Phospholipase activation is either through direct interaction of agonists with membrane-bound G-protein coupled receptors, or by increasing intracellular calcium influx. This cell-specific activation leads to translocation of $cPLA_2$ to the nuclear envelope, endoplasmic reticulum or the Golgi apparatus, to ensure AA release in a regulated fashion.[90, 91, 94-96]

At the ER and nuclear membrane, the released AA is acted upon by the enzyme prostaglandin H synthase (PGHS), commonly known as cyclooxygenase (COX). PGHS is present in 2 isoforms, PGHS-1 (COX-1) and PGHS-2 (COX-2). Both COX isoforms are known to catalyze the two reactions that result in PG synthesis. The name cyclooxygenase was used to describe the first of these two enzymatic activities of the protein. The first reaction catalyzes the addition of molecular oxygen to AA thereby cyclizing the molecule to the intermediate endoperoxide compound prostaglandin G_2 (PGG_2). The second reaction catalyzes the conversion of PGG_2 to PGH_2, which constitutes the peroxidase activity of the enzyme. PGH_2 is highly unstable and is acted upon by tissue-specific synthases to form the different PGs.[90, 91, 94, 96, 97]

Metabolism of PGH_2 by the downstream synthases is the critical step which imparts cell specificity to the different PGs and TXA_2. Differences in localization and cell concentration of these enzymes correlate to the production of a predominant PG being produced by a specific cell type, as well as being responsible for differences in PG production by various tissues. For example, TXA_2 synthase is expressed in platelets and macrophages, prostacyclin synthase is expressed in endothelial cells, PGF synthase expression has been identified in the uterus, PGD_2 synthase is widely expressed in the brain and mast cells, and PGE_2 synthase (mPGES) is expressed by a variety of cell types.[90, 91, 98]

PGs are not stored inside the cell, and are released immediately after their synthesis. PGs act in an autocrine or paracrine manner, i.e., they act locally at the site, or adjacent to the site of their synthesis. They are known to bind to specific G-protein coupled receptors (GPCRs) on the cell surface to produce their biological effects. Although the receptors are structurally similar, they are usually specific for a PG of a given class. There are at least 9 known PG receptors: DP1 and DP2 bind PGD_2, EP1-EP4 bind PGE_2, FP binds $PGF_{2\alpha}$, IP binds PGI_2 and TP binds TXA_2. All of these receptors, except DP2, are GPCRs. IP, DP1, EP2 and EP4 are typically known as the "relaxant" receptors and signal through G_s-mediated increases in cyclic adenosine monophosphate (cAMP). In contrast, the "contractile" receptors, EP1, FP and TP signal thorough Gq-mediated increases in intracellular calcium. The EP3 receptor is the "inhibitory" receptor which decreases cAMP by activating G_i. The major functions of the different PGs and TXA_2 are listed below in Table 1.1. Since some prostanoids are labile, it is often difficult to measure the active compounds in the circulation under physiological conditions. Thus,

quantitation of the inactive metabolites in urine or other body fluids, for example, TXB_2 for TXA_2, PGJ_2 for PGD_2, is commonly used to assess the amount of PGs produced.[90, 91, 99, 100]

Table 1.1 Major functions of prostanoids in different organ systems[91]

The major functions of the prostanoids in different organ systems are broadly classified below.

System	Mediator	Effect
Cardiovascular	PGI_2	Vasodilation
	TXA_2	Vasoconstriction
Hematologic	PGI_2	Platelet de-aggregation
	TXA_2	Platelet aggregation
Gastrointestinal	PGE_2	Protection of gastric mucosa
Renal	PGI_2	Vasodilation
	PGE_2	Salt and water excretion
Reproductive	$PGF_{2\alpha}$	Contraction of uterine smooth muscle, parturition
Respiratory	PGI_2	Vasodilation
	PGD_2	Allergic asthma
Neurologic	PGE_2	Fever, hyperalgesia

1.3.2. Cyclooxygenases

Cyclooxygenase (COX) or prostaglandin H synthase (PGHS) is an enzyme that catalyzes the first 2 steps in the biosynthesis of prostaglandins (PGs) from its substrate arachidonic acid (AA). COX activity has been studied since the 1980s, originally using preparations from sheep seminal vesicles.[101] In 1990, Fu et al. were the first to report the de novo synthesis of a new COX protein synthesized by human monocytes when stimulated by cytokines and other inflammatory mediators.[102] The novel COX protein was shown to be a distinct isoform of the previously identified COX enzyme, although there was sequence homology between the two isoforms. Thus, the 2 isoforms of COX were named COX-1 and COX-2, and are encoded by different genes located on different chromosomes.[91, 103]

Both COX-1 and COX-2 have similar tertiary structures and are known to carry out the same catalytic reaction.[104] However, while COX-1 is considered to be responsible for basal, constitutive PG synthesis, COX-2 is known to synthesize primarily the pro-inflammatory PGs when induced by certain stimuli. Thus, COX-1 has been described to maintain the physiological “housekeeping” functions of the body, whereas COX-2 regulates the inflammatory response under “induced” conditions. This correlates to expression of COX-1 in a variety of organs in the body which usually show low to undetectable levels of COX-2 under normal conditions. In particular, a high level of COX-1 is found to be constitutively expressed in vascular endothelial cells, platelets, kidney and the stomach, and is thereby responsible for synthesizing the prostanoids which would maintain the homeostatic functions of these tissues and organs. In contrast, COX-2 is primarily an inducible enzyme and is traditionally thought to play an important

role in the production of prostanoids that contribute to pathological conditions. COX-2 is localized in the nuclear envelope, and a variety of pro-inflammatory stimuli are known to increase expression of COX-2 in various cells and tissues. These stimuli include cytokines such as interleukin-1 (IL-1), IL-2, tumor necrosis factor-α (TNF-α), INF-□, lipopolysaccharide (LPS), TPA, hormones such as FSH and LH, and several growth factors such as EGF, PDFG and FGF, among others.[91, 104-106]

Both isoforms of COX have a molecular weight of 71 kDa, and are approximately 600 amino acids in length. COX-1 and COX-2 are known to share 63% sequence homology.[106] X-ray crystallographic studies have revealed much about the 3-D structures of COX-1 and COX-2. Thus 3 distinct domains are known to comprise the structure of the COX proteins: an N-terminal epidermal growth factor domain, a membrane binding motif, and a C-terminal catalytic domain that consists of the COX and peroxidase active sites.[107, 108]

1.3.3. Inhibition of COX activity

In 1971, Vane was the first to demonstrate that the anti-inflammatory activity of aspirin, which had been used as an anti-inflammatory, anti-pyretic and analgesic since 1889, and other non-steroidal anti-inflammatory drugs (NSAIDs), was due to the inhibition of COX, and subsequent PG synthesis.[109] NSAIDs are known to inhibit both COX-1 and COX-2 non-specifically in a variety of ways. While aspirin covalently impairs the AA binding site to COX, ibuprofen is a reversible competitive inhibitor of COX that competes with AA for the COX active site, whereas indomethacin causes a slow irreversible inhibition of COX.[110, 111]

Chronic use of the traditional NSAIDs, which inhibit both COX-1 and COX-2, is associated with severe side effects which may include gastrointestinal ulceration and bleeding, renal damage and platelet dysfunction. Since COX-1 is a constitutive enzyme maintaining "housekeeping" functions in these tissues, the side-effects of NSAIDs were considered to be due to inhibition of COX-1. Also, since COX-1 is not upregulated during inflammatory conditions, when COX-2 plays a major role, the beneficial effects of the NSAIDs during pain and inflammation were attributed primarily to inhibition of COX-2. This concept provided the rationale for the development of a new series of compounds which would selectively inhibit COX-2, and thereby maintain the therapeutic efficacy of the traditional NSAIDs, without adverse effects resulting from the inhibition of COX-1.[106, 112]

Although the structures of COX-1 and COX-2 were found to be similar, X-ray crystallography revealed that there is a single amino acid difference between the 2 enzymes, which could offer selectivity during drug development for isoform-selective inhibitors. At position 523, COX-1 has the amino acid isoleucine, whereas, COX-2 contained valine, which is smaller than isoleucine by a single methyl group. Thus, the smaller valine in COX-2 is thought to create a side-pocket and thereby provide additional access to the drugs. On the other hand, the bulkier isoleucine in COX-1 blocks this access to the side-pocket. Thus, bulkier molecules, such as celecoxib and rofecoxib, which could bind to the COX-2 side-pocket, but could not access the active site of COX-1, were subsequently developed to attain COX-2-selective inhibition.[113, 114]

1.3.4. Celecoxib

Celecoxib (Celebrex®) was the first of the "coxibs", the group of COX-2-selective inhibitors that were introduced more than a decade ago. Celecoxib is approved worldwide primarily for the treatment of inflammatory conditions such as arthritis, spondylitis and acute or chronic musculoskeletal pain. Celecoxib has similar efficacy as the traditional NSAIDs in reducing arthritis, but provides improved GI tolerability due to selective inhibition of COX-2. The molecule is a 1, 5-diaryl pyrazole-based compound that is 30 times more potent at inhibiting COX-2 than COX-1. The selectivity of celecoxib for COX-2 is approximately 10 to 15 times less than that of other coxibs.[115] The analgesic and anti-inflammatory actions of celecoxib, at the therapeutic dose of 200-400 mg/day, are mediated through COX-2 inhibition, and not COX-1, as assessed by TXA_2 formation. Celecoxib has been reported to have no significant effect on platelet function in healthy volunteers, and effects on renal function are comparable to other NSAIDs.[114-117]

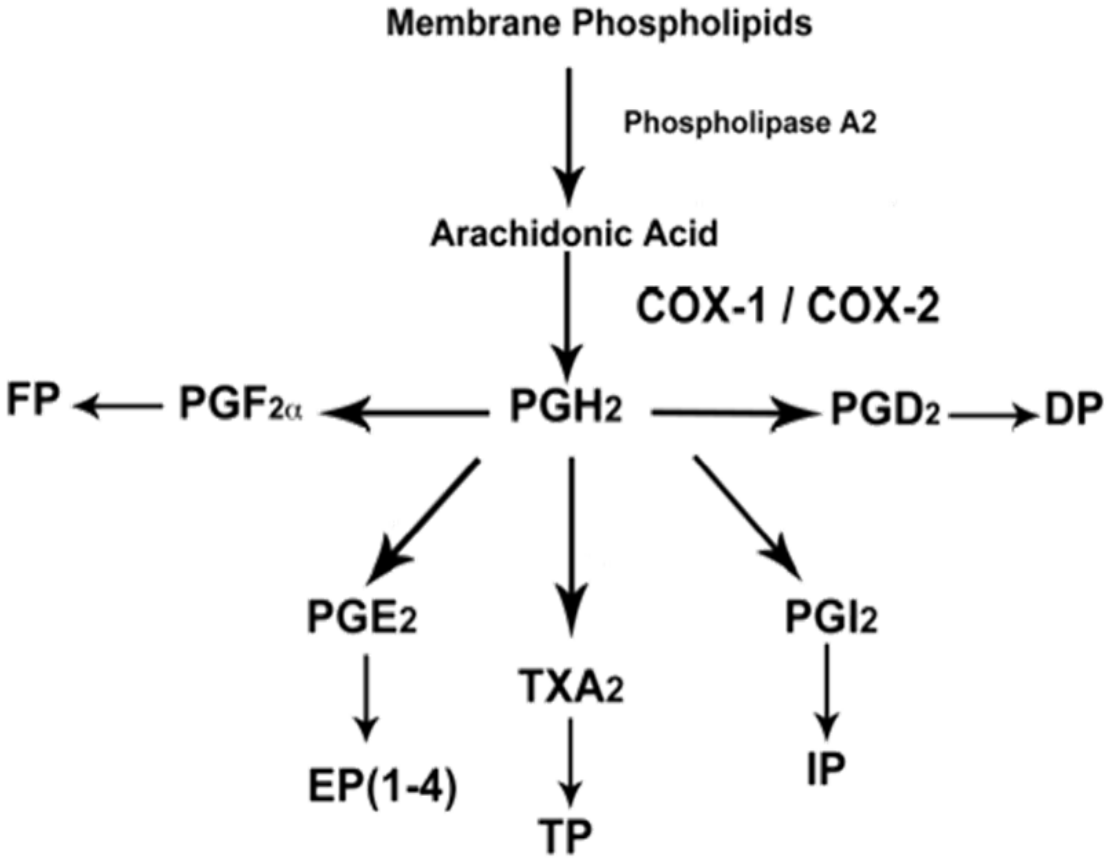
COX-2-selective inhibitors have been shown to produce less gastrointestinal toxicity, although their chronic use is associated with an increased risk of serious adverse cardiovascular events.[118] Although the COX-2-selective inhibitor rofecoxib was withdrawn from the market due to an increase in these adverse cardiovascular effects following chronic administration, the level of risk associated with celecoxib use is reported to be significantly lower than that observed for rofecoxib.[119] In addition, a number of reports have determined the level of cardiovascular risk associated with celecoxib use to be comparable to, or lower than nonselective NSAIDs.[120, 121] However, chronic administration of celecoxib doses higher than those approved for treating osteoarthritis was shown to increase adverse cardiovascular events when

compared to placebo, and the risk was greater for patients with preexisting cardiovascular disease.[122] A prospective trial designed to compare the cardiovascular safety of celecoxib with two other commonly used NSAIDs in patients with preexisting cardiovascular disease is currently in progress.[123] The therapeutic value of celecoxib as a potential treatment for AAAs may ultimately depend on the level of adverse cardiovascular effects that result from this medication.

1.3.5. COX-2 and abdominal aortic aneurysms

Human abdominal aortic aneurysms (AAAs) are characterized by a systemic increase in inflammatory mediators, as well as localized inflammation in the vascular wall.[4, 124] The prostanoids are believed to play an important role in regulating these inflammatory processes.[4, 125] While both COX-2 and the resultant PGE_2 have been found to be induced in the aneurysmal tissue, COX-1 was not significantly altered.[4] Using the AngII-induced model of AAAs, we have reported that the incidence and severity of AAAs following AngII infusion are significantly reduced in hyperlipidemic Apoe-/- mice, as well as non-hyperlipidemic COX-2 wild type mice, pre-treated with COX-2 selective inhibitor, celecoxib[55]. Genetic deficiency of COX-2 is also found to significantly reduce AAA formation in the mice. While AngII was found to induce COX-2 mRNA in the aorta, significant COX-2 protein expression was also detected in the medial smooth muscle cells (SMCs) of the abdominal aorta.[56] These findings demonstrate that COX-2 plays an important role in the development of AAAs.

Figure 1.1 Illustration of the cyclooxygenase pathway



1.4. SMOOTH MUSCLE CELL PHENOTYPE

1.4.1. The smooth muscle cell

The normal blood vessel is composed of three basic layers: the intima, the media and the adventitia. The intima is composed of a single continuous layer of endothelial cells and is separated from the medial layer by the internal elastic lamina. The media is composed exclusively of smooth muscle cells (SMCs), within concentric layers of elastic lamina. The external elastic lamina separates the media from the adventitia which comprises of connective tissue, fibroblasts, capillaries and fat cells.[126]

The major function of the mature smooth muscle cell (SMC) is to contract and thereby regulate blood vessel tone and diameter, blood pressure and distribution of blood flow. For maintaining these contractile functions, SMCs are known to express a variety of contractile proteins, receptors and ion channels, and signal transduction molecules, many of which are unique from those expressed by other cell types.[127, 128]

1.4.2. Phenotypic plasticity of SMCs

Arterial SMCs are known to originate from the mesoderm. During development, mesenchymal cells in the embryo accumulate and begin a gradual differentiation into SMCs which initially develop an appearance similar to fibroblasts. At this stage, the cytoplasm of these SMCs contains large amounts of rough endoplasmic reticulum (RER), a prominent Golgi complex, and free ribosomes, with only a few myofilaments, and their main functions are to proliferate and to produce extracellular matrix components which include collagen and elastin. These cells also typically express β-actin as the major actin

isoform, and vimentin as the intermediate filament protein. This phenotype of SMCs which is the state most associated with formation of the vessel wall is known as the "synthetic" phenotype.[129]

As the blood vessels mature, the SMCs are known to undergo a change in their phenotype. This is evident by a progressively decreasing size of the synthetic organelles, with the myofilaments occupying the majority of the cytoplasm. The cells tend to lose their synthetic and proliferative capabilities, and instead attain the ability to contract. Moreover, there is a significant decrease in the content of β-actin and these SMCs instead begin expressing α-actin as the major actin isoform, along with a host of other proteins of the contractile apparatus, including desmin, myosin, tropomyosin, h-caldesmon and others. Thus, the SMCs comprising the fully developed media are known to attain a phenotype referred to as the "contractile" or the "differentiated" phenotype.[129]

Although the developmental changes involving the differentiation of SMCs are very similar to those of cardiac or skeletal muscle cells, these cells typically undergo a terminal and irreversible differentiation.[130, 131] In contrast, vascular SMCs are thought to retain considerable plasticity and can undergo extensive, as well as, reversible changes in their phenotype in response to physiological and pathological stimuli. Thus, although its major function is contraction, the SMC, by virtue of its phenotypic plasticity, can execute a variety of other functions at different developmental stages, during vascular repair, or in case of certain vascular diseases.[127, 132] This characteristic of the SMC is probably most evident in response to vascular injury, when fully differentiated SMCs have the capacity to undergo "de-differentiation" and revert back to a synthetic phenotype. At this stage, these de-differentiated SMCs have drastically increased rates of

proliferation and migration, together with synthesis of the extracellular matrix components which are essential for vascular repair. Upon resolution of the injury, the factors which initially stimulated the repair process return to normal, thereby prompting the SMCs to reassume their contractile phenotype.[127, 128]

A major drawback of the high degree of plasticity displayed by SMCs is their ability to respond to abnormal environmental signals, which can lead to adverse phenotypic switching, aiding in the development of major vascular diseases. Vascular SMC proliferation and de-differentiation have been found to contribute to a variety of pathological processes including atherosclerosis, hypertension, and coronary restenosis.[132-134]

1.4.3. Markers of SMC phenotypes

The diversity of SMC phenotypes are studied based on changes in morphology, expression levels of SMC marker genes, and the proliferative and migration potential of the SMCs. However, it is important to note that the contractile and synthetic SMCs represent two opposing ends of a broad phenotypic spectrum, and SMCs may exist as intermediate phenotypes. Morphology of cultured SMCs is considered to be an important parameter to describe the different SMC phenotypes. While contractile SMCs are elongated and spindle-shaped cells, the synthetic SMCs are less elongated and are known to display an epithelioid or rhomboid appearance. Several studies have also shown that the synthetic or de-differentiated cells have higher rates of proliferation and migration compared to the differentiated cells.[135] However, in addition to these qualitative

parameters, the quantitative analysis of levels of marker proteins has been suggested to be the ideal method for characterization of the different SMC phenotypes.[136]

1.4.3.1. Markers of contractile SMC phenotype

A variety of SMC-selective or specific genes have been identified which are utilized as markers to classify the SMC phenotypes. A majority of these are contractile proteins, the levels of which represent the relative state of differentiation of SMCs. These proteins include smooth muscle (SM) α-actin, SM myosin heavy chain (MHC), calponin, SM22α, desmin and smoothelin among others.[128] Since the expression of these proteins are regulated at the gene levels, such as transcription and splicing, they are suitable for use as molecular markers for SMC phenotypes.[137]

The actin isoforms are usually classified into two major groups: muscle and non-muscle actin, and are indicated by α, β or based on their isoelectric points. Of these, SM α-actin, a major protein for SMC contraction, is highly selective for and is the most abundant protein of adult SMCs in the contractile phenotype, making up approximately 40% of the total protein content.[128, 138] SM α-actin is the first known protein expressed during differentiation of the SMCs in the course of development, undergoing significant increases in expression even later during vascular development.[127, 139] In contrast, aortic SMCs in proliferating cultures undergoing phenotypic modulation have been shown to have a decreased mRNA as well as protein content of α-actin, whereas non-muscle β-actin expression is increased.[129, 140] Thus, α-actin is one of the most widely utilized markers to define the contractile SMC phenotype.

Another marker of differentiated SMC phenotype that has been used extensively is the 22kDa protein, SM22α. Of the three isoforms of SM22, α isoform is the most dominant, and is expressed abundantly in SMCs of the adult. It is one of the earliest markers of differentiated SMC, and is expressed in a pattern very similar to SM α-actin. Although the functions of SM22α remain largely unclear, some studies have suggested its role in the regulation of SMC contraction.[127, 128, 136, 137, 141]

Desmin is the principal intermediate filament protein expressed in differentiated SMCs of the adult. Although detectable levels of desmin are not found in large mammalian arteries, rodent aortas have been reported to express low levels of desmin.[142] Thus, while vimentin is the intermediate filament protein typically expressed in the synthetic SMCs, desmin has been utilized as a useful marker to assess the relative state of differentiation of vascular SMCs existing in the contractile phenotype.[127, 143]

Calponin is a calcium regulatory protein expressed selectively in differentiated SMCs. It is known to interact with F-actin and tropomyosin in a calcium independent manner, but with calmodulin in a calcium dependent manner, and thereby regulates contraction of SMCs.[127] Calponin can exist in two isoforms: acidic and basic calponin.[137] While acidic calponin is expressed in both smooth muscle and non-muscle tissues, basic calponin is exclusively expressed in contractile smooth muscles, and has been shown to be down-regulated in de-differentiated SMCs.[144] Based on these reports, calponin is considered an important marker to identify differentiated SMCs.

Myosin is a major protein of the contractile apparatus of SMCs multiple isoforms of which have been identified. Of these, two variants, SM1 and SM2 are expressed

specifically in mature contractile SMCs, towards the later part of the developmental stage. Vascular SMCs are also known to express two non-muscle isoforms, NMHC-A and NMHC-B. Of these, NMHC-B is similar to SMemb which is expressed in synthetic SMCs of the embryonic aorta and in neointimal lesions.[145] Thus, myosin heavy chains, with their high degree of specificity for expression in contractile SMCs, are suggested to be one of the most discriminating markers of differentiated SMCs identified to date.[127, 136, 137]

Smoothelin has been suggested to be selectively expressed in differentiated SMCs in two isoforms: type A and type B. Of these, type B isoform of smoothelin is thought to be expressed only in contractile vascular SMCs.[127] The expression of smoothelin has also been shown to be down-regulated in cultured SMCs modulating towards a de-differentiated phenotype.[146] Thus, being even more sensitive than the myosin heavy chains, smoothelin has been suggested to be a well-characterized marker of contractile SMC phenotype.[136]

1.4.3.2. Markers of synthetic SMC phenotype

As SMCs alter their phenotype from a contractile to a synthetic state, the expression of the above mentioned contractile markers which are up-regulated during the differentiated state, are in turn decreased.[127, 137, 147] However, the extent of reduction in expression of these markers varies. Since the expression of markers which are found to be increased in the synthetic phenotype is rare, the decrease in expression of the contractile markers is generally assumed to be a characteristic of the synthetic phenotype of SMCs.[127, 136]

Alteration of SMCs to a de-differentiated phenotype is also associated with increased rates of proliferation and migration of the cells, together with synthesis of components of the extracellular matrix (ECM) such as collagen, elastin and hyaluronic acid.[127, 129, 136] Studies with primary culture of arterial SMCs show that rapid proliferation of phenotypically modulated SMCs are associated with high rates of collagen and elastin synthesis. Type I is the main type of collagen reported to be produced in these studies, with smaller amounts of type III and type V also being detected.[148] Similarly, Ang et al. have also reported an increase in expression of nearly 25-30 times more collagen by phenotypically altered SMCs, compared to levels produced by cells in the contractile state.[149]

Apart from collagen, alteration of the SMC phenotype is also associated with increased synthesis of glycosaminoglycans which are long unbranched polysaccharides.[150] Hyaluronan or hyaluronic acid (HA) is an unsulphated glycosaminoglycan and is a principal component of the extracellular matrix. HA is a linear high molecular weight polymer consisting of repeating disaccharide units of (β1→ 3)D-glucuronate-(β1→ 4)N-acetyl-D-glucosamine. HA is synthesized by three glycosyltransferases on the plasma membrane known as hyaluronic acid synthases (HAS1, HAS2 and HAS3). HAS2 is the major HAS isoform found to be expressed in vascular SMCs, which express very low levels of HAS1 and HAS3.[151] HAS2 protein is localized at the cell surface and extrudes HA directly to the extracellular space following synthesis. Volume expansion of the extracellular space is also observed due to binding of HA with salt and water.[152, 153]

HA has been implicated in several vascular diseases including atherosclerosis, restenosis and other models of vascular injury which involve an increase in expression of HA, HA synthases, or the HA receptor CD44. These vascular diseases have also been shown to be associated with changes of the SMC phenotype from that of a contractile cell to one which migrates to the intimal space, proliferates rapidly to produce the neointima, and synthesizes abundant extracellular matrix, and HA has been suggested to be a principal component of this extracellular matrix.[154-157] A study utilizing human vascular SMCs has reported that in response to platelet-derived growth factor (PDGF), a potent mitogen, HA secretion is significantly increased in the proliferating cells.[158] This was followed by studies by Evanko et al. who showed that PDGF stimulates the formation of hyaluronan and versican-rich pericellular matrices around arterial SMCs. They also reported an increase in expression of hyaluronan synthase 2 as well as HA in these cells, suggesting a link between ECM expansion and alterations in SMC phenotype.[151] Thus, the above reports suggest that HA is one of the major ECM components synthesized by SMCs of de-differentiated phenotype, and thus, can be used as an effective marker for identifying the synthetic SMCs. In addition, since HAS2 is the major isoform among HA synthases expressed in SMCs, for our current studies, we have utilized HAS2 as a marker of SMC de-differentiation in the aorta.

1.5. PURPOSE OF STUDY

AAA is a chronic inflammatory disease with both COX-2 and PGE_2 being induced in human aneurysmal tissues. Using a mouse model of the disease, previous studies in our laboratory were the first to report the importance of COX-2 in AngII-

induced AAA formation in mice by utilizing mice genetically deficient in COX-2 or pre-treating them with a COX-2-selective inhibitor celecoxib. With no pharmacological treatments currently available for AAAs, the purpose of the current studies was to further illustrate the role of COX-2 inhibition during AngII-induced AAA progression in mice, as well as elucidate the mechanisms contributing to disease formation in the mouse abdominal aorta.

1.6. SPECIFIC AIMS

1.6.1. Specific aim 1

The 1st aim of the current studies was to determine the role of COX-2 inhibition with celecoxib in attenuating progression of AngII-induced AAAs in mice, when treatment is begun after initiation of the disease. We hypothesized that beginning treatment with celecoxib after initiation of aneurysmal pathology, will reduce the incidence and severity of AngII-induced AAAs in mice at different stages of disease progression.

1.6.2. Specific aim 2

The 2nd aim of the current studies was to elucidate the mechanisms contributing to the increased susceptibility of the abdominal region of the aorta towards AngII-induced aneurysm formation in mice. We hypothesized that increased COX-2 expression in the abdominal region, as compared to the other regions of the aorta, causes the abdominal region of the aorta to become more susceptible towards AngII-induced aneurysm development in mice.

1.6.3. Specific aim 3

The 3rd aim of the current studies was to determine the mechanisms by which COX-2 inhibition regulates the development of AngII-induced AAAs in mice. We hypothesized that COX-2 inhibition with celecoxib maintains smooth muscle cells of the aorta in a more differentiated phenotype during attenuation of AngII-induced AAA progression in mice.

CHAPTER 2

METHODS

The experiments described in the later chapters of this dissertation are based on utilization of common techniques. Thus, to avoid repetition, the general experimental methods used in all the subsequent chapters have been outlined below.

2.1. Animals

Fourteen week old male nonhyperlipidemic mice of a mixed C57BL/6J X 129/Ola genetic background (Taconic Farms Inc.) were used for the current studies. The mice were housed under barrier conditions at the University of Kentucky, College of Pharmacy animal facility, with food and water provided *ad libitum*. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee and conform with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Angiotensin II infusion

For subcutaneous osmotic pump implantation, mice were anesthetized with isoflurane at a concentration of 2% in oxygen that was delivered using a precision vaporizer and an induction box. Saline or Angiotensin II (AngII, Sigma, St. Louis, MO) were then filled into Alzet osmotic minipumps (model 2004; ALZA Scientific Products, Mountain View, CA) and delivered subcutaneously at a rate of 1000 ng/kg/min for 3, 7, 14, 21 or 28 days. Pumps were placed into the subcutaneous space of anesthetized mice through a small incision in the back of the neck that was closed with wound clips. The

wound clips were removed after 2 weeks and all incision sites healed rapidly without the need for additional medication.[51]

2.3. Study designs

2.3.1. COX-2 inhibitor studies

This section describes the COX-2 inhibitor studies in Chapters 3 and 6. Fourteen week old male nonhyperlipidemic mice were infused with 1000 ng/kg/min AngII, as described above in section 2.2. Five days after beginning the AngII infusion, the mice were divided into two groups. One group received a control diet (normal chow diet; Research Diets Inc., New Brunswick, NJ), while the other group received a diet containing COX-2-selective inhibitor celecoxib (normal chow diet re-pelleted with 1000 ppm celecoxib, LKT Laboratories, St Paul, MN). The mice were subsequently sacrificed and abdominal aortas were collected after 14, 21 and 28 days of AngII infusion. Tissues from these experiments were used for analysis of vascular pathology (as described in Chapter 3), as well as gene expression, and protein expression of smooth muscle cell phenotypic markers (as described in Chapter 6) by real-time PCR and histochemical techniques, respectively, which are described below in sections 2.8 and 2.9.

2.3.2. Studies with different aortic segments

This section describes the studies with the different aortic segments in Chapter 4. Fourteen week old male nonhyperlipidemic mice were infused with saline or 1000 ng/kg/min AngII, as described above in section 2.2. The mice were subsequently sacrificed after 3, 7, 14, 21 and 28 days of the infusion. The arch, thoracic and abdominal

regions of the aorta were collected and analyzed for COX-2 and AT1a receptor gene expression by real-time PCR, and protein expression by immunohistochemistry, as described below in sections 2.8 and 2.9 respectively.

2.3.3. Cell proliferation studies

This section describes the smooth muscle cell proliferation studies in Chapter 5. Fourteen week old male nonhyperlipidemic mice were infused with saline, or 1000 ng/kg/min AngII by osmotic mini-pumps, as described above in section 2.2. Following the implantation of the pumps, the mice were intraperitoneally injected with bromodeoxyuridine (BrdU) (Invitrogen, CA) at a dose of 100 mg/kg, dissolved in saline, every 24 hours. The mice were subsequently sacrificed after 7 or 14 days of AngII infusion. The abdominal segments of the aorta and skin samples (for positive control) were collected and analyzed for cell proliferation by BrdU immunohistochemistry, as described below in section 2.9.1.

2.3.4. COX-2 knockout studies

This section describes the COX-2 knockout studies in Chapter 6. Fourteen week old non-hyperlipidemic COX-2 wild-type (WT) or COX-2 knockout (KO) mice were infused with AngII at a dose of 1000 ng/kg/min, as described above in section 2.2. The mice were subsequently sacrificed after 7 days of AngII infusion. The abdominal aortas were then collected and analyzed for gene expression of markers of the SMC phenotypes by real-time PCR, which is described below in section 2.8.

2.4. Tissue collection

For performing euthanasia, mice were first anesthetized by intra-peritoneal injection of ketamine (100 mg/kg) (Fort Dodge Animal Health, Iowa) and xylazine (10 mg/kg) (Ben Venue Laboratories, Bedford, OH) until unresponsive to pain, followed by thoracotomy and exsanguination under anesthesia. The aortas were excised and divided into 3 segments, with the arch section beginning from adjacent to the heart to the left subclavian artery, the thoracic beginning from the left subclavian artery to the last set of intercostals, and the abdominal section beginning from the end of the intercostals to the bifurcation of the aorta into the common iliac arteries.

For RNA experiments, the aortas were excised and divided into arch, thoracic and abdominal segments, and stored in RNALater (Sigma, St Louis, MO) at -20ºC to maximize RNA stability.

For histochemical experiments, the mice were perfused with saline, followed by 1:10 neutral buffered formalin (Fisher, Kalamazoo, MI) solution. The aortas were excised and divided into segments as described above. The tissues were then fixed in 1:10 neutral buffered formalin overnight, followed by 70% ethanol (Fisher, Kalamazoo, MI), and subsequently stored in 70% ethanol at 4ºC.

2.5. Vascular pathology

AAAs were quantified as percentage incidence of aneurysms, with a maximal external diameter of $\geq$ 1.5 mm of the abdominal section of the aorta, considered as an incidence of aneurysm. The mice which had a ruptured aorta and subsequent death due to hemorrhage before the completion of the study were not included in calculating the AAA

incidence. Ruptured aneurysms were quantified as percentage incidence of rupture of the aorta between day 6 and day 17 of AngII infusion. AAA severity was determined by classifying the AAAs as Type 1, where the external diameter of the abdominal aorta was between 1.5 – 1.9 mm, and those as Type 2, where the external diameter was ≥ 2 mm.

2.6. RNA isolation

Total RNA was extracted from the tissues collected using RNEasy Mini kits (Qiagen, Valencia, CA). The RNEasy method utilizes a combination of binding properties of a silica-based membrane with the speed of microspin technology. The aorta tissue samples were first lysed in a glass micro-mortar and pestle apparatus, and then homogenized using a highly denaturing guanidine-thiocyanate containing buffer, which also inactivates RNAses, to ensure purification of intact RNA. The lysate was then diluted with an ethanol solution, which allowed the RNA to bind the silica membrane in the RNEasy Mini spin column. The solution was passed through the spin column, which effectively binds the RNA. The spin column was washed 3 times using wash buffer to allow removal of contaminating materials. RNA was then eluted using RNAse-free water.

2.7. cDNA preparation

Reverse transcription was used to synthesize complimentary DNA (cDNA) from the total RNA. RNA was reverse transcribed using the following components: random hexamers (used at a 1:60 dilution), 5X reverse transcription buffer, deoxynucleotide triphosphate mix (dNTPs), RNAse inhibitor, Superscript II reverse transcriptase and

diethyl pyrocarbonate (DEPC) water. The reverse transcription was carried out using a thermal cycler (Eppendorf) with the following conditions: RNA + hexamers + dNTPs at 65°C for 5 min followed by placing the mixture on ice for 5 min. This was followed by addition of the remaining components with continued incubation in the thermal cycler with the following conditions: 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes to terminate the reaction. This cDNA was stored at -20ºC until utilized for gene expression analysis.

2.8. Quantification of mRNA expression

Gene expression quantitation was performed in a two-step quantitative real time PCR (qRT-PCR) (ABI Prism 7000 system) in which the PCR step is coupled with fluorogenic 5' nuclease chemistry (Taqman chemistry). To account for variability in the starting material between samples, mRNA encoding the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) was quantitated for an internal normalizing control. Primer/probe assays (COX-2, AT1a, α-actin, desmin, SM22α, Cnn1, Myh11, Smtn, Has2 and HPRT) were purchased from Applied Biosystems (Assays-on-Demand). A relative standard curve using RNA from lung tissue was run within the same reaction. The quantity of mRNA for the unknown gene was extrapolated from the standard curve, followed by normalization with the HPRT levels.

2.9. Histology

The stored tissues were processed in an automated tissue processor which exposes the tissues through a series of ethanols and xylenes. The tissues were then embedded in

paraffin and sectioned on a microtome at a thickness of 6μm. The sections were deparaffinized, and then subjected to histological analysis. At least 10 sections from each animal, with a minimum of 3 animals per group, were analyzed for each experiment.

2.9.1. BrdU immunohistochemistry

The tissue sections were deparaffinized and hydrated by treatment with xylene (Fisher) and ethanols (Fisher) respectively. In order to expose the antigen to the antibody, the sections were treated 2N hydrochloric acid (Sigma, St Louis, MO) at 37ºC for 30 minutes. The sections were then treated with boric-acid-borate buffer for 1 minute and with 0.01% trypsin (Invitrogen, CA) and incubated at 37ºC for 10 minutes. The sections were subsequently blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 minutes at room temperature and incubated with a biotinylated primary antibody against BrdU (Invitrogen, CA), at a concentration of 1:400 overnight at 4ºC. The sections were then processed as recommended in the Vectastain ABC Elite Kit (Vector Labs) for detection with 3, 3-diaminobenzidine (DAB) (Vector Labs), which produced a brown staining, followed by hematoxylin (Sigma, St Louis, MO) for counterstaining.

2.9.2. COX-2 and α-actin immunohistochemistry

Deparaffinized and hydrated tissue sections were heated with antigen retrieval buffer, containing citrate, at 96ºC, in a microwave oven, in order to expose the antigen to the antibody. The sections were cooled for 30 minutes at room temperature, and subsequently blocked with 1% BSA in PBS for 30 minutes at room temperature. Primary

antibodies against COX-2 (Vector Labs) and α-actin (Dakocytomation, CA), at concentrations of 1:200, were used for immunohistochemical detection of COX-2 and α-actin, respectively, with subsequent use of an anti-rabbit secondary antibody. The sections were incubated with the primary antibody overnight at 4ºC, and then with the secondary antibody for 30 mins at room temperature. The sections were then processed as recommended in the Vectastain ABC Elite Kit (Vector Labs) for detection with 3, 3-diaminobenzidine (DAB) (Vector Labs), which produced a brown staining, followed by hematoxylin (Sigma, St Louis, MO) for counterstaining.

2.9.3. Histochemical analysis of hyaluronic acid

Deparaffinized tissue sections were incubated with a solution containing 1:200 hyaluronic acid binding protein (HABP), overnight at 4ºC. Since the HABP is biotinylated, the sections were subsequently processed as recommended in the Vectastain ABC Elite Kit for detection with DAB, which produced a brown staining, followed by hematoxylin for counterstaining.

2.9.4. Collagen staining

Collagen content in the aortic tissues was evaluated by staining with Masson's trichrome stain (Newcomer Supply, WI). This stain is typically used to differentiate between collagen and smooth muscle cells, by utilizing 3 dyes. Masson's trichrome stain employs a very basic rule of staining: the less porous tissues are colored by the smallest dye molecule. The sections were stained first with an acid dye, Biebrich Scarlet, which binds with the acidophilic tissue components, including cytoplasm, smooth muscle and

collagen, and stains these tissues red. The sections were then treated with phosphotungstic and phosphomolybdic acids. Because the smooth muscle is much less permeable than collagen, the phospho acids cause the Biebrich scarlet to diffuse out of the collagen, but not out of the muscle. Finally a large dye, such as aniline blue was used to stain the collagen. Thus, the smooth muscle cell layer stained red, while the collagen stained blue.[159]

2.10. Statistical analysis

The mean and SEM were calculated for each parameter with an individual aorta from a single mouse considered as an *n* of 1. Significant differences in AAA incidence among different groups were determined using χ^2 analysis. Statistical significance of quantitative PCR data was determined using the unpaired t-test. Differences were considered statistically significant at $p < 0.05$. Data were analyzed using the GraphPad Prism software.

CHAPTER 3

COX-2 INHIBITION IMPROVES SURVIVAL AND LIMITS EXPANSION OF ABDOMINAL AORTIC ANEURYSMS IN MICE

Introduction

Abdominal aortic aneurysms (AAAs) are a major health concern in the ageing population mostly affecting men over the age of 55 years.[2] AAAs are a chronic condition that begin as minor vessel dilation that progress over years to produce a weakened aorta with increased susceptibility for rupture, which has greater than a 90% incidence of mortality.[4, 5] Surgery is the only treatment option for AAAs and is reserved for the most severe cases with a high probability of rupture because of significant risk associated with surgical repair.[6, 40, 160] Although AAAs are readily detected by non-invasive imaging methods at various stages of progression, no pharmacological treatments are currently available to slow progression of the disease.[6, 161]

Human AAAs are characterized by inflammation of the involved aorta and increased production of prostaglandin (PG) mediators of inflammation.[38] The synthesis of PGs requires the activity of a prostaglandin G/H synthase, also known as cyclooxygenase (COX), of which exist the constitutively expressed isoform COX-1 and the inducible isoform COX-2.[162] Human aneurysmal tissue expresses predominantly the COX-2 isoform, and COX-2-selective inhibitors have been proposed as a potential

pharmacological treatment for AAAs.[4, 38]

Using a mouse model of AAAs induced by chronic AngII infusion, we previously determined that pre-treatment with the COX-2-selective inhibitor celecoxib prior to disease initiation reduces AAA formation, whereas a COX-1-selective inhibitor is not effective.[55] Furthermore, we have shown that AngII-induced AAA formation is significantly reduced in mice deficient in COX-2.[56] The genetic deficiency of microsomal prostaglandin E synthase-1, which contributes to PGE_2 synthesis downstream COX-2, also reduces AngII-induced AAA formation.[163] These findings demonstrate that the inactivation of COX-2 or PGE_2 synthesis prior to initiating AAA formation reduces subsequent development of the disease.

In addition to COX-2, the AngII infusion model in mice has been utilized to explore a variety of mechanisms that contribute to AAAs. Previous reports have suggested the potential benefit of pre-treatments administered before AngII infusion in reducing AAA formation in mice.[66, 164] An additional study examining leukotriene receptor antagonism showed the utility of treatment when first administered at the beginning of the AngII infusion. However, treatment with the leukotriene receptor antagonist was not effective when it was administered after beginning the AngII infusion.[165] Therefore, medications that are shown to be effective in reducing AngII-induced AAAs following pre-treatment with the agent, may not produce similar effectiveness in reducing AAA progression when treatment is started after initiation of the disease. This suggests the importance of identifying an effective pharmacological treatment that can reduce progression of the disease after it has been diagnosed.

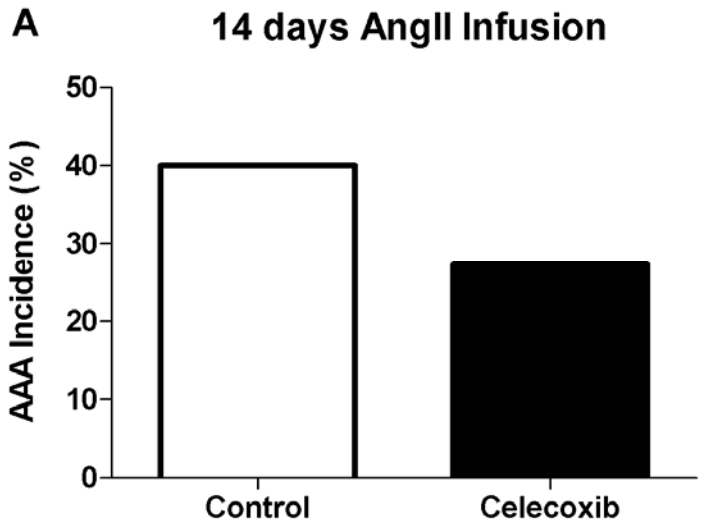
We therefore hypothesized that COX-2 inhibition can effectively attenuate AngII-induced AAA progression in mice, when treatment is begun after initiation of the disease. In the current study, we utilized mice infused with AngII, and treated with a control diet or diet containing COX-2-selective inhibitor celecoxib, to investigate the role of COX-2 in AngII-induced AAA development.

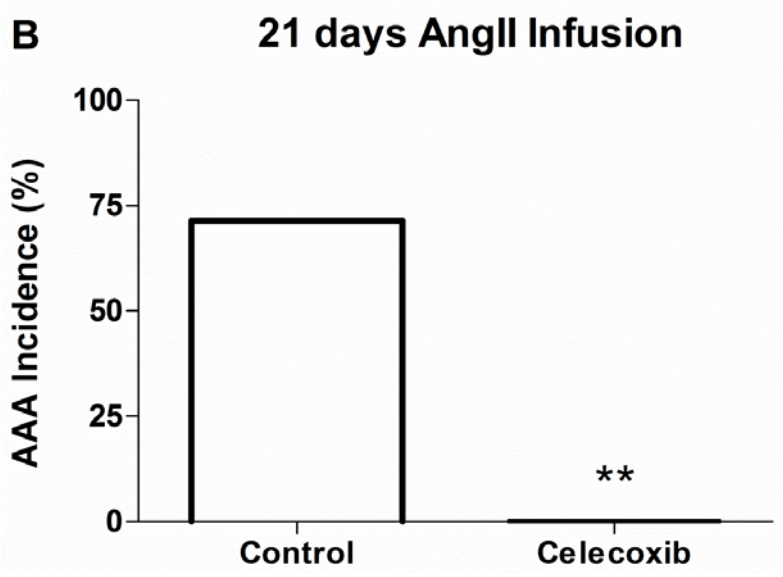
Results

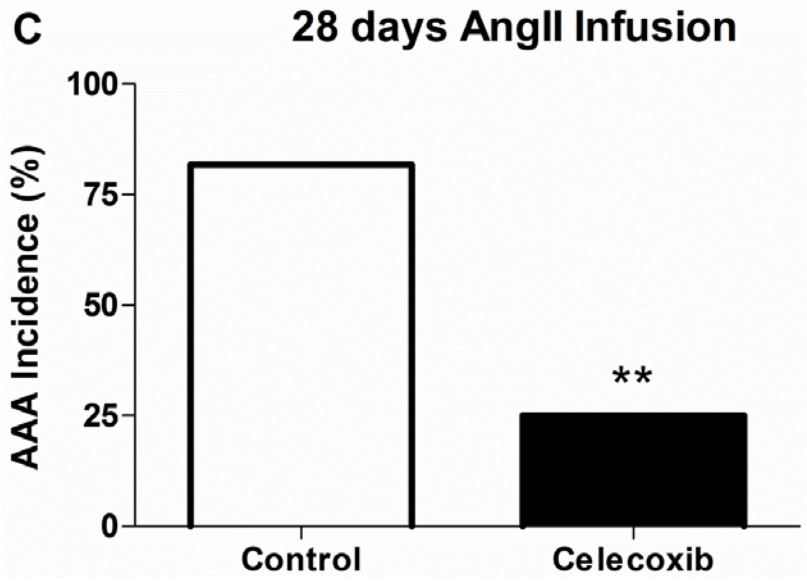
3.1. COX-2 inhibitor treatment begun after initiation of the disease effectively reduces AAA incidence

The infusion of AngII produces rapid development of aneurysmal pathology in the abdominal aorta with the earliest effects occurring during the first 3 days of the infusion.[54, 56] To examine the effect of COX-2 inhibition when started after the initiation of AAA formation, celecoxib treatment was begun 5 days after beginning the AngII infusion. The mice were then sacrificed at different time-points during the AngII infusion for analysis of the abdominal aorta during AAA progression. The analysis of AAA incidence after 14 days of AngII infusion was utilized to allow for examining the effects of COX-2 inhibition during early-stage of AAA progression. Although celecoxib treatment from day 5 to day 14 of the AngII infusion resulted in a trend for reduced AAA incidence, the difference was not statistically significant (Figure 3.1A). As compared to the 40% AAA incidence in mice on control diet at the 14 day time-point, AAA incidence was increased to 71% in control mice when analyzed after 21 days of AngII infusion (Figure 3.1B). Analysis of this 21 day time-point showed that celecoxib administration significantly reduced AAA incidence when treatment began on day 5 of the AngII infusion, as compared to mice on control diet (Figure 3.1B). As compared to mice on control diet infused with AngII for 28 days, celecoxib treatment from day 5 to day 28 of the infusion also significantly reduced AAA incidence (Figure 3.1C). These findings indicate that COX-2 inhibitor treatment was effective for attenuating AAA progression when treatment was begun after the disease had been initiated.

Figure 3.1: Effectiveness of celecoxib on AAA incidence after beginning AngII infusion. AAA incidence of mice on control or celecoxib diet was determined after **(A)** 14 days; **(B)** 21 days; and **(C)** 28 days of AngII infusion. Data represented as percent incidence; n ≥ 8; ** represent *p value* < *0.01*; Chi-squared test.




B
21 days AngII Infusion
AAA Incidence (%)
100
75
50
25
0
**
Control
Celecoxib


C
28 days AngII Infusion
AAA Incidence (%)
100
75
50
25
0
**
Control
Celecoxib

3.2. AAA severity is effectively attenuated by COX-2 inhibitor treatment begun after initiation of the disease

The external aortic diameter of the supra-renal region of the aorta was measured as a quantitative method of analyzing the effect of celecoxib treatment on AAA severity. Abdominal aortas were categorized as no AAA with diameters < 1.5 mm, as Type 1 AAA with diameters from 1.5 – 1.9 mm, or Type 2 AAA with diameters ≥ 2 mm. As shown in Figure 3.2A, celecoxib treatment from day 5 to day 14 of the AngII infusion did not significantly alter the mean aortic diameter and resulted in a level of AAA severity similar to mice on control diet (Figure 3.2B). In contrast, analysis of abdominal aortas after 21 days of AngII infusion shows a significant reduction in mean aortic diameter in mice treated with celecoxib (Figure 3.3A). The reduction in mean aortic diameter in mice after 21 days of AngII infusion resulted from decreased incidence of both Type I and Type II AAAs (Figure 3.3B). Similar results were obtained after 28 days of AngII infusion, when celecoxib treatment started on day 5 of the infusion significantly decreased the mean aortic diameter (Figure 3.4A), which resulted from a reduced incidence of both Type 1 and Type 2 AAAs (Figure 3.4B). Therefore, COX-2 inhibitor treatment effectively reduced the severity of AAAs during progression of the disease.

Figure 3.2: Effectiveness of celecoxib on AAA severity 14 days after beginning AngII infusion. External abdominal aortic diameter of mice on control or celecoxib diet was determined after 14 days of AngII infusion **(A)**. Data represented as mean ± SEM; n ≥ 8; unpaired t-test. AAA severity of mice on control or celecoxib diet was determined by classification as Type 1 or Type 2 AAAs after 14 days of AngII infusion **(B)**. Data represented as percent incidence; n ≥ 8.

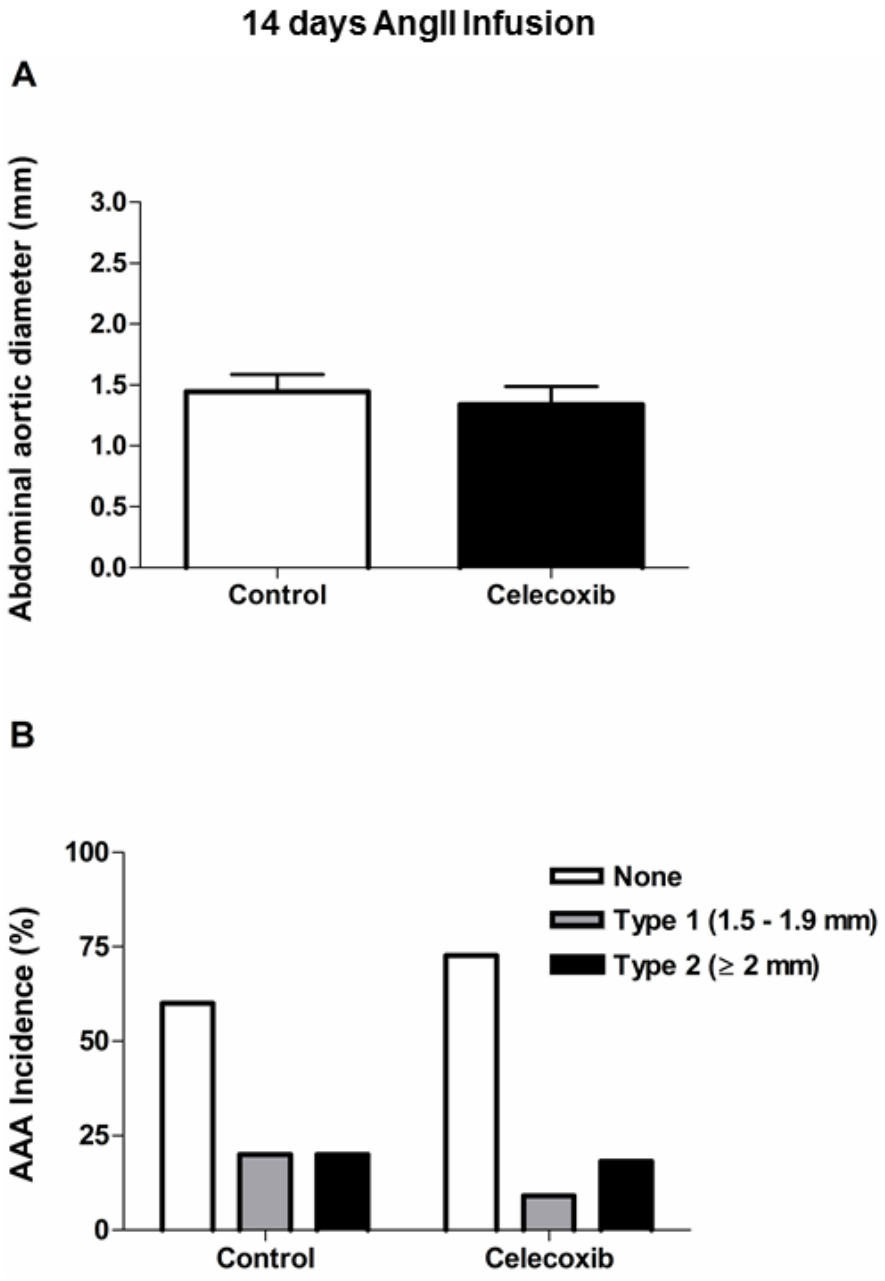


Figure 3.3: Effectiveness of celecoxib on AAA severity 21 days after beginning AngII infusion. External abdominal aortic diameter of mice on control or celecoxib diet was determined after 21 days of AngII infusion **(A)**. Data represented as mean ± SEM; n ≥ 8; "**" represent *p value < 0.01*; Unpaired t-test. AAA severity of mice on control or celecoxib diet was determined by classification as Type 1 or Type 2 AAAs after 21 days of AngII infusion **(B)**. Data represented as percent incidence; n ≥ 8.

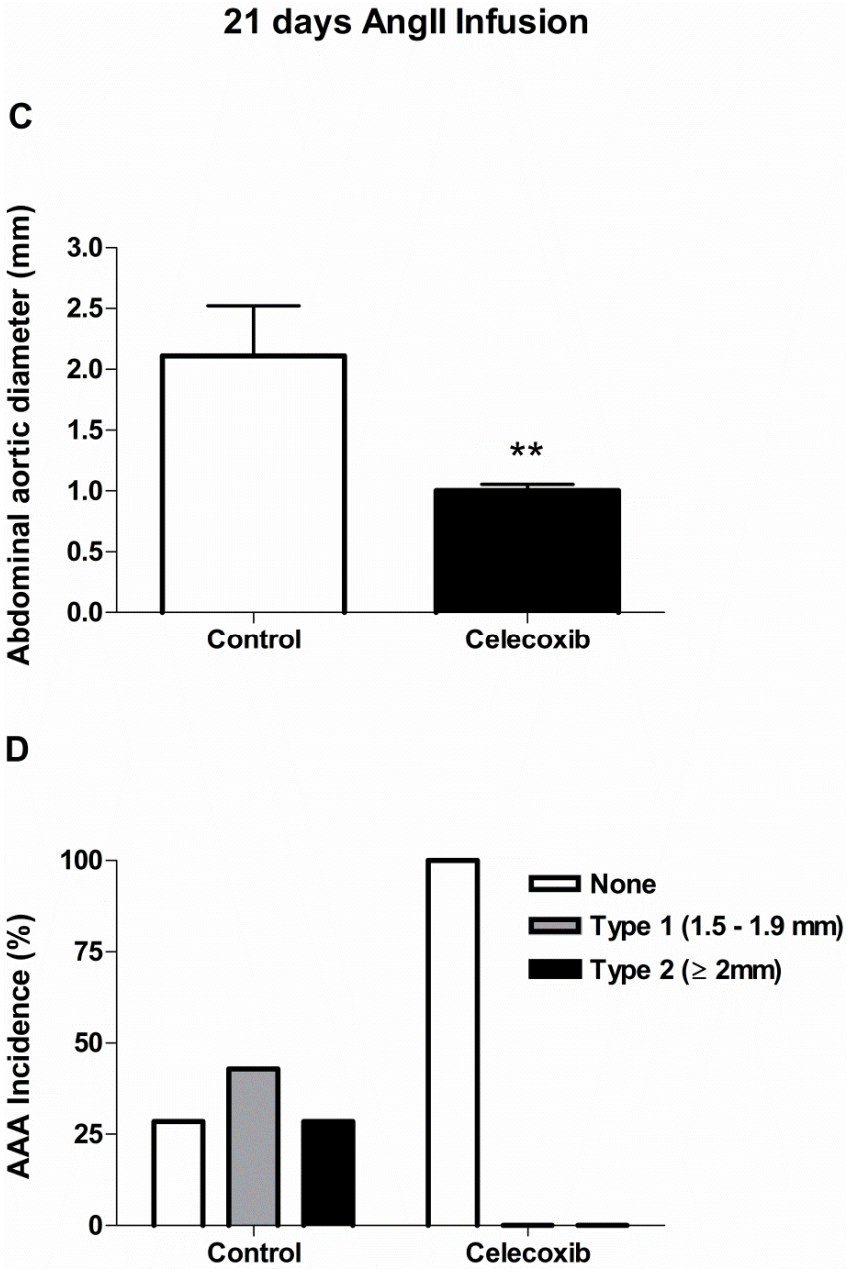
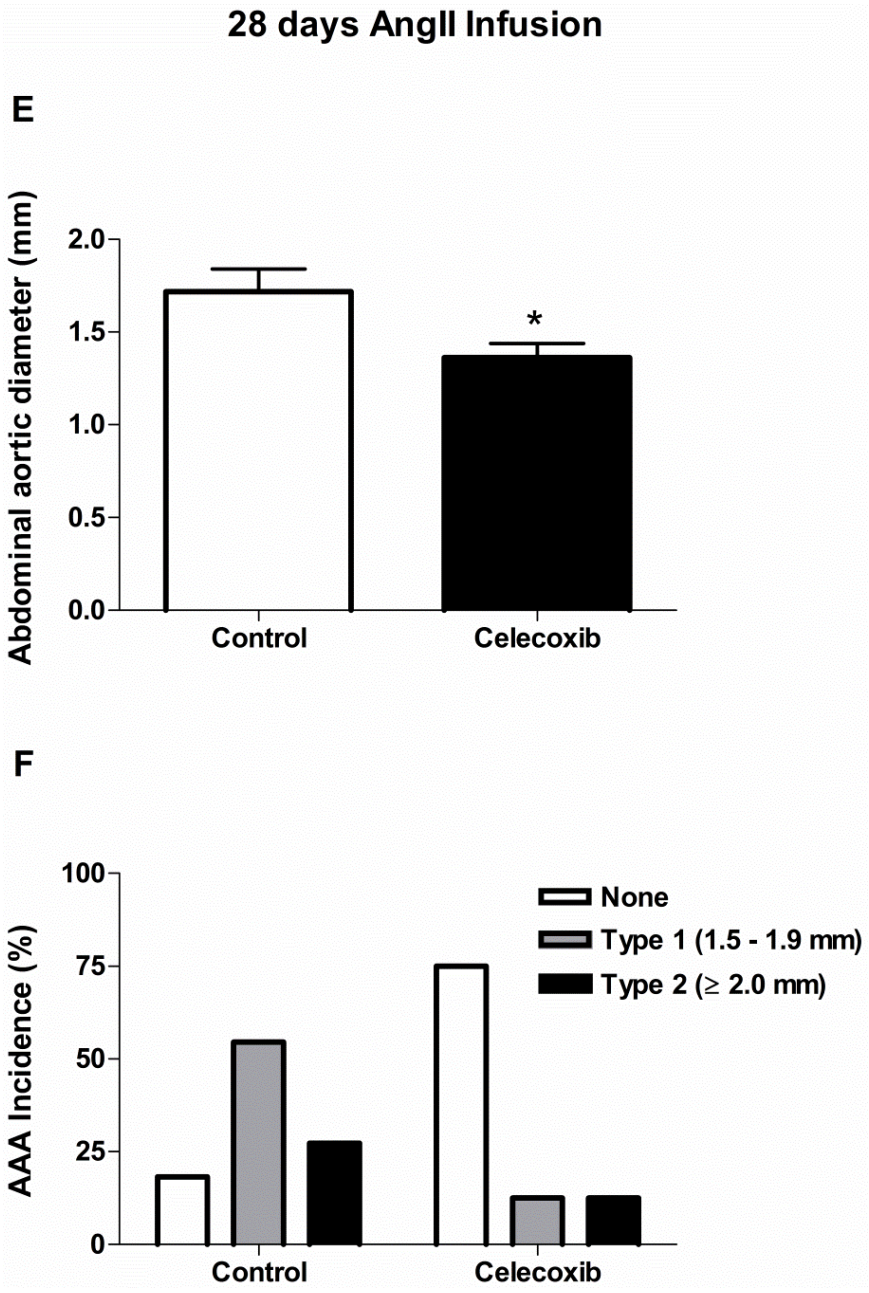


Figure 3.4: Effectiveness of celecoxib on AAA severity 28 days after beginning AngII infusion. External abdominal aortic diameter of mice on control or celecoxib diet was determined after 28 days of AngII infusion **(A)**. Data represented as mean ± SEM; n ≥ 8; "*" represent *p value < 0.05*; Unpaired t-test. AAA severity of mice on control or celecoxib diet was determined by classification as Type 1 or Type 2 AAAs after 28 days of AngII infusion **(B)**. Data represented as percent incidence; n ≥ 8.



3.3. COX-2 inhibitor treatment protects against AngII-induced aortic rupture

The AngII infusion model of AAAs results in an increased incidence of aortic rupture and resulting mortality.[4] In the current study, we performed necropsy on mice that died prior to completion of the 28-day AngII infusion and utilized the detection of a hemorrhage adjacent to the aorta as confirmation of a ruptured aorta. As is evident from the survival curve (Figure 3.5A), the death of the mice from aortic rupture occurred throughout the AngII infusion. The time to death during the course of AngII infusion was observed to be delayed in the celecoxib treated mice. Celecoxib treatment also significantly decreased the overall incidence of mortality that occurred during the AngII infusion (Figure 3.5B). Therefore, COX-2 inhibitor treatment initiated 5 days after beginning the AngII infusion effectively protected mice from aortic rupture and death.

Figure 3.5A: Effect of celecoxib on time to death of AngII-infused mice. The effect of control or celecoxib treatment started 5 days after beginning AngII infusion was determined on survival of mice after 28 days of AngII infusion. Data represented as percent survival; $n \geq 19$.

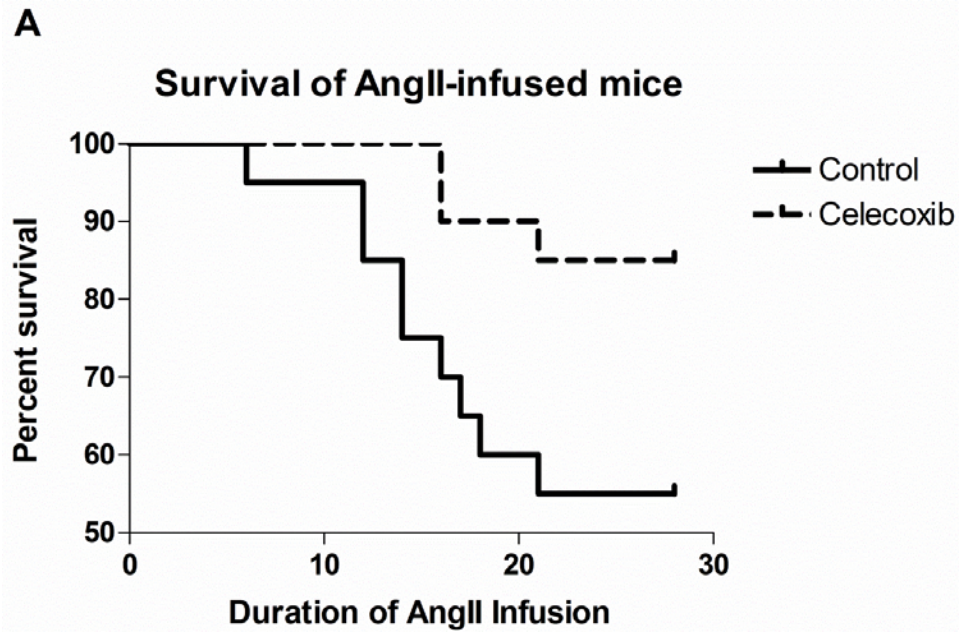
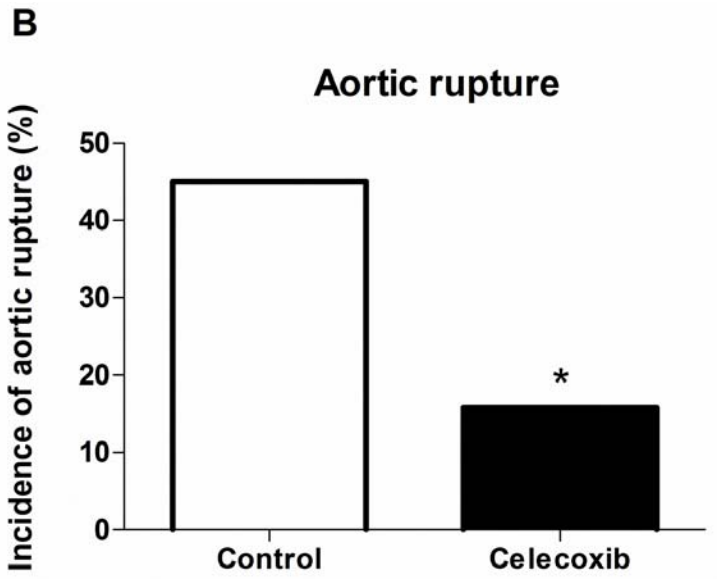


Figure 3.5B: Effectiveness of celecoxib on AngII-induced aortic rupture and death. The effect of control or celecoxib treatment started 5 days after beginning AngII infusion was determined on incidence of aortic rupture in mice after 28 days of AngII infusion. Data represented as percent incidence; n ≥ 19; "*" represent *p value* < *0.05*; Chi-squared test.



Discussion

AAAs are a chronic condition with a prevalence of approximately 5% in men 65 years or older.[14] AAAs often progress slowly over years or decades leading to increased susceptibility of aortic rupture later in life.[4] Although these AAAs are readily identified by non-invasive imaging methods even at an early stage of development, most are below the size threshold required for surgical intervention.[40, 161] Human studies examining the progression of AAAs show a predictable annual growth rate with the majority of early AAAs eventually requiring surgery, which is associated with a significant risk of mortality.[4, 6, 160] However, currently there are no pharmacological treatments to slow progression of this disease.

The AngII infusion model of AAAs was established by Daugherty et al. as being highly effective in producing AAAs in apoE (-/-) mice when infused with 1000 ng/kg/min of AngII for 28 days, and has since then been a widely accepted model for characterizing AAA development.[51] Similar to the apoE (-/-) mice, studies from our laboratory, using this model, have shown that AngII infusion induces a high incidence of AAAs in mice of a non-hyperlipidemic background as well.[55] In the current studies, we have utilized this AngII infusion model in non-hyperlipidemic mice to characterize the role of COX-2 during AAA progression.

The aim of the current study was to characterize a potential treatment to attenuate AAA progression after the disease has been initiated. Aneurysmal pathology has been shown to occur as early as 48 hours after beginning AngII infusion, as manifested by medial macrophage infiltration into the suprarenal aorta. This was accompanied by

disruption of elastin fibers at these sites of macrophage accumulation.[54] We have also previously reported that development of early AAAs is observed in mice 3 days after beginning AngII infusion.[56] Therefore, the AngII-induced AAA model results in a robust and rapid development of aneurysmal pathology during the first few days of AngII infusion.

In the current study, we examined the effect of beginning treatment with the COX-2-selective inhibitor, celecoxib, after the development of early aneurysmal pathology in mice. We found that beginning celecoxib treatment after 5 days of AngII infusion significantly decreased AAA incidence as compared to mice on control diet, after completion of a 28 day infusion. The 28 day AngII infusion model has been shown to result in AAAs of varying severity as determined by the mean external diameter of the abdominal aorta.[56] The current studies show that compared to control mice, celecoxib treatment significantly reduced the mean external diameter of the abdominal aorta. The reduction in mean aortic diameter in mice resulted from decreased incidence of both Type I (external aortic diameter of 1.5 – 1.9 mm) and Type II AAAs (external aortic diameter of $\geq$ 2 mm). Thus, celecoxib, administered during AAA progression, effectively reduced both incidence and severity of AAAs when analyzed after 28 days of AngII infusion.

Our previous studies have shown that AAA incidence increases during the course of the 28 day AngII infusion.[56] To identify the stage of AAA progression when celecoxib produces its benefit, we analyzed the effect of COX-2 inhibition at time-points prior to completion of the 28 day infusion. When analyzed 14 days after AngII infusion, celecoxib treatment, started 5 days after the infusion, reduced the incidence of AAAs

compared to the control mice, although this difference was not statistically significant. The AAA incidence in the control mice at this 14 day time-point was considerably lower (40%) than those observed at later time-points of 21 (71%) and 28 days (82%). Therefore, the lack of statistical significance for the effect of celecoxib treatment after 14 days of AngII infusion may have resulted from limited disease development in the control mice at this early time-point. With the higher AAA incidence (71%) in control mice after 21 days of AngII infusion, celecoxib treatment did significantly reduce AAA incidence at this time-point. These results suggest that beginning COX-2 inhibitor treatment, after initiation of the disease, effectively attenuates AAA expansion during progression of the disease.

The primary clinical significance of AAA development is the increased susceptibility of aortic rupture. AAAs are characterized by extensive remodeling of the vessel wall and eventual degradation of the extracellular matrix leading to a ruptured aorta. Aortic rupture associated with AAAs is reported to result in approximately 90% mortality in humans.[5] The AngII-induced mouse model of AAAs is also associated with an increased incidence of aortic rupture and resulting death. Saraff et al. were the first to report that approximately 10% of the mice treated with AngII died due to ruptured aorta during early AngII infusion.[54] In another study utilizing the AngII infusion model, approximately 25% of the mice were reported to have died due to hemorrhage around the aortic arch as well as the suprarenal aorta during the first 7 days of AngII infusion.[166] However, using the same model, AAA studies by Wang et al. have reported sudden death of mice due to rupture of the abdominal aorta for longer periods of AngII infusion.[163] Similarly, in the current study, we observed aortic rupture and

resulting death in mice on control diet throughout the AngII infusion. Previous studies have shown that decreased AAA formation in mice is associated with an improved survival by reducing AngII-induced aortic rupture. Genetic deletion of mPGES-1 was found to reduce AAA development in mice and decrease both the incidence as well as the time to aortic rupture.[163] Recently, treatment with rosiglitazone has been reported to reduce AngII-induced aortic rupture in apoE (-/-) mice.[167] Similarly in the current study, celecoxib treatment reduced the rate of mortality during the course of AngII infusion, thereby resulting in a significant decrease in the overall incidence of aortic rupture and death. Thus, in addition to effectively reducing AAA incidence, COX-2 inhibition also significantly improves survival following AngII infusion in mice.

Several other studies have been carried out using the AngII infusion model in mice to identify potential pharmacotherapies for AAAs. Similar to our previous findings with celecoxib, pre-treatment with fasudil, a Rho-kinase inhibitor, as well as simvastatin, a HMG-CoA reductase inhibitor was found to reduce AngII-induced AAA formation in mice.[56, 66, 164] An additional study showed the usefulness of a JNK inhibitor in reducing AAAs when treatment was initiated after completion of the AngII infusion.[168] Further studies show that pharmacological antagonism of the leukotriene (LT) B4 receptor, BLT1, concomitant with AngII infusion, reduces AngII-induced AAAs, although, administration of the drug post AngII infusion failed to protect the mice from AAAs[165]. However these studies using the AngII infusion model indicate the dearth of pharmacological inhibitors which limit AAA progression after initiation of the disease. In our current studies, we show for the first time, that treatment with a COX-2-

selective inhibitor, initiated after early development of the disease, is effective in attenuating the progression of AngII-induced AAAs in mice.

Previous studies have utilized hyperlipidemic mice to investigate the use of genetic deletion or pharmacological inhibition of involved molecules in reducing AAAs. However, expansion of small AAAs in humans has been shown to be independent of their cholesterol levels.[13] Thus, our current finding that celecoxib displayed effectiveness in attenuating AngII-induced AAAs in a non-hyperlipidemic mouse model may be relevant to using celecoxib in human studies in the absence of atherosclerosis.

Chronic administration of both non-selective and COX-2-selective inhibitors has been associated with increased adverse cardiovascular events.[118, 169] Although the COX-2-selective inhibitor rofecoxib was withdrawn from the market due to increased adverse cardiovascular events, the level of risk for developing these adverse effects is considered lower with celecoxib use and comparable to other NSAIDs.[123, 170, 171] While there has also been considerable interest in identifying effective anti-inflammatory agents which lack these adverse cardiovascular effects, the therapeutic potential of medications which inhibit novel targets downstream of COX-2 is not known.[172] Hence, our current findings describing the effectiveness of celecoxib in an AAA model, together with the lack of approved medications, emphasize the need for further studies to evaluate celecoxib as a potential treatment for AAAs.

CHAPTER 4

COX-2 CONTRIBUTES TO INCREASED SUSCEPTIBILITY OF ABDOMINAL AORTA TOWARDS ANEURYSM FORMATION IN MICE

Introduction

The aorta can be divided into three broad regions: the arch, thoracic and abdominal regions. In humans, the most common location for aneurysm development is the abdominal region of the aorta.[161, 173] These are known as abdominal aortic aneurysms (AAAs), and are specifically localized in the infra-renal abdominal aorta, i.e., just below the renal arteries.[Ref] A similar effect is observed in the AngII-induced mouse model of AAAs where the majority of aneurysm development is specific to the abdominal aorta.[51] Although these different regions of the aorta have diverse biochemical properties, the reasons as to why aneurysms are primarily localized in the abdominal region of the aorta, are not well-defined.[173]

Human AAAs are characterized by both an increase in systemic inflammatory mediators and vascular inflammation localized in the aortic wall. Prostanoids are believed to be important regulators of these inflammatory processes.[4, 124, 125] Both COX-2 and the resultant PGE_2 have been found to be induced in the human aneurysmal tissue as compared to the normal aorta.[38, 125, 174] Using the AngII infusion model of AAAs, we have previously reported that genetic deficiency as well as pharmacological inhibition

of COX-2 can reduce AAA formation in mice.[55, 56] These reports indicate that COX-2 plays an important role during AAA formation.

AngII is known to increase COX-2 expression in cultured vascular smooth muscle cells.[78, 175-177] This increase in COX-2 expression by AngII has been found to be mediated by the AT1 subtype of AngII receptors.[78, 175] Expression of these AT1a receptors has also been reported to be higher in the abdominal region as compared to the thoracic region of the aorta of mice in absence of AngII infusion.[178] However, comparison of AT1a receptor expression in different segments of the aorta following AngII infusion has not been previously reported.

Based on the above reports, we therefore hypothesized that a greater induction of COX-2 coupled to AT1a receptor expression contributes to the increased susceptibility of the abdominal aorta towards AngII-induced aneurysm formation in mice.

Results

4.1. AngII infusion results in prolonged induction of COX-2 in the abdominal aorta

To examine the role of COX-2 in the increased susceptibility of the abdominal aorta, the induction of COX-2 expression in response to AngII infusion was compared between the arch, thoracic and the abdominal aortic segments. In the aortic arch, the infusion of AngII for 3 days resulted in a significant induction of COX-2 mRNA expression, as compared to saline-infused mice (Figure 4.1A). In contrast, COX-2 expression in the arch significantly decreased in response to AngII after 7 and 21 days of the infusion (Figure 4.1A). After 28 days of infusion, there was no significant difference in COX-2 expression in the aortic arch of saline and AngII -infused mice (Figure 4.1A).

In the thoracic segment of the aorta, AngII induced a significant increase in COX-2 mRNA expression when analyzed after 3 days of infusion (Figure 4.1B). Similar to the aortic arch, the initial induction of COX-2 expression in the thoracic segment was followed by reduced expression of COX-2 in response to 7 days of AngII infusion, with a statistically significant reduction observed after 21 days of infusion (Figure 4.1B). After 28 days of infusion, COX-2 mRNA levels in the thoracic aorta of mice infused with AngII were not significantly different from those infused with saline (Figure 4.1B).

Analysis of the abdominal segment of the aorta shows that AngII infusion for 3 days significantly increased COX-2 expression, as compared to saline-infused mice (Figure 4.1C). In contrast to the arch and thoracic segments, 7 days of AngII infusion significantly increases COX-2 expression in the abdominal aorta (Figure 4.1C). The AngII-induced increase in COX-2 expression in the abdominal aorta continued through

the 21 day time-point (Figure 4.1C). After 28 days of infusion, COX-2 expression in the abdominal aorta was not significantly increased in response to AngII (Figure 4.1C).

The above results for COX-2 mRNA expression in the arch, thoracic and abdominal aortic segments are represented in a combined graph in Figure 4.1D. Figure 4.1D shows COX-2 mRNA levels in the three aortic segments in response to AngII infusion for different time-periods expressed as a percentage of saline mRNA levels under the corresponding condition. Saline infusion was used as the control condition for these experiments. Thus, Figure 4.1D shows that 3 days of AngII infusion resulted in the induction of COX-2 mRNA which was more than 200% of the corresponding saline expression in the arch and thoracic segments of the aorta. However, by 7 days of AngII infusion, these COX-2 mRNA levels in the arch and thoracic aorta decreased to almost 50% of the basal levels expressed in saline-infused mice and remained lower through the 21 days infusion time-point. At the end of 28 days of AngII infusion, the COX-2 mRNA expression in the arch and thoracic segments were comparable to those expressed in saline-infused mice. In the abdominal segment, AngII infusion for 3 days resulted in approximately 350 fold greater induction of COX-2 mRNA, compared to the saline-infused mice. However, in contrast to the arch and thoracic segments, the AngII-induced COX-2 expression in the abdominal aorta remained higher for the 7 and 21 day time-points as well, till approximately 150% more than saline mRNA levels after 28 days of infusion.

These findings indicate that in the abdominal aorta COX-2 gene expression remains induced during the majority of the AngII infusion, in contrast to the transient induction that occurs in the arch and thoracic segments.

Figure 4.1A: Effect of AngII infusion on COX-2 mRNA expression in the aortic arch segment

COX-2 mRNA expression was determined in the aortic arch of mice following 3, 7, 21 and 28 days of saline or AngII infusion. COX-2 expression in the aortas of AngII-treated mice was standardized to corresponding saline values at each time-point. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 8; * represent *p value < 0.05*; *** represent *p value < 0.001*; Unpaired t-test.

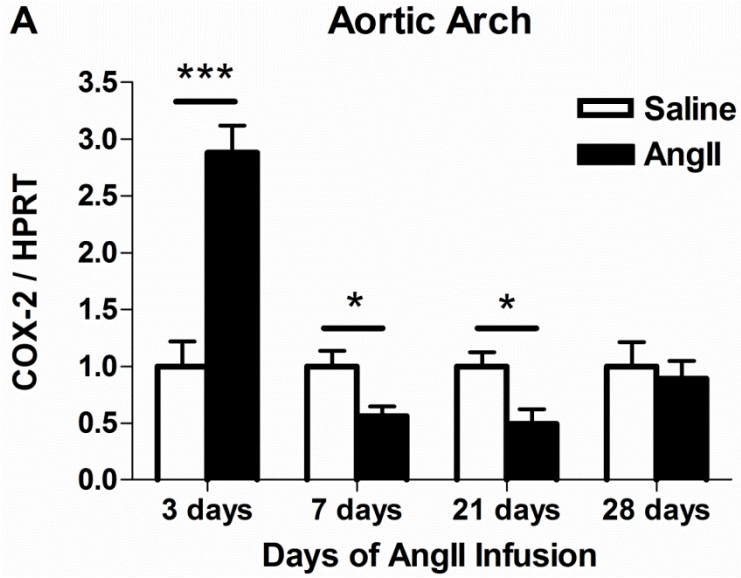


Figure 4.1B: Effect of AngII infusion on COX-2 mRNA expression in the thoracic aorta segment

COX-2 mRNA expression was determined in the thoracic aorta of mice following 3, 7, 21 and 28 days of saline or AngII infusion. COX-2 expression in the aortas of AngII-treated mice was standardized to corresponding saline values at each time-point. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 8; * represent *p value* < *0.05*; ** represent *p value* < *0.01*; Unpaired t-test.

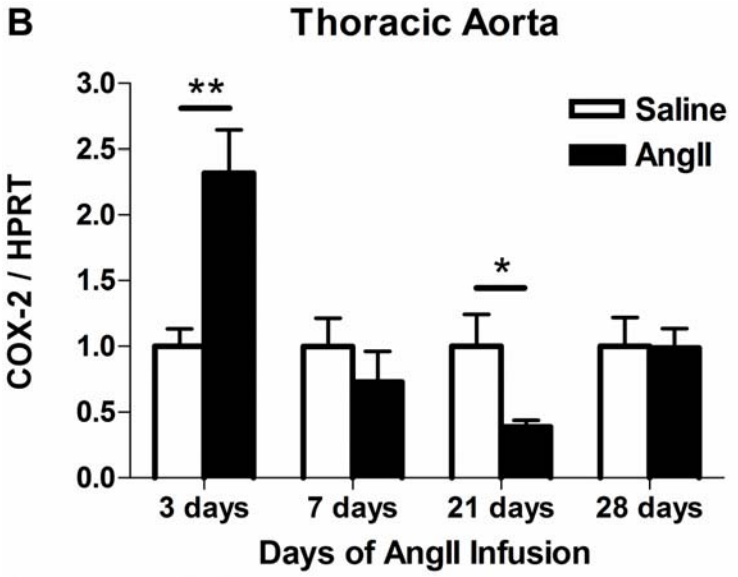


Figure 4.1C: Effect of AngII infusion on COX-2 mRNA expression in the abdominal aorta segment

COX-2 mRNA expression was determined in the abdominal aorta of mice following 3, 7, 21 and 28 days of saline or AngII infusion. COX-2 expression in the aortas of AngII-treated mice was standardized to corresponding saline values at each time-point. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 8; * represent *p value* < *0.05*; Unpaired t-test.

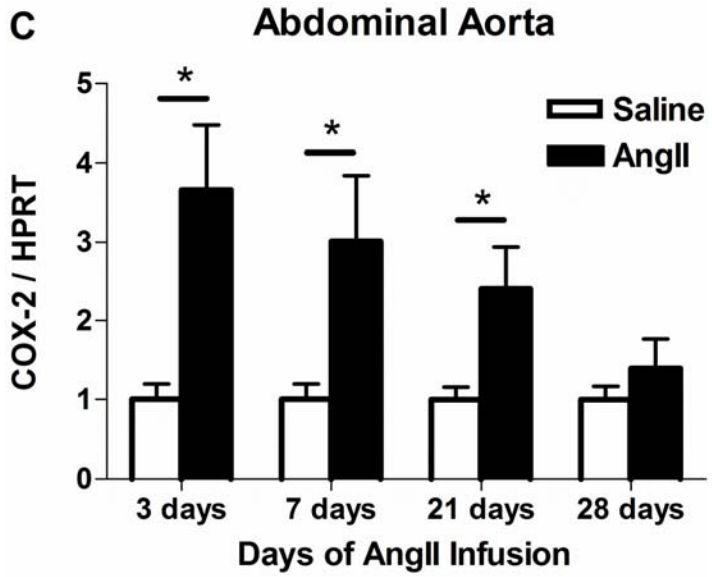
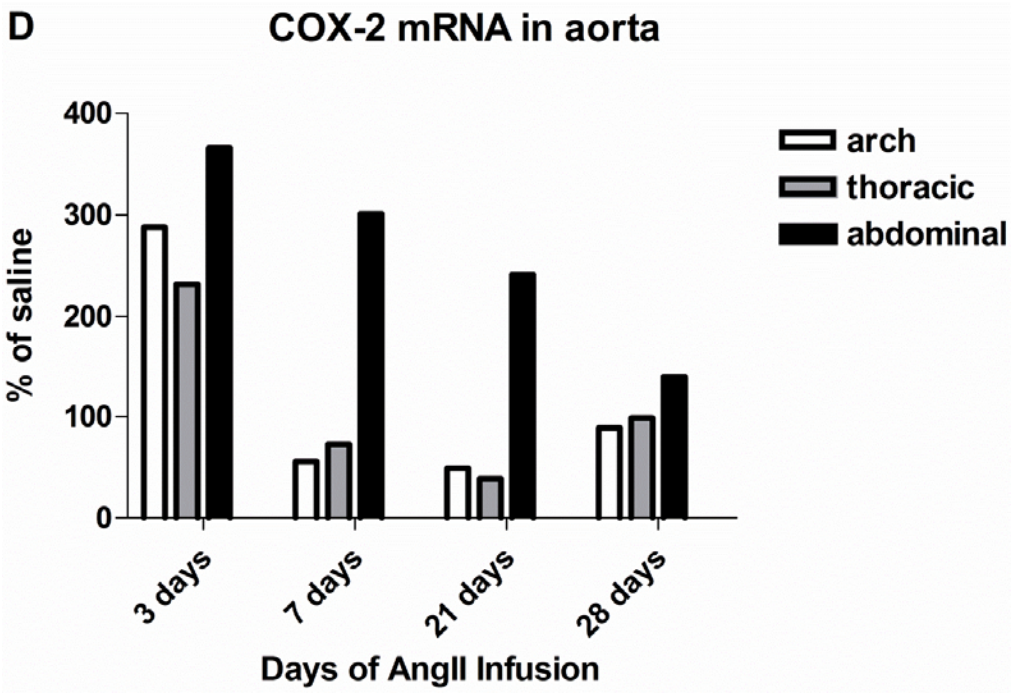


Figure 4.1D: Effect of AngII infusion on COX-2 mRNA expression in the arch, thoracic and abdominal segments of the aorta

COX-2 mRNA levels were expressed as percentage of expression levels in aortas of saline-infused mice. COX-2 expression was analyzed in the arch, thoracic and abdominal segments of the aorta after 3, 7, 21 and 28 days of infusion. Data represented as percentage of saline-infused control; $n \geq 8$.

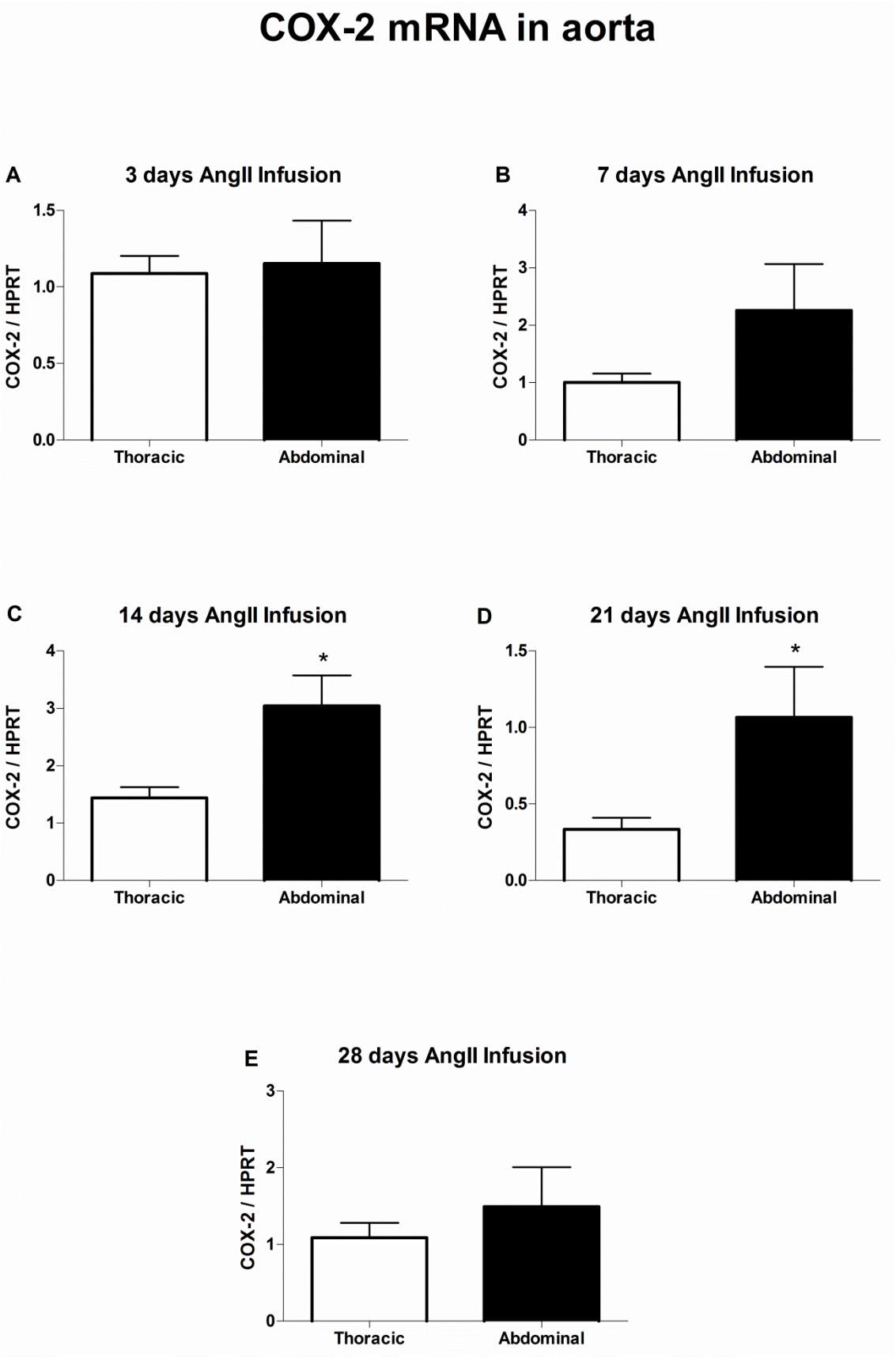


4.2. Comparison of COX-2 expression between the thoracic and abdominal aorta following AngII infusion

With the induction of COX-2 in response to AngII infusion being greatest in the abdominal segment, a direct comparison of COX-2 mRNA expression between the abdominal and thoracic segments was performed. After 3 days of AngII infusion, there was an equivalent level of COX-2 mRNA between the thoracic and abdominal segments (Figure 4.2A). By 7 days of the AngII infusion, there was a trend for COX-2 to be greater in the abdominal aorta, although not statistically significant (Figure 4.2B). COX-2 mRNA expression was significantly greater in the abdominal segment after 14 days of AngII infusion, as compared to thoracic aorta (Figure 4.2C). The significantly greater level of COX-2 expression in the abdominal aorta remained at the 21 day time-point (Figure 4.2D), whereas after 28 days of AngII infusion, expression returned to comparable levels between the two segments (Figure 4.2E). These results indicate that COX-2 gene expression in the abdominal aorta was significantly greater than the thoracic region during the majority of AngII infusion.

Figure 4.2: Comparison of COX-2 mRNA expression between thoracic and abdominal aortic segments.

COX-2 mRNA expression was determined in thoracic (clear bars) and abdominal (filled bars) segments of the aorta of mice following **(A)** 3 days; **(B)** 7 days; **(C)** 14 days; **(D)** 21 days and **(E)** 28 days of AngII infusion. Data represented as mean ± SEM; $n \geq 8$; * represent *p value* < *0.05*; Unpaired t-test.

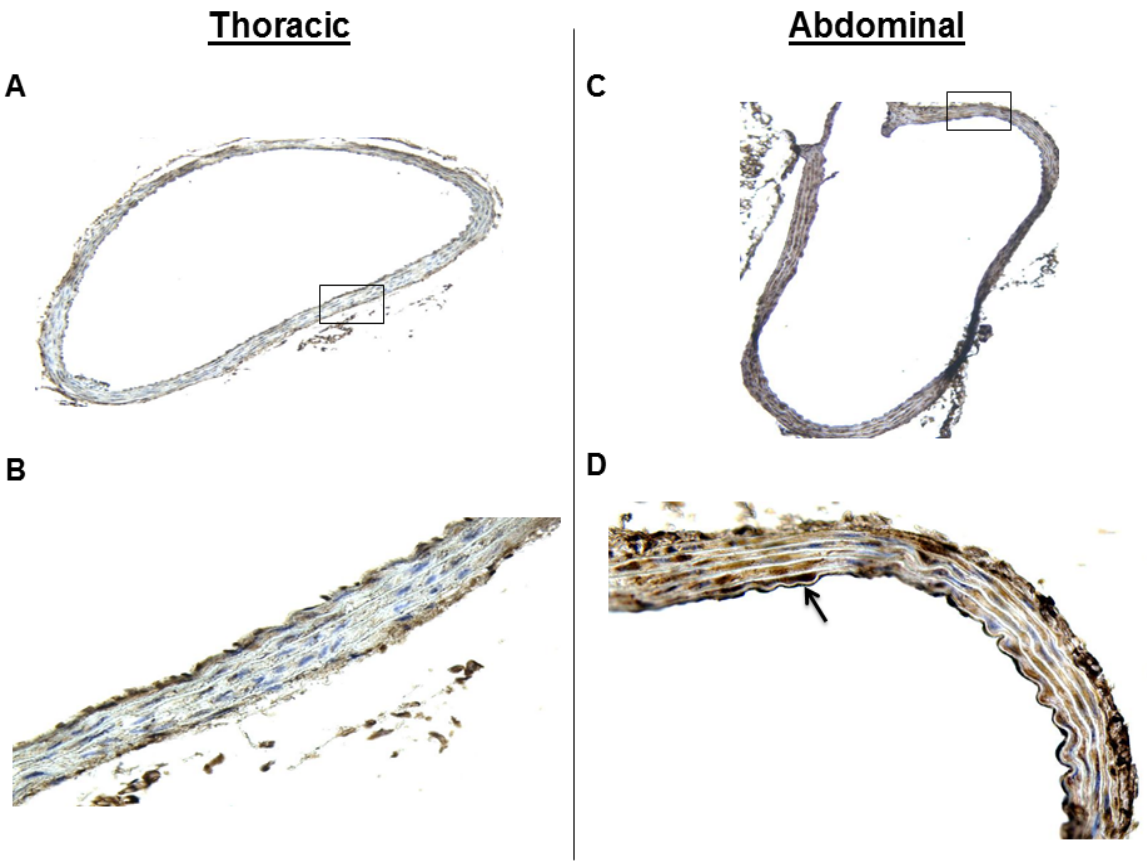

COX-2 mRNA in aorta
A
3 days AngII Infusion
COX-2 / HPRT
1.5
1.0
0.5
0.0
Thoracic
Abdominal
B
7 days AngII Infusion
COX-2 / HPRT
4
3
2
1
0
Thoracic
Abdominal
C
14 days AngII Infusion
COX-2 / HPRT
4
3
2
1
0
*
Thoracic
Abdominal
D
21 days AngII Infusion
COX-2 / HPRT
1.5
1.0
0.5
0.0
*
Thoracic
Abdominal
E
28 days AngII Infusion
COX-2 / HPRT
3
2
1
0
Thoracic
Abdominal

4.3. Comparison of COX-2 protein expression between thoracic and abdominal aortic segments.

To compare the expression of COX-2 protein between the thoracic and abdominal aortic segments, mice were infused mice with 1000 ng/kg/min AngII and sacrificed after 14 days. The thoracic and abdominal segments of the aortas were collected and analyzed for COX-2 protein expression by immunohistochemistry. COX-2 protein expression was not observed in cells of the vascular wall of the thoracic aorta (Figures 4.3A and 4.3B). In contrast, abundant COX-2 expression was observed in the abdominal segment of the aorta (Figure 4.3C). Higher magnification of the abdominal aorta shows a concentration of staining for COX-2 around the nuclei of medial smooth muscle cells (Figure 4.3D). These findings indicate that after AngII infusion COX-2 protein expression is readily detected in SMCs of the abdominal aorta but is not observed in cells of the thoracic aorta.

Figure 4.3: Comparison of COX-2 protein expression between thoracic and abdominal aortic segments.

Immunohistochemical analysis of COX-2 protein in thoracic aorta with **(A)** 100X and **(B)** 400X magnification and in abdominal aorta with **(C)** 100X and **(D)** 400X magnification. Brown staining indicates detection of COX-2 protein.

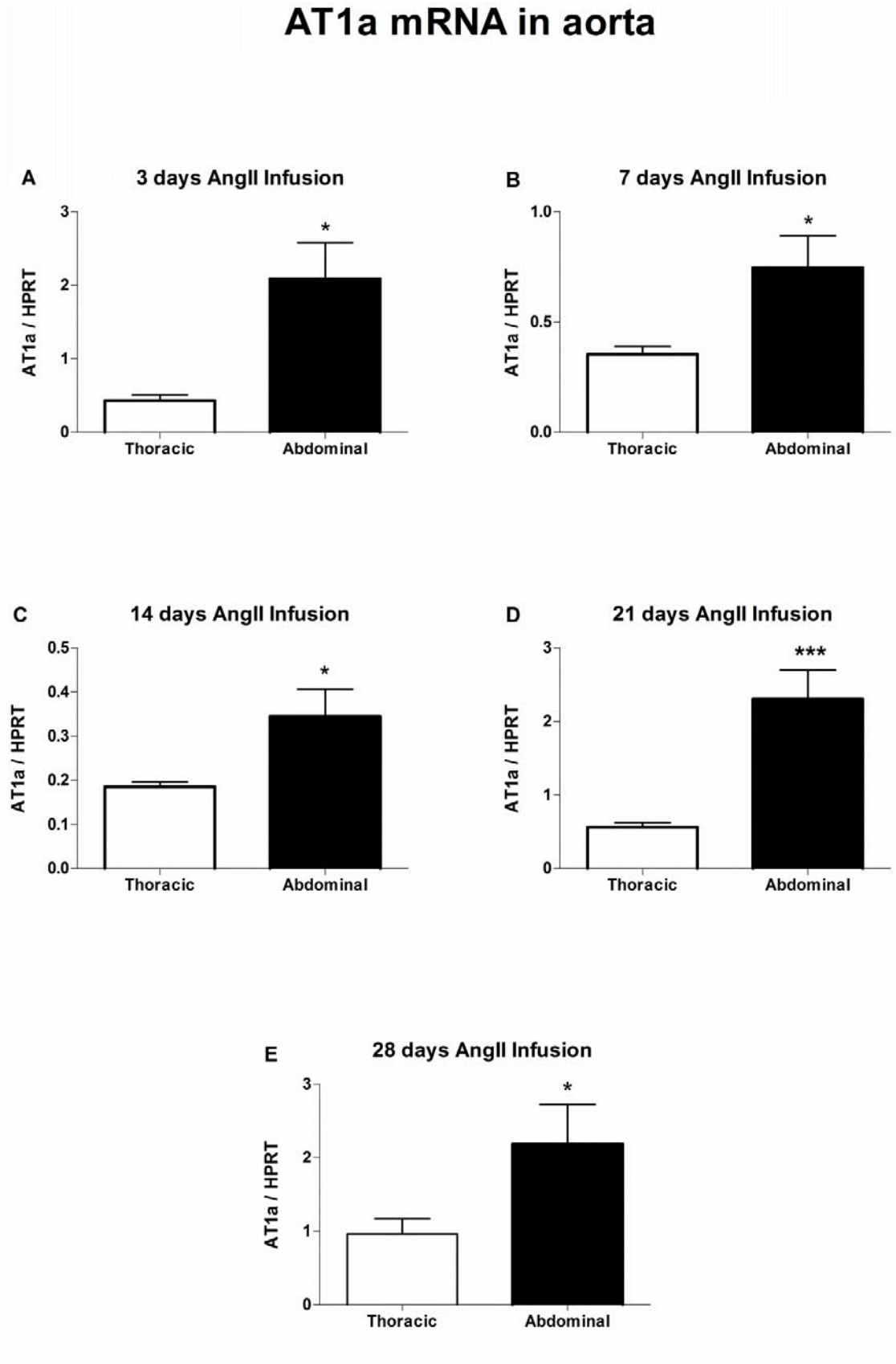


4.4. Abdominal aorta shows greater AngII receptor expression than the thoracic aorta

To account for increased levels of COX-2 induction in the abdominal aorta, mice were infused with AngII and sacrificed after 3, 7, 14, 21 and 28 days of infusion. The aortas were collected and the mRNA expression of the AT1a receptor was compared between the thoracic and abdominal segments during the course of AngII infusion. As shown in Figure 4.4A, after 3 days of AngII infusion, AT1a gene expression in the abdominal segment was significantly greater than the corresponding thoracic segment. After 7 days of AngII infusion, AT1a mRNA levels remained significantly greater in the abdominal aorta as compared to the thoracic region (Figure 4.4B). This increased AT1a expression was maintained at subsequent time-points of 14 days (Figure 4.4C), 21 days (Figure 4.4D) and 28 days (Figure 4.4E) of AngII infusion. These results indicate that AT1a receptor gene expression in the abdominal aorta remained significantly greater than expression in the thoracic region throughout the AngII infusion.

Figure 4.4: Comparison of AT1a mRNA expression between thoracic and abdominal aortic segments.

AT1a mRNA expression was determined in thoracic (clear bars) and abdominal (filled bars) segments of the aorta of mice following **(A)** 3 days; **(B)** 7 days; **(C)** 14 days; **(D)** 21 days and **(E)** 28 days of AngII infusion. Data represented as mean ± SEM; n ≥ 8; * represent *p value < 0.05*; ** represent *p value < 0.01*; *** represent *p value < 0.001*; Unpaired t-test.


AT1a mRNA in aorta
A
3 days AngII Infusion
AT1a / HPRT
3
2
1
0
*
Thoracic
Abdominal
B
7 days AngII Infusion
AT1a / HPRT
1.0
0.5
0.0
*
Thoracic
Abdominal
C
14 days AngII Infusion
AT1a / HPRT
0.5
0.4
0.3
0.2
0.1
0.0
*
Thoracic
Abdominal
D
21 days AngII Infusion
AT1a / HPRT
3
2
1
0
***
Thoracic
Abdominal
E
28 days AngII Infusion
AT1a / HPRT
3
2
1
0
*
Thoracic
Abdominal

Discussion

In the current study, we examined the increased expression of COX-2 as a mechanism contributing to the increased susceptibility of the abdominal segment towards AngII-induced aneurysm formation in mice. Various studies have reported the induction of COX-2 in smooth muscle cells in response to AngII treatment. Ohnaka et al. were the first to show in cultured rat vascular smooth muscle cells (SMCs) that AngII markedly increased the expression of COX-2 mRNA in a time and dose-dependent manner.[176] In addition to mRNA, COX-2 protein was also found to be transiently increased by AngII in these cultured SMCs of rat aorta.[78] Subsequent studies with human vascular SMCs showed that the AngII-induced increase in expression of COX-2 involved transcriptional activation of the COX-2 gene.[175] More recently, Morinelli et al. have also shown up-regulation of the COX-2 protein in response to AngII treatment.[177] However, these studies involved exposure of cultured aortic SMCs in vitro to an acute treatment of AngII. In contrast to acute exposure, a chronic infusion of AngII in mice for two weeks was found to significantly down-regulate COX-2 mRNA in mesenteric arteries.[179] Similarly, in the current studies, after the initial induction of COX-2 mRNA at the 3 day time-point, we observed a down-regulation of COX-2 in the arch and thoracic segments at later time-points during the AngII infusion. In contrast to the other segments, the abdominal aorta was resistant to the down-regulation and showed a prolonged induction of COX-2 that occurred during the majority of AngII infusion.

To better characterize the region-specific COX-2 expression, a direct comparison was performed between the thoracic and abdominal aortas. We determined that COX-2 gene expression was significantly greater in the abdominal region at both 14 and 21 days

after beginning the AngII infusion. No difference in COX-2 expression was observed between the thoracic and abdominal regions at the early 3 day time-point which may be attributed to both aortic segments showing significant AngII-induced COX-2 expression at this initial time-point. Additional analysis by immunohistochemistry revealed that COX-2 protein was readily detected in the smooth muscle cells of the abdominal, but not thoracic aorta. These observations suggest that a greater and more prolonged induction of COX-2 in the abdominal region may contribute to the increased susceptibility of this aortic segment to aneurysm progression.

The AngII type 1 (AT1) receptor is mainly responsible for mediating the various physiological and pathological actions of AngII in vascular SMCs.[180] Previous studies have shown that the AT1 receptor contributes to AngII-induced COX-2 expression in vascular SMCs. Young et al. showed that pre-incubation with AT1 receptor antagonist, losartan, but not the AT2 receptor antagonist PD123319, attenuated AngII mediated increase in COX-2 mRNA in rat aortic SMCs.[78] Similar results were reported by Hu et al. when they showed that AngII-mediated COX-2 protein expression was also mediated by the AT1 receptors.[175] These previous findings with cultured SMCs suggest that the induction of COX-2 that was observed following AngII infusion in mice is dependent on AT1a receptor expression.

In the absence of AngII infusion, the abdominal region of the aorta in mice has been shown to express a higher level of AT1a receptors as compared to the thoracic region.[178] However, comparison of AT1a receptor expression in different segments of the aorta following AngII infusion has not been previously reported. The current studies show for the first time that, in mice infused with AngII, AT1a receptor mRNA expression

in the abdominal aorta was significantly greater than the thoracic region throughout the AngII infusion. This suggests that AT1a receptor expression contributes to the increased susceptibility of the abdominal aorta to AngII-induced COX-2 expression.

Thus, our current findings indicate that a greater and prolonged induction of COX-2, coupled to an increased expression of the AT1a receptors, leads to an increased susceptibility of the abdominal region of the aorta towards AngII-induced aneurysm formation in mice.

CHAPTER 5

ANGII TREATMENT INDUCES CHANGE IN AORTIC SMOOTH MUSCLE CELL PHENOTYPE DURING AAA FORMATION IN MICE

Introduction

The arterial media in adults is made up of differentiated smooth muscle cells (SMCs) arranged in concentric layers allowing the cells to provide contractile function to the vessel. However, these fully differentiated cells have the capacity to undergo de-differentiation and revert back to a synthetic and proliferative phenotype in response to mitogenic stimulation and vascular injury. Vascular SMC proliferation and de-differentiation have been found to contribute to a variety of pathological processes including atherosclerosis, hypertension and coronary restenosis.[132-134]

A role for altered differentiation of SMCs has recently been identified as a mechanism contributing to the development of thoracic aortic aneurysms. However, compared to AAAs, the prevalence of these thoracic aortic aneurysms (TAAs) is considered to be significantly lower.[161] A number of studies have suggested the role of phenotypic modulation of SMCs during the development of TAAs. Mutations in genes of a number of proteins of the SMC contractile apparatus have been reported to contribute to aneurysms of the thoracic aorta.[181-186] In addition, studies utilizing animal models have also suggested the role of altered SMC phenotype in the formation of TAAs.[187-190] Thus, although the previous reports suggest that SMC de-differentiation is a key

factor in the formation of TAAs, the role of altered SMC phenotype in a model of AAAs has not been previously characterized.

Chronic infusion of AngII has been widely utilized to study AAA development in mice.[51] Among its other effects, AngII is well known to induce proliferation of cultured aortic SMCs.[77-79] However, significantly less is known about the role of AngII in regulating the phenotypic plasticity of SMCs. Pannu et al. have recently reported that the expression of angiotensin-converting enzyme as well as the markers of Ang II-induced vascular inflammation (macrophage inflammatory protein-1alpha and beta) are upregulated in certain thoracic aneurysms which result from a mutation in a contractile protein.[186] AngII antagonism has also been shown to down-regulate markers of the de-differentiated phenotype of SMCs in the spontaneously hypertensive rat aorta.[191, 192] Thus, although proliferation of SMCs has been a well-characterized effect of AngII, the role of AngII in regulating the phenotype of vascular SMCs has not been well defined.

With a role for altered SMC phenotype being suggested in other types of aneurysms, we hypothesized that AngII treatment modifies aortic SMCs to a proliferative or de-differentiated phenotype during AAA progression in mice. In the current studies, we utilized mice infused with either saline or AngII to investigate the role of AngII in regulating SMC phenotype *in vivo*, as well as characterize the phenotype of aortic SMCs during aneurysmal development.

Results

5.1. Increased proliferation of aortic SMCs is not detected during AngII-induced AAA formation

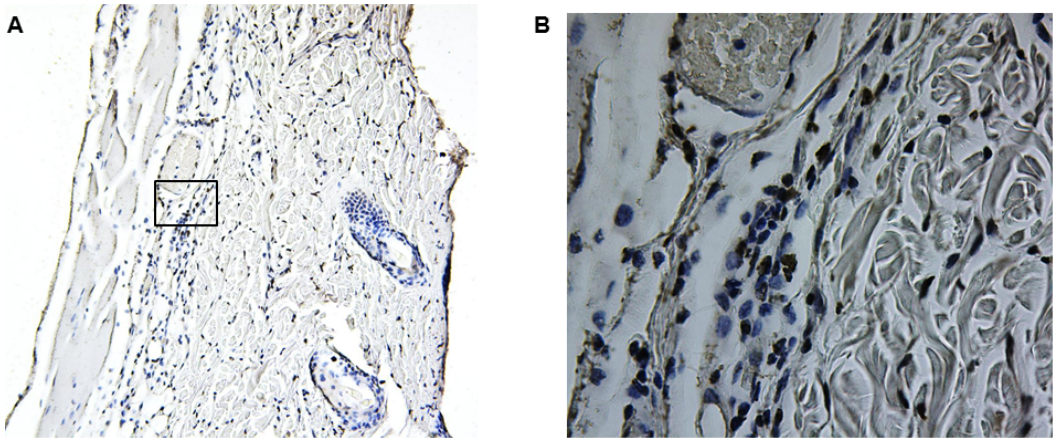
To investigate the role of AngII in regulating aortic SMC proliferation during AAA development, we utilized the BrdU labelling method, which is a highly sensitive and specific histological procedure for detection of proliferating cells *in vivo*. Following the implantation of saline or AngII-containing osmotic mini-pumps, mice were intraperitoneally injected with BrdU at a dose of 100mg/kg every 24 hours. After 7 or 14 days of infusion, the mice were sacrificed. The abdominal segments of the aorta were dissected, processed and subsequently sectioned to perform analysis for cell proliferation by BrdU immunohistochemistry.

Proliferating cells are evident by brown staining with an anti-BrdU antibody which detects BrdU that has been incorporated into DNA during S phase of cell cycle during proliferation. As a positive control for BrdU incorporation, skin samples were obtained from the BrdU-treated mice. The skin of these mice showed significant detection of BrdU in cells of the epidermis (Figures 5.1A-B). In contrast, in the aortas of the saline-treated mice, no significant labeling of SMCs with BrdU was observed (Figures 5.1C-D). Similar results were obtained for the AngII treatments, where no significant proliferation was detected in the SMCs comprising the medial layer of the aorta after both 7 (Figures 5.1E-F) and 14 days of AngII infusion (Figures 5.1G-H). These results suggest that proliferation of the SMCs of the aorta may not play an important role during AngII-induced AAA development in mice.

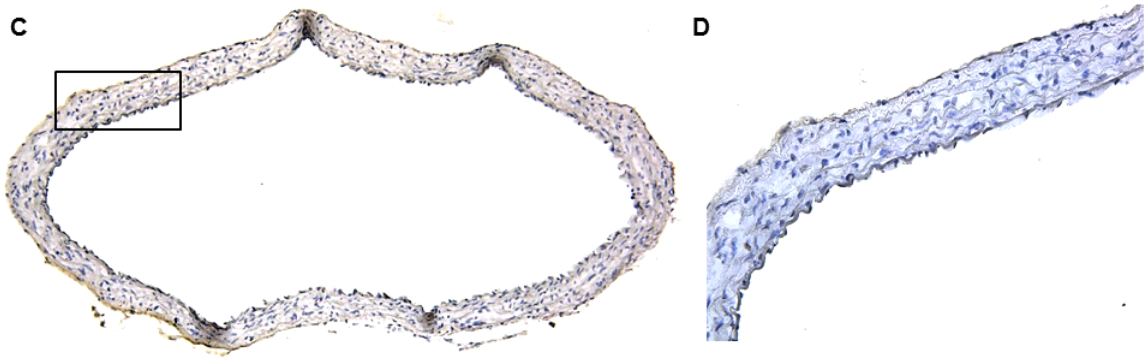
Figure 5.1: SMC proliferation in the abdominal aorta

Immunohistochemical analysis of SMC proliferation in the positive control skin tissue at **(A)** 100X magnification and **(B)** 400X magnification, and in the abdominal aorta of saline-infused mice at **(C)** 100X magnification, and **(D)** 400X magnification. For AngII-infused mice, immunohistochemical analysis of proliferating SMCs after 7 days of AngII infusion at **(E)** 100X magnification, and **(F)** 400X magnification, and after 14 days of AngII infusion at **(G)** 100X magnification, and **(H)** 400X magnification Brown staining indicates detection of BrdU-positive proliferating cells.

Skin

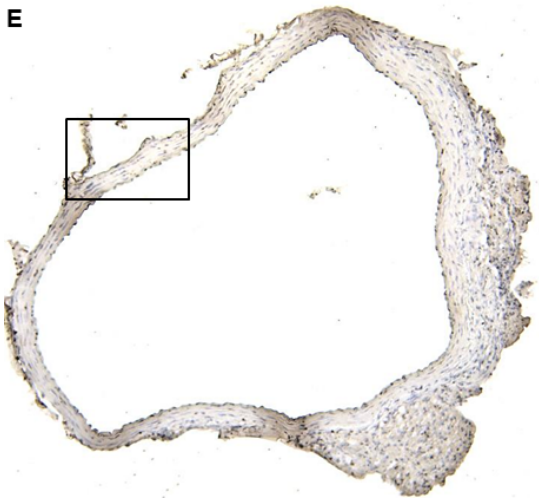


Saline Infusion

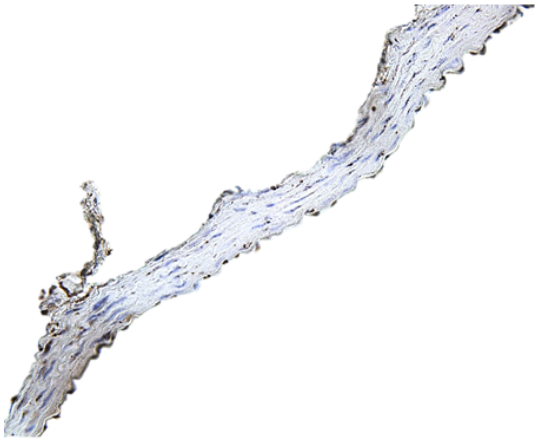


7 days AngII Infusion

E



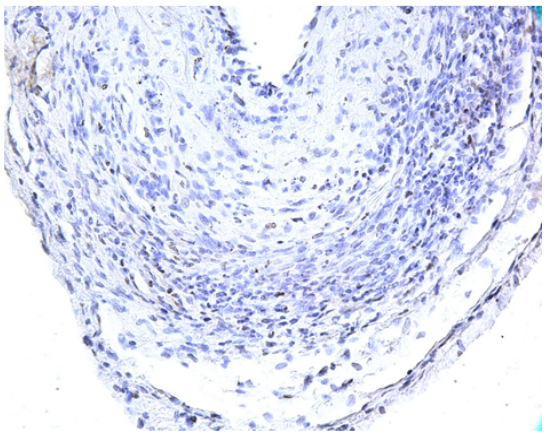
F



14 days AngII Infusion

G

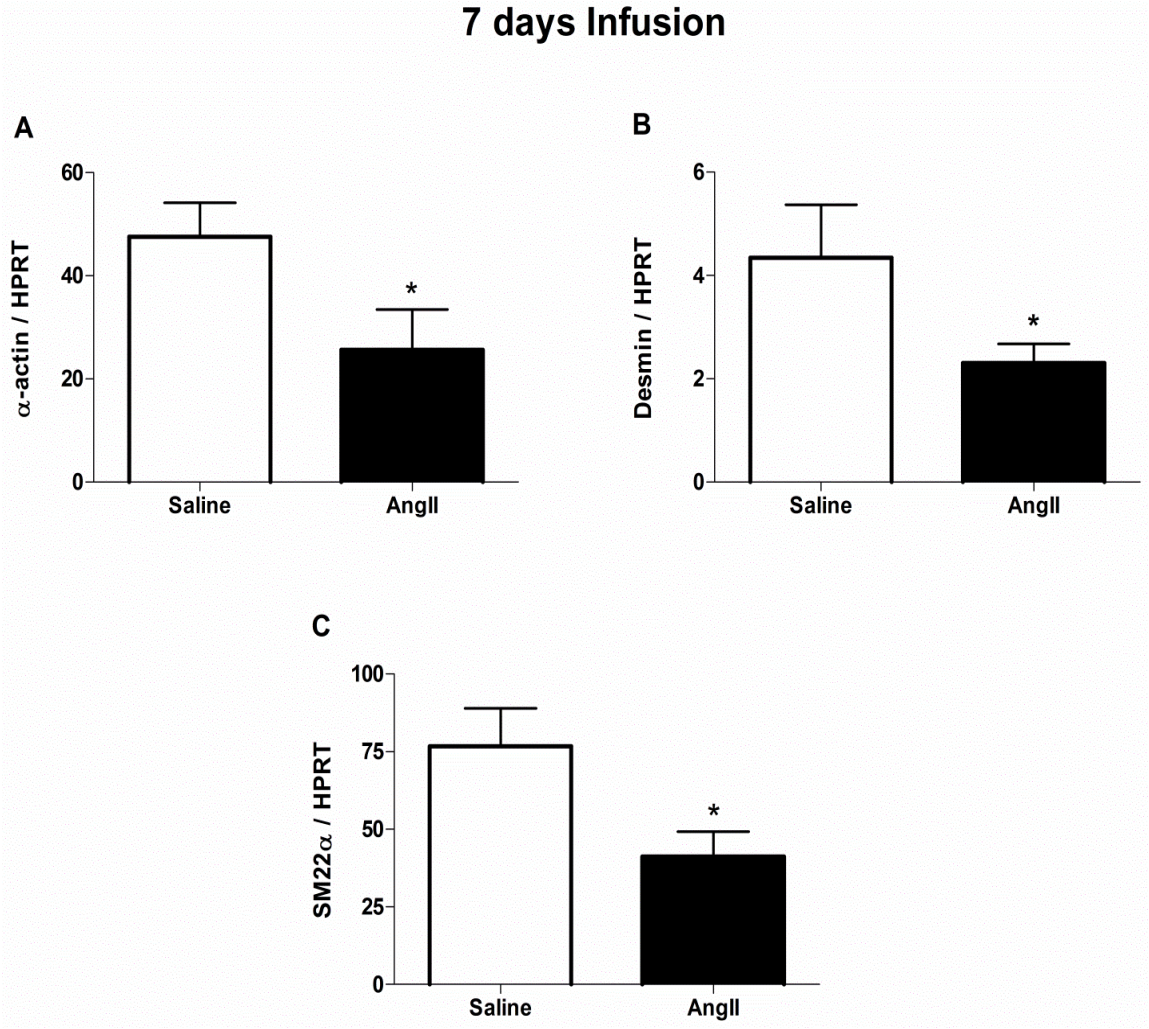
H



5.2. AngII decreases markers of a differentiated SMC phenotype

To elucidate the role of AngII in regulating the vascular smooth muscle cell (SMC) phenotype, male non-hyperlipidemic mice were infused with saline, as control, or AngII at a dose of 1000 ng/kg/min. The mice were sacrificed after 7 days of saline or AngII infusion to examine SMC phenotypic changes during AngII-induced early aneurysmal development. The aortas were dissected and analyzed for mRNA expression of α-actin, SM22α and desmin, which are markers of a differentiated smooth muscle cell phenotype, by real-time PCR. Seven days of AngII infusion was found to significantly decrease the mRNA expression of α-actin (Figure 5.2A), desmin (Figure 5.2B) and SM22α (Figure 5.2C) in the abdominal aorta, as compared to saline infusion. These findings suggest that gene expression of markers of a differentiated SMC phenotype is significantly reduced in response to AngII infusion in mice.

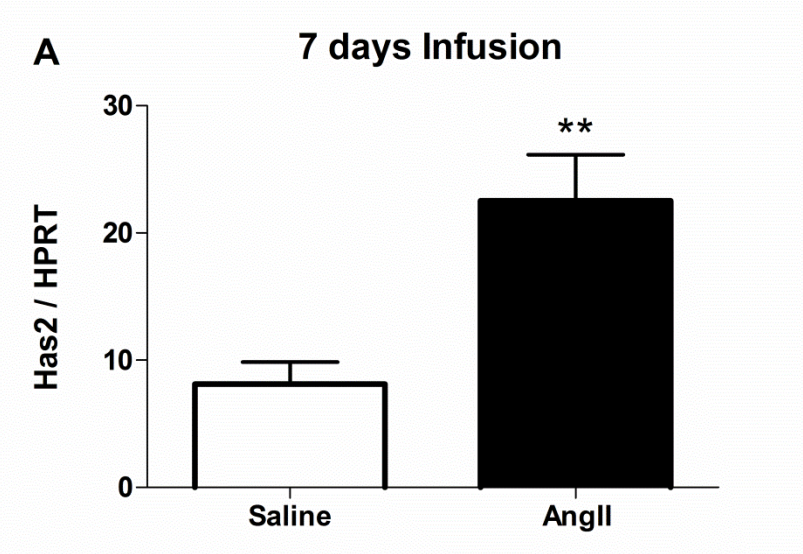
Figure 5.2: Effect of AngII infusion on mRNA expression of markers of differentiated SMC phenotype. mRNA expression of **(A)** α-actin, **(B)** desmin and **(C)** SM22α was determined in the abdominal aortas of mice infused with saline or AngII for 7 days. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 9; "*" represent *p value < 0.05*; Unpaired t-test.



5.3. AngII increases a marker of a de-differentiated vascular smooth muscle cell phenotype

With AngII infusion reducing the expression of markers of a differentiated SMC phenotype, we examined the effect of AngII on expression of a de-differentiated phenotype marker. Male mice were infused with either saline, as control, or AngII at a dose of 1000 ng/kg/min, and sacrificed after 7 days. The aortas were dissected and analyzed for mRNA expression of hyaluronic acid synthase 2 (Has2), which has been shown to be expressed by SMCs of a de-differentiated phenotype. Compared to control, AngII infusion significantly increased the gene expression of Has2 in the abdominal aortas of mice (Figure 5.3). This finding suggests that in response to AngII treatment, SMCs of the aorta show increased expression of a gene that is characteristic of a synthetic phenotype.

Figure 5.3: Effect of AngII infusion on mRNA expression of a marker of de-differentiated SMC phenotype. mRNA expression of Has2 was determined in the abdominal aortas of mice infused with saline or AngII for 7 days. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 9; "*" represent *p value < 0.05*; Unpaired t-test.



5.4. Characterization of SMC phenotype in the aorta during AngII-infused AAA development

5.4.1 Aortic SMCs lose differentiated phenotype during AAA development

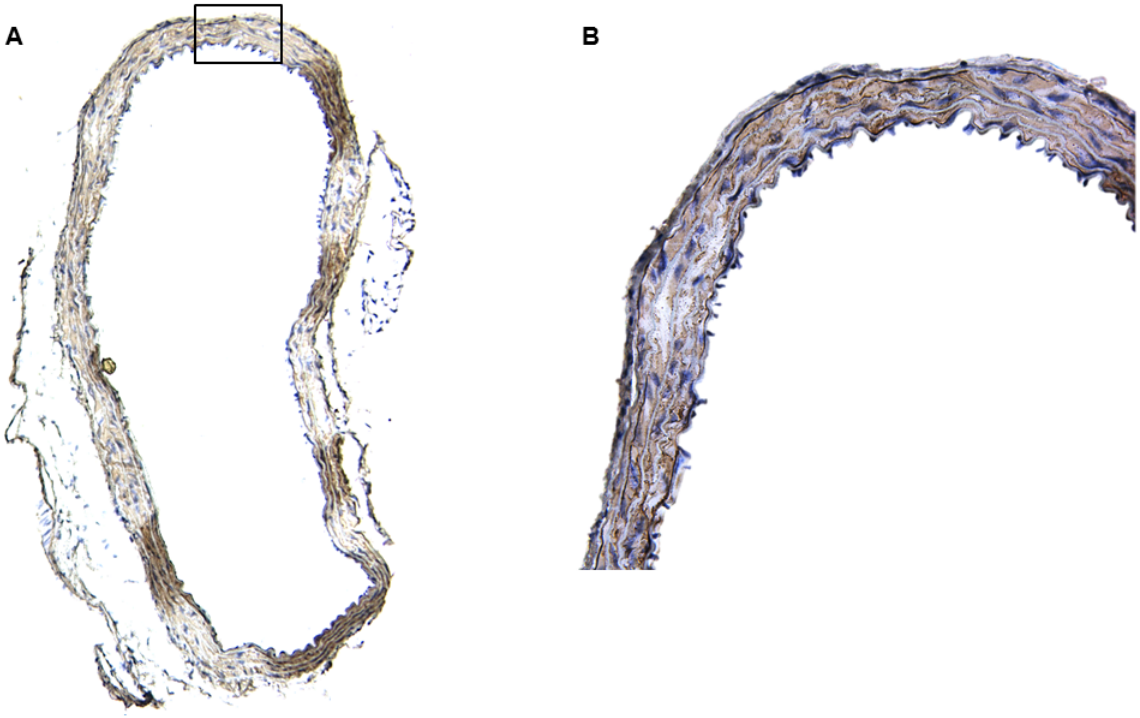
To characterize the phenotype of SMCs during AAA development, mice were infused with either saline or AngII and sacrificed after 7 or 14 days of infusion. The abdominal segments of the aorta were dissected, processed and subsequently sectioned to perform immunohistochemical analysis for protein expression of α-actin. Abundant α-actin expression, indicated by brown staining was observed in the medial SMCs of the aortas of mice treated with saline (Figures 5.4A-B). α-actin protein expression was also detected in the SMCs of the medial layer of the aorta of mice infuse with AngII for 7 days (Figure 5.4C). However, analysis of the aorta at higher magnification revealed an initiation of aneurysmal pathology characterized by aortic dilation in response to AngII infusion for 7 days. Interestingly, this region of the aorta undergoing dilation, exhibited a distinct loss of expression of α-actin (Figure 5.4D). A similar pattern of α-actin expression was observed in the aortas of mice infused with AngII for 14 days. While abundant α-actin expression was observed in the thinner "non-aneurysm" segment of the aorta, (Figure 5.4E), significant α-actin expression was not detected in the region of the aorta developing into an aneurysm. Higher magnification of the non-aneurysmal region of the aorta shows a concentration of brown staining for α-actin in the SMCs comprising the medial layer (Figure 5.4F), which was absent in the cells comprising the growing aneurysm (Figure 5.4G). These findings suggest that during AngII-induced AAA

development in mice, the SMCs of the abdominal aorta undergo a loss of their differentiated phenotype.

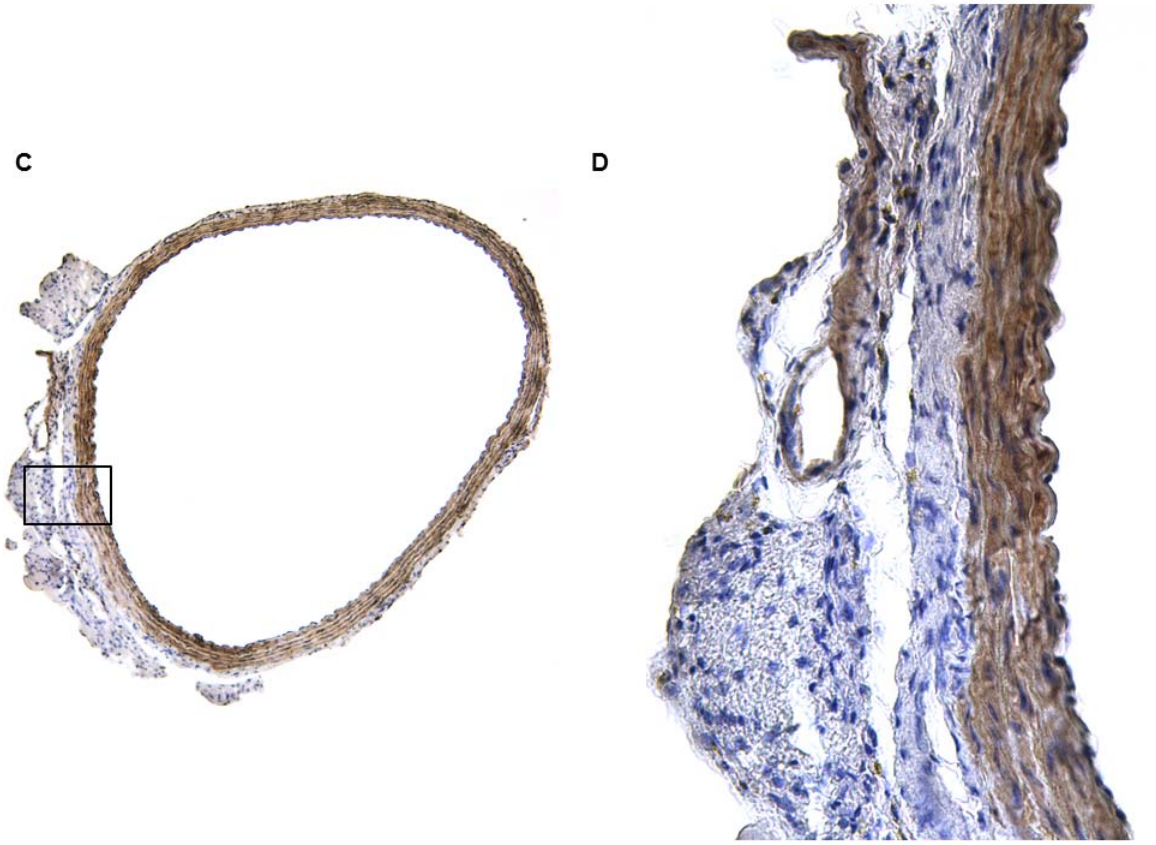
Figure 5.4: α-actin protein expression in the abdominal aorta

Immunohistochemical analysis of α-actin protein in the abdominal aorta of mice infused with saline at **(A)** 100X magnification, and **(B)** 400X magnification. Analysis for α-actin protein expression was also performed in the abdominal aorta of mice infused with AngII for 7 days at **(C)** 100X magnification, and **(D)** 400X magnification, and subsequently in mice infused with AngII for 14 days at **(E)** 100X magnification, and at 400X magnification in **(F)** the "non-aneurysm" region of the aorta and **(G)** the "aneurysm" region of the aorta. Brown staining indicates detection of α-actin protein.

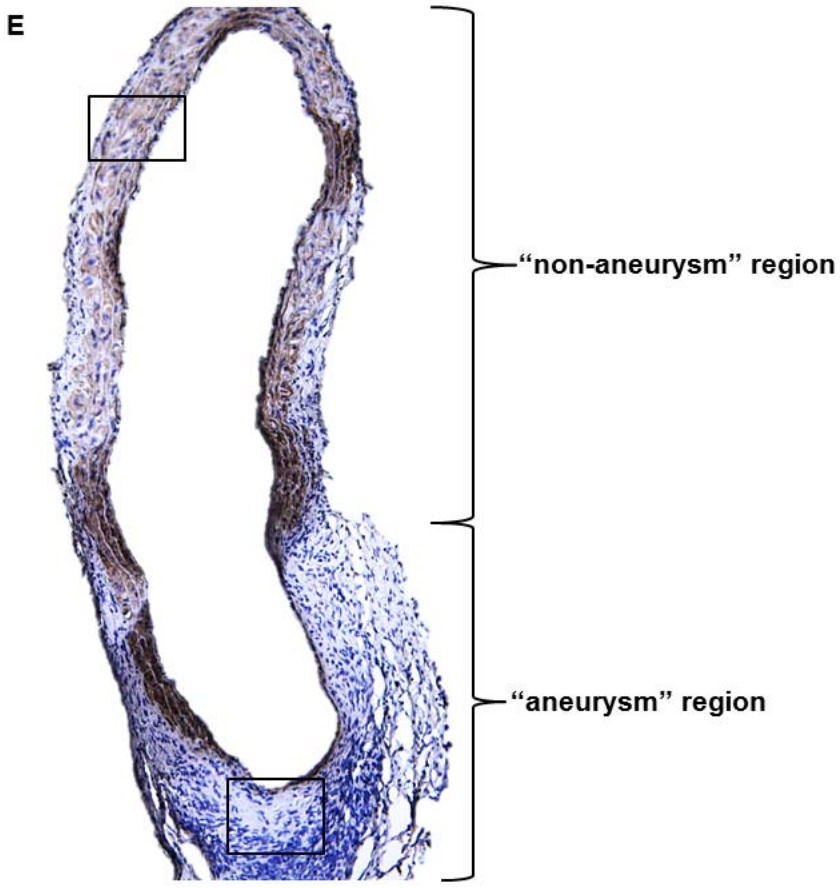
Saline Infusion

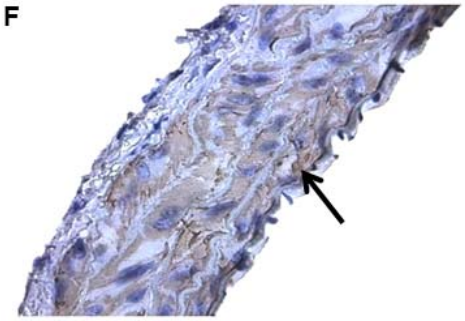


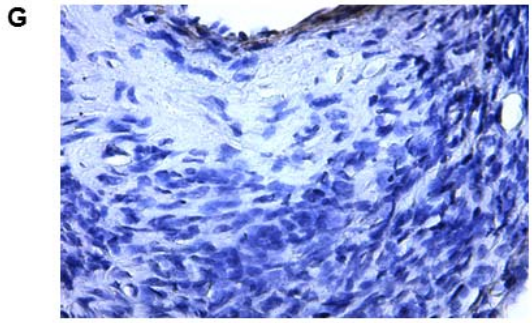
7 days AngII Infusion



14 days AngII Infusion







5.4.2 Increased extracellular matrix production is associated with AngII-induced AAA development

To further characterize aortic SMC phenotype during AngII-induced AAA development, mice were infused with saline or AngII and sacrificed after 7 and 14 days of AngII infusion. The abdominal segments of the aorta were dissected, processed and subsequently sectioned to perform analysis for expression of collagen and hyaluronic acid (HA), which are components of the extracellular matrix.

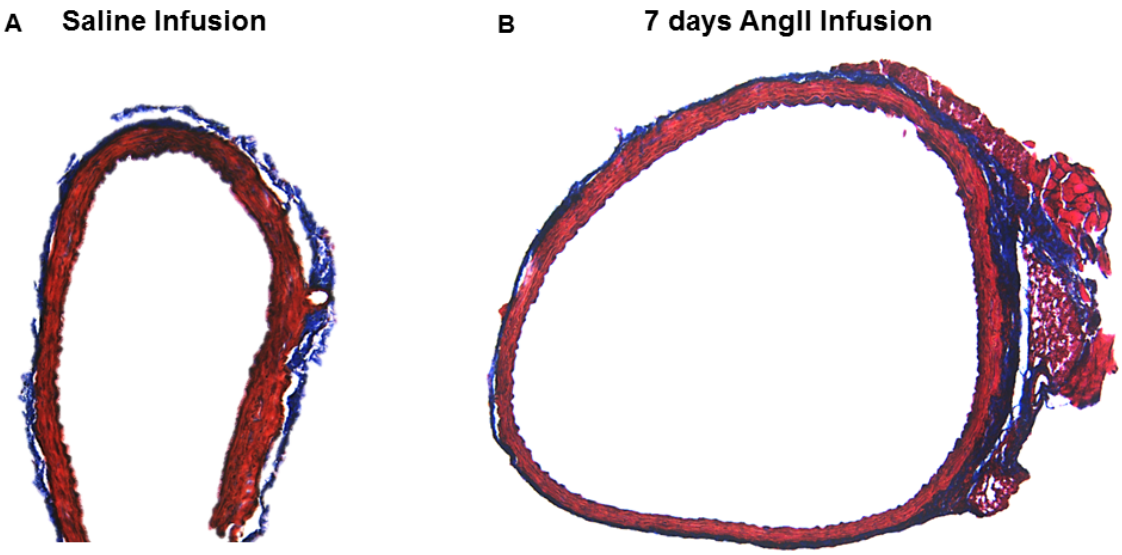
Collagen content in the aortic tissues was evaluated by staining with Masson's trichrome stain. Aortas of saline-infused mice stained red, indicating detection of smooth muscle cells, without any significant expression of collagen (Figure 5.5A). After 7 days of AngII infusion, the aortas continued to stain red in most regions, indicative of SMC expression, with a moderate amount of collagen also expressed in the region of the aorta which was characteristic of early aneurysmal pathology (Figure 5.5B) However, after 14 days of AngII infusion, abundant collagen expression was observed in the "aneurysm" region of the aorta, as indicated by the blue stain (Figure 5.5C). In contrast, cells comprising the medial layer of the "non-aneurysm" region of the aorta stained red, indicating that they are SMCs.

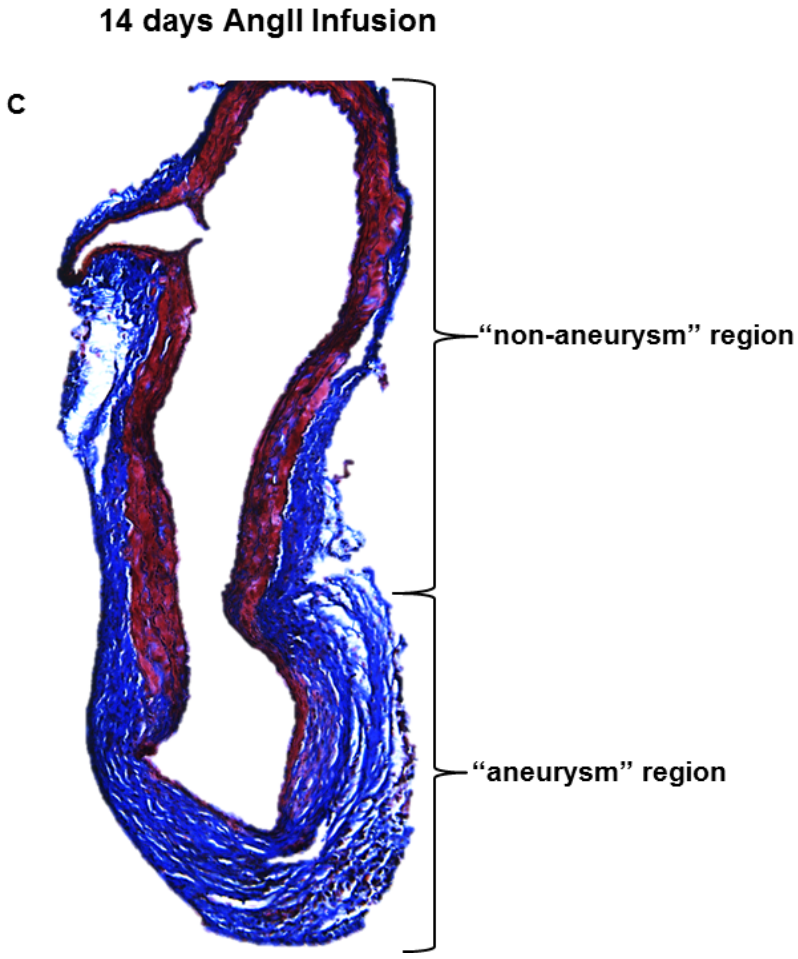
Further analysis of the abdominal aortas with hyaluronic acid binding protein (HABP) resulted in undetectable levels of HA in the saline-infused mice (Figures 5.5D-E). The expression of HA after 7 days of AngII infusion was similar to the pattern exhibited by collagen, with a few cells on the outer edge of the medial layer beginning to show evidences of HA expression (Figures 5.5F-G). After 14 days of AngII infusion, the cells of the growing aneurysm were found to express large amounts of HA, as confirmed

by abundant brown staining (Figure 5.5H). However, HA expression was not observed in the medial layer of the "non-aneurysm" region of the aorta. Higher magnification of the abdominal aorta showed that SMCs on the outer edge of the medial layer adjacent to the aneurysm express HA, as indicated by the arrow in the figure (Figure 5.5I). These results suggest that during AngII-induced AAA development, aortic SMCs undergo phenotypic change to become de-differentiated and synthesize increased amounts of extracellular matrix.

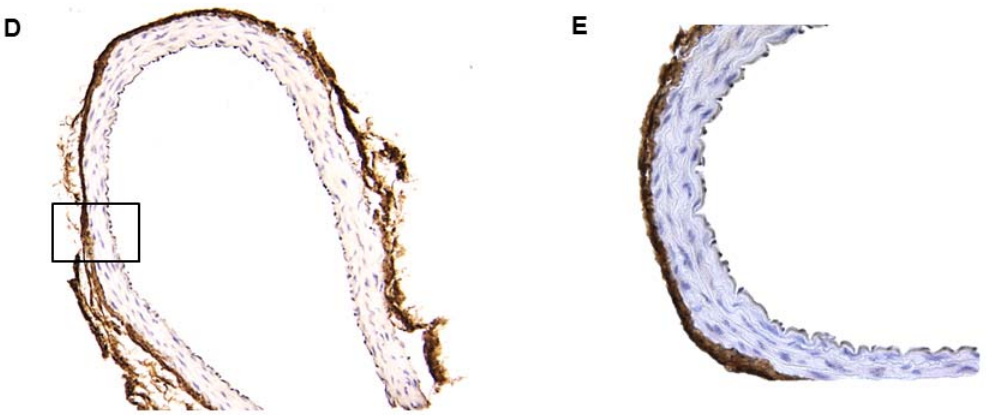
Figure 5.5: Extracellular matrix production in the abdominal aorta

Histological analysis of collagen in the abdominal aorta in mice infused with **(A)** saline, **(B)** 7 days of AngII infusion and **(C)** 14 days of AngII infusion at 100X magnification. Blue staining indicates detection of collagen, and red staining indicates detection of smooth muscle. Detection of HA in the abdominal aortas of saline-infused mice at **(D)** 100X magnification, and **(E)** 400X magnification, in mice infused with AngII for 7 days at **(F)** 100X magnification, and **(G)** 400X magnification, and in mice infused with AngII for 14 days at **(H)** 100X magnification, and **(I)** 400X magnification Brown staining indicates detection of HA due to binding with HA-binding protein.

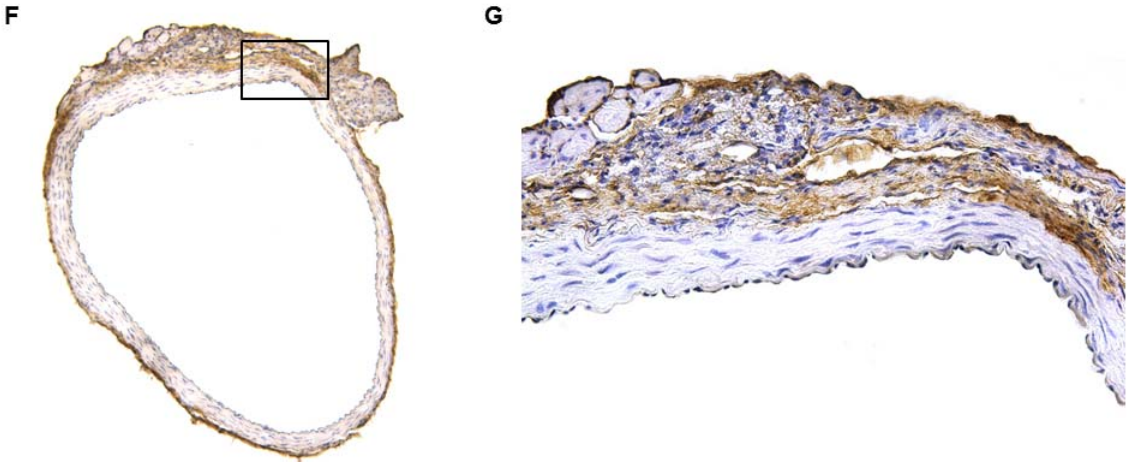



14 days AngII Infusion
C
"non-aneurysm" region
"aneurysm" region

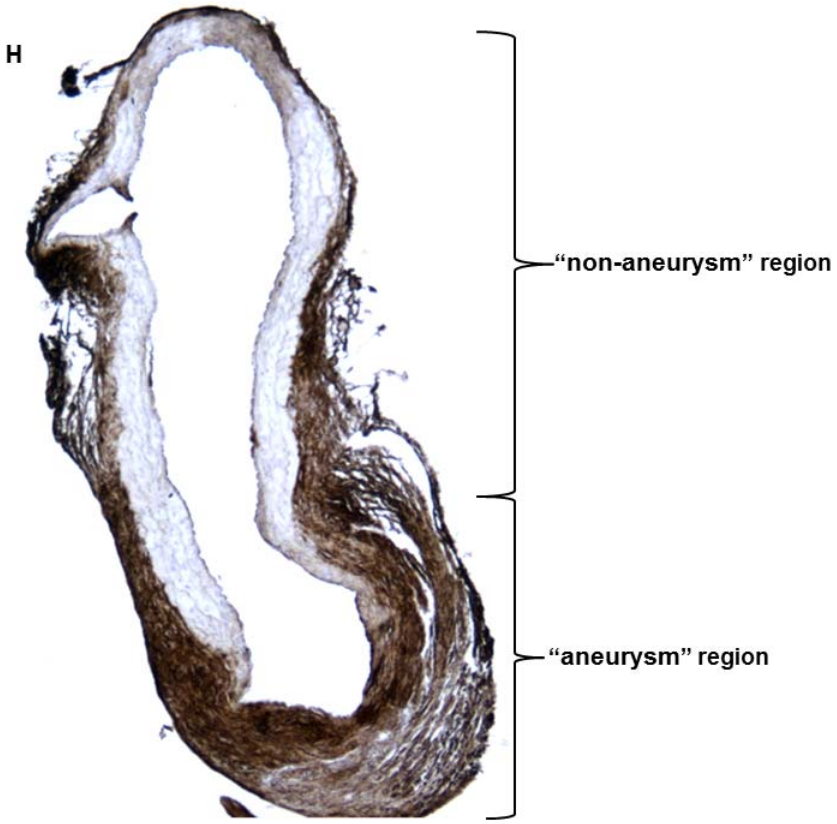
Saline infusion

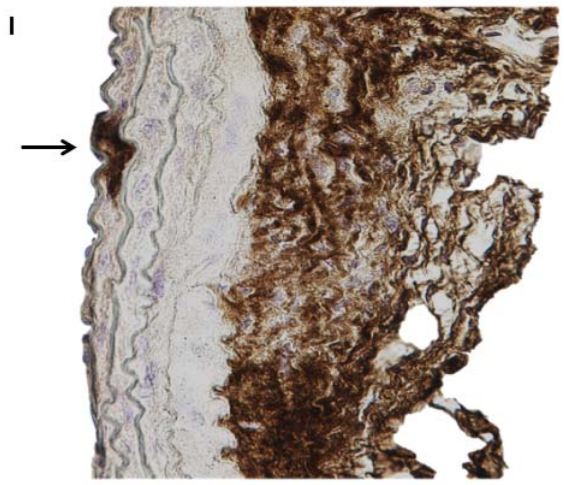


7 days AngII infusion



14 days AngII infusion





Discussion

Vascular SMCs are highly specialized cells which exhibit considerable phenotypic plasticity. Alteration of SMCs from a contractile to a synthetic phenotype is known to play a major role in the development of several vascular diseases such as atherosclerosis, restenosis and hypertension. In the current studies, we show for the first time that, SMCs of the aorta show evidence of characteristics consistent with an altered phenotype during AngII-induced AAA development in mice.

AngII is a potent mitogen and several studies have established AngII as an effective inducer of vascular SMC proliferation in cell culture.[77-79] However, only a few studies have investigated the role of AngII infusion in regulating SMC proliferation in vivo. Schwartz et al. were the first to show that AngII infusion in rats induces proliferation of the SMCs, as measured by [^{3}] thymidine incorporation. The effect of AngII, however, was more pronounced in the neointimal SMCs of the injured artery, than in the underlying media or the SMCs of the normal arterial wall.[193] Similar results were obtained by the same group when AngII infusion was found to increase SMC proliferation of carotid and mesenteric arteries of rats, as determined by BrdU incorporation.[194] More recently, Schiffrin et al have also reported the induction of DNA synthesis by AngII in rat blood vessels.[195] Thus, although proliferation is well-established in cultured SMCs, the role of AngII in inducing proliferation in the SMCs of the aorta, especially in mice, have not been well-defined.

The current study investigated the role of AngII in inducing proliferation of aortic SMCs during AngII-induced AAA development in mice. Mice were infused with saline

or AngII for 7 or 14 days together with an i.p. injection of BrdU every 24 hours. Proliferation of aortic SMCs was then determined by immunohistochemical analysis of BrdU incorporated into the S phase of the replicating DNA. AngII infusion, for both 7 and 14 days, was not found to induce detectable level of BrdU incorporation in the aortic SMCs of the mice. However, BrdU incorporation was sufficiently detected in the positive control skin tissue. Thus, this study shows for the first time that SMC proliferation of the aorta may not be a significant mechanism contributing to AngII-induced AAA development in mice.

Although previous studies have reported that AngII infusion induces SMC proliferation, our findings suggest that SMCs of the aortas of mice may not show significant induction of proliferation in response to AngII treatment.[193-195] In the current study, proliferation of SMCs was analyzed in the abdominal region of the aorta, whereas a majority of the previous studies involved either a thoracic aorta or carotid artery after induction of vascular injury. This suggests that the effect of AngII induced proliferation of SMCs in animals could be more pronounced only under conditions of vascular injury. Moreover, mice have been reported to exhibit diminished responses to AngII infusion compared to rats, which could explain the differences observed with our AngII infusion results in mice.[196] Thus, in contrast to other vascular diseases such as atherosclerosis, restenosis and hypertension, SMC proliferation does not appear to play a significant role during AngII-induced AAA formation in mice.

Limited evidence is available on the role of AngII during phenotypic conversion of SMCs. Attenuating the effect of AngII by selective antagonism of the AT1 receptor has been shown to restore expression of the contractile-type myosin heavy chain isoform

SM2 as well as the expression of calponin in the spontaneously hypertensive rat aorta[192, 197]. The AT1 receptor antagonist has also been found to down-regulate the non-muscle myosin heavy chain isoform, a marker of a de-differentiated SMC phenotype, in these rat aortas[191]. Recently, AngII has been reported to play an important role in the development of thoracic aortic aneurysms (TAAs) in humans, which are linked to a mutation in myosin heavy chain 11, a marker of a contractile SMC phenotype.[186] Thus, with findings that AngII contributes to thoracic aneurysm formation which is a disease where altered SMC differentiation is a causative factor, in the current studies, we investigated the effect of AngII infusion in modulating SMC phenotype in the abdominal aorta of mice.

Mice were infused with either saline, as control, or AngII for 7 days and phenotypic change of the SMCs of the abdominal region of the aorta was examined by analyzing the changes in gene expression of markers characterizing both the contractile and synthetic phenotype. AngII infusion was found to significantly decrease the mRNA expression of α-actin, desmin and SM22α, which are well-known markers of the differentiated SMC phenotype, while increasing the mRNA levels of Has2, which is expressed by a de-differentiated SMC phenotype. These results indicate for the first time that 1 week of AngII infusion may affect abdominal aortic SMCs of mice to change from a contractile to a synthetic phenotype.

A variety of genetic studies were carried out in humans to reveal that SMC contractile dysfunction can lead to thoracic aortic aneurysms (TAAs).[181] Several groups have shown that patients with mutations in the transforming growth factor-β receptor-1 and -2 have an altered SMC phenotype and are predisposed to aneurysms of

the thoracic aorta.[198-200] Also, mutations in genes expressed specifically by differentiated SMCs, including smooth muscle α-actin, myosin light chain kinase, SMC-specific myosin heavy chain and Fibulin-4 have all been associated with increased susceptibility to TAAs.[182-186] In addition, phenotypic alteration of vascular SMCs has previously been reported to precede elastolysis in a mouse model of TAAs.[187] Owens et al have also shown that SMCs of the aorta have reduced expression of contractile proteins following the development of aneurysms induced by elastase perfusion in mice. This study also showed minimal detection of contractile proteins by immunohistochemistry in human AAAs.[188] Vascular SMCs have also been found to undergo remodeling from a contractile to a synthetic phenotype during progression of elastase-induced aneurysms in the right internal carotid artery of rabbits.[189] More recently, deficiency of integrin-linked kinase, a protein associated with the contractile phenotype of SMCs, has been reported to cause TAAs in mice.[190] Therefore, although a number of reports indicate a role for an altered SMC phenotype in contributing to the formation of aneurysms that form at locations other than the abdominal aorta, previous reports have not shown a role for altered SMC differentiation in contributing to the development of AAAs.

With our finding that 7 days of AngII infusion in mice is associated with an alteration of aortic SMCs to a de-differentiated phenotype, in the current studies, we examined whether SMCs of the abdominal aorta could potentially change their phenotype during AngII-induced AAA development in mice. Mice were infused with saline or AngII for 7 or 14 days, and subsequently analyzed for immunohistochemical expression of makers of the contractile and synthetic phenotypes in the SMCs of the abdominal

aorta. AngII infusion was found to cause significant aneurysmal pathology with a loss of the differentiated phenotype as manifested by decreased expression of α-actin in the "aneurysmal" region of the aorta, when compared to the "non-aneurysm" region where no pathology was observed. Since collagen and HA are extracellular matrix components typically synthesized by de-differentiated SMCs, and AngII is known to stimulate collagen synthesis in human vascular SMCs, we also analyzed the deposition of collagen and HA in these abdominal aortas.[201] The AAAs were specifically found to produce abundant collagen and hyaluronic acid, indicating that, similar to other vascular diseases, the tissue composing the AAAs produces significant levels of extracellular matrix components known to be produced by SMCs expressing a synthetic phenotype.

De-differentiated vascular SMCs are usually thought to have an increased rate of proliferation together with synthesis of ECM components, and a decrease in the expression of smooth muscle specific contractile proteins. However, several reports have suggested that differentiation and proliferation of SMCs are not mutually exclusive, and that factors regulating one process may not affect the other.[202] For example, vascular SMCs during embryogenesis and postnatal development exhibit high rates of proliferation as well as expression of differentiation proteins.[203, 204] Alternatively, some agents which inhibit vascular SMC proliferation do not affect their differentiation.[205] In the current studies, we found that, although SMCs of the abdominal aorta show evidence of an altered phenotype in response to AngII treatment, proliferation of these cells is largely undetected during AAA development in mice. This is similar to atherosclerosis where vascular SMCs in the advanced lesions are phenotypically modulated, but demonstrate very low levels of proliferation.[206, 207]

This suggests that, although considered to constitute the same synthetic phenotype, vascular SMCs under certain conditions may not proliferate, but retain their de-differentiation properties.

Our current findings suggest that vascular SMCs of the abdominal aorta show evidence of reduced differentiation characteristics and revert to a more de-differentiated phenotype during AngII-induced AAA formation in mice.

CHAPTER 6

COX-2 REGULATES VASCULAR SMOOTH MUSCLE CELL PHENOTYPE DURING AAA DEVELOPMENT IN MICE

Introduction

COX-2 is known to play an important role during AAA formation in mice. Using an AngII-infusion model in mice, we have previously reported that genetic deficiency of COX-2 significantly reduces AAA formation.[56] Furthermore, we have shown that pre-treatment with the COX-2-selective inhibitor celecoxib prior to disease initiation reduces AAA formation in mice.[55] As described previously in Chapter 3, we have also recently found that celecoxib treatment, even when begun after disease initiation, can effectively attenuate the progression of AAAs in mice. However, the mechanism by which COX-2 can regulate the development of AngII-induced AAAs in mice has not been described.

Prostaglandins (PGs) have been suggested to play a key role in regulating vascular SMC phenotype. Similar to their diverse pathophysiological role in health and disease, PGs have been reported to exhibit differential regulation of SMC phenotypic plasticity. Prostaglandin I_2 (prostacyclin) has been shown to promote a differentiated phenotype of human vascular SMCs in culture.[208] In contrast, PGE_2 production has been suggested to enhance the de-differentiation of rat aortic SMCs.[209] In addition, both prostacyclin analogue iloprost, as well as PGE_2 have been shown to increase synthesis of hyaluronic acid (HA), the extracellular matrix component typically

expressed by SMCs of synthetic phenotype, by inducing expression of HA synthases Has1 and Has2 in SMCs.[210-212] More recently, the non-selective COX inhibitor indomethacin, and the COX-2-selective inhibitor rofecoxib have been shown to reduce vascular HA accumulation by inhibiting Has1 and Has2 mRNA expression in mouse models of atherosclerosis and neointimal hyperplasia.[213] Thus, although the role of COX-2 in regulating SMC phenotype is not well defined, the above studies suggest that PGs could be important factors in the regulation of phenotypic modulation of SMCs.

Although there is limited data on the role of SMC phenotype on AAAs, other types of aneurysms have been associated with an altered phenotype of vascular SMCs. In addition to genetic mutations in the contractile proteins predisposing to thoracic aortic aneurysms and dissections, several animal models of thoracic aneurysms have suggested that change in SMC phenotype is an important factor contributing to the development of the disease[181-185, 187-190, 214]. Moreover, our data in Chapter 4 show that, in a mouse model of AAAs, AngII infusion can induce vascular SMCs to change from contractile to a synthetic phenotype during AAA development in mice.

Based on the role of PGs in regulating the phenotype of cultured SMCs, and that SMC phenotypic modulation is an important phenomenon contributing to thoracic aneurysm formation, we hypothesized that the effectiveness of COX-2 inhibition in reducing AngII-induced AAA development in mice is associated with maintaining a differentiated phenotype of aortic SMCs. In the current studies, we utilized COX-2-selective inhibitor celecoxib, to investigate the role of COX-2 in regulating SMC phenotype.

Results

6.1. COX-2 inhibition maintains aortic SMCs in a differentiated phenotype during AngII-induced AAA development in mice

6.1.1. COX-2 inhibition increases mRNA expression of markers of differentiated SMC phenotype

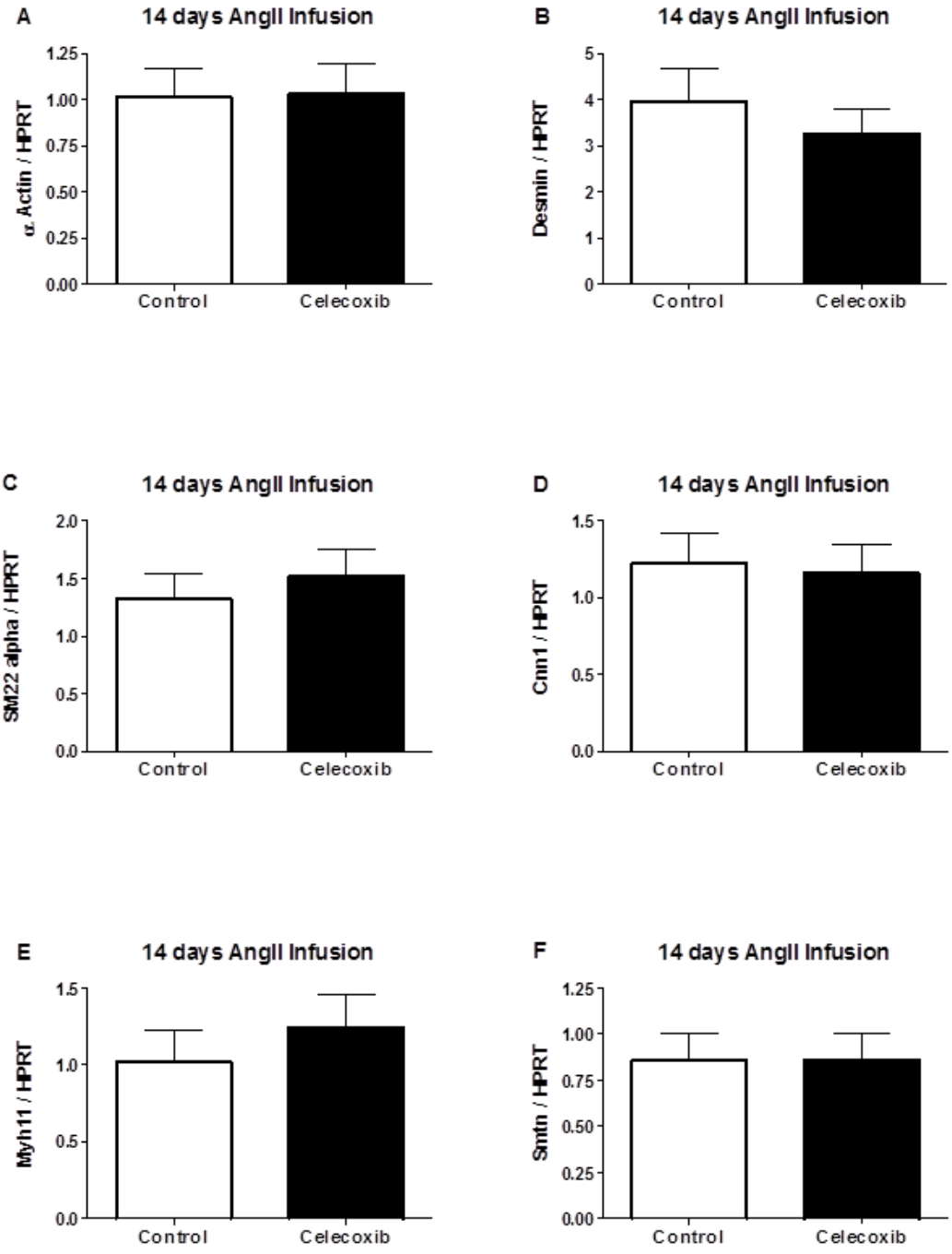
To elucidate the role of COX-2 in regulating aortic SMC phenotype during AngII-induced AAA formation, male non-hyperlipidemic mice were infused with AngII at a dose of 1000 ng/kg/min. Five days after AngII infusion, the mice were divided into two groups; one group received a control diet (normal chow diet), and the other group received a diet containing the COX-2-selective inhibitor celecoxib (normal chow diet repelleted with 1000 ppm celecoxib). The mice were subsequently sacrificed and abdominal aortas were collected at different time-points of AngII infusion for examining the effect of celecoxib at the early, intermediate and late stages of AAA development. The aortas were analyzed for mRNA expression of different markers of differentiated and de-differentiated SMCs by real-time PCR. α-actin, desmin, smooth muscle 22α (SM22α), calponin (Cnn1), myosin heavy chain 11 (Myh11) and smoothelin (Smtn) were used as contractile markers, while hyaluronic acid synthase 2 (Has2) was utilized as a marker of a de-differentiated SMC.

Analysis of abdominal aortas after 14 days of AngII infusion was utilized to allow for examining the effects of COX-2 inhibition on SMC phenotypic change during the early stage of AAA development. We found that celecoxib treatment from day 5 to day

14 of the AngII infusion did not significantly alter the mRNA expression of any of the differentiated SMC markers, α-actin, desmin, SM22α, Cnn1, Myh11 and Smtn, as compared to the mice on control diet (Figures 6.1A-F). Thus, COX-2 inhibition with celecoxib had no detectable change on the expression of the SMC differentiation genes at this early time-point of AngII-induced AAA development in mice.

Figure 6.1: Effect of celecoxib on gene expression of markers of differentiated SMC phenotype during early AAA development. mRNA expression of **(A)** α-actin, **(B)** desmin, **(C)** SM22α, **(D)** calponin, **(E)** Myh11 and **(F)** smoothelin was determined in the abdominal aortas of mice infused with AngII for 14 days, and treated with control or celecoxib diet from day 5 to day 14 of AngII infusion. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; $n \geq 8$; unpaired t-test.

14 days AngII Infusion

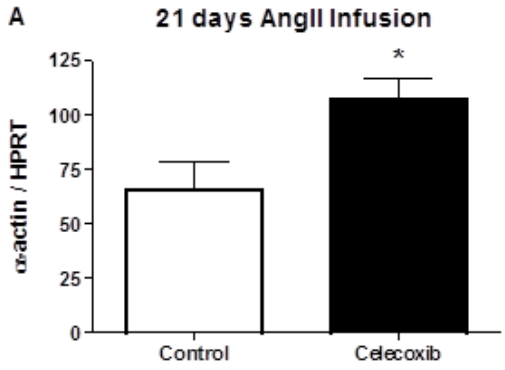


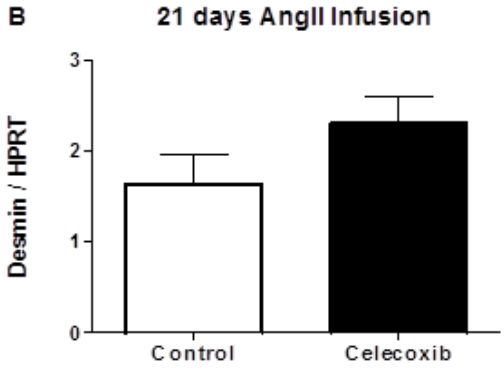
With our early time-point analysis showing no significant difference in the contractile markers, we analyzed subsequent time-points of AngII infusion to elucidate the role of COX-2 on SMC differentiation during the intermediate and late stages of AAA development. The mice were thereby sacrificed after 21 and 28 days of AngII infusion and the abdominal aortas of the control or celecoxib-treated mice were analyzed for gene expression of the SMC differentiation markers. Celecoxib treatment from day 5 to day 21 of AngII infusion was found to significantly increase the mRNA expression of α-actin, SM22α, Cnn1, Myh11 and Smtn, as compared to control treatment (Figures 6.2A, 6.2C-F). Although there was a trend towards an increased expression of desmin, one of the earliest contractile markers, in the celecoxib treated mice than the control group, this difference was not statistically significant (Figure 6.2B). Thus, COX-2 inhibition with celecoxib was found to significantly decrease the markers of SMC differentiation during the intermediate phase of AAA formation in mice.

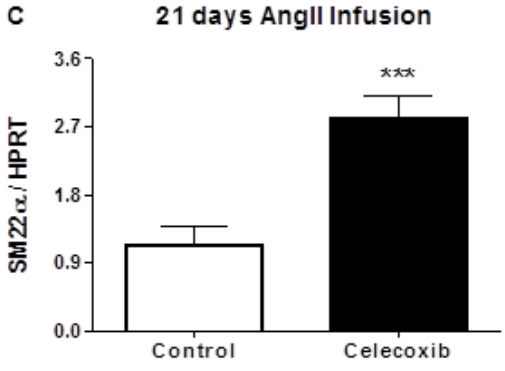
Similar results were obtained even during the late phase of AAA development, when abdominal aortas of mice on celecoxib diet from day 5 to day 28 of AngII infusion displayed a significant increase in mRNA expression of all the SMC differentiation markers, α-actin, desmin, SM22α, Cnn1, Myh11 and Smtn compared to the control mice (Figures 6.3A-F). Thus, the above findings indicate that increased differentiation of aortic SMCs during the intermediate and late time-points of AngII infusion is associated with the effectiveness of celecoxib in reducing AngII-induced AAA progression in mice.

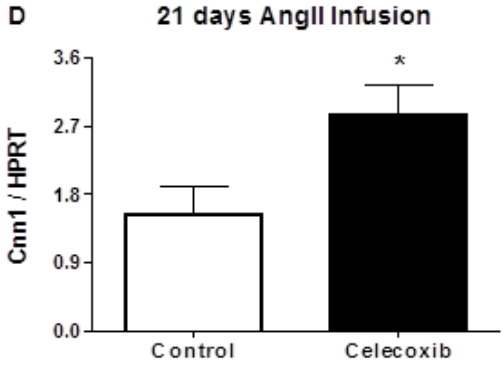
Figure 6.2: Effect of celecoxib on gene expression of markers of differentiated SMC phenotype during the intermediate phase of AAA development. mRNA expression of **(A)** α-actin, **(B)** desmin, **(C)** SM22α, **(D)** calponin, **(E)** Myh11 and **(F)** smoothelin was determined in the abdominal aortas of mice infused with AngII for 21 days, and treated with control or celecoxib diet from day 5 to day 21 of AngII infusion. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 8; "*" represent *p value* < *0.05*; "***" represent *p value* < *0.001*; unpaired t-test.

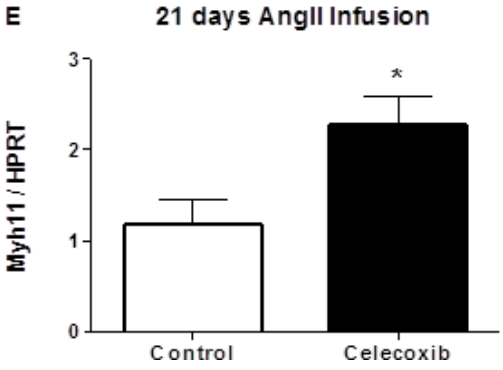
21 days AngII Infusion











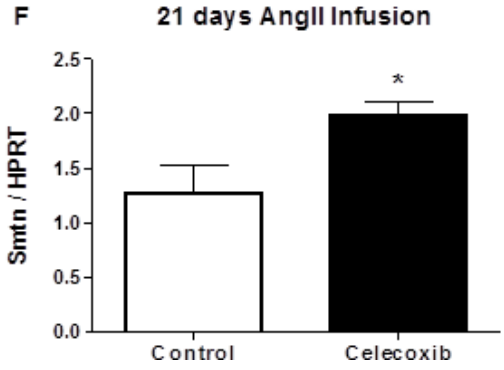
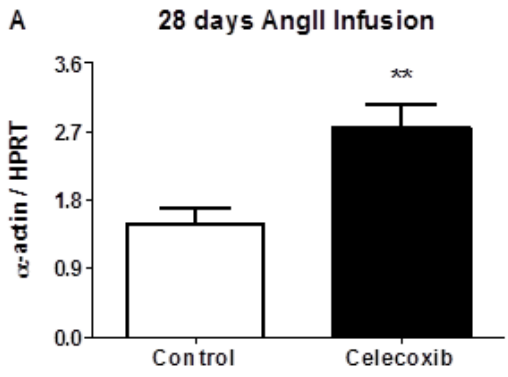
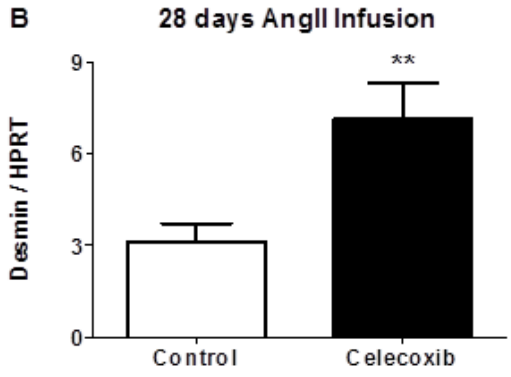
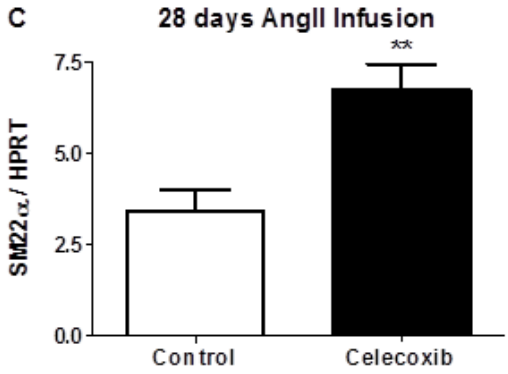


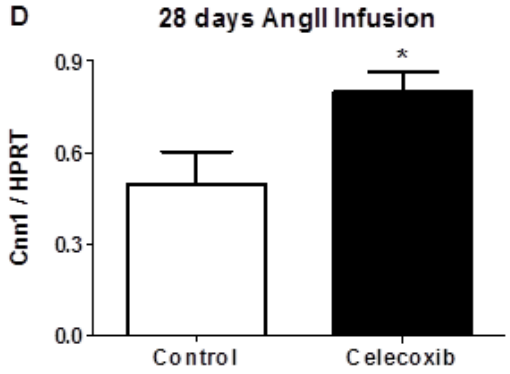
Figure 6.3: Effect of celecoxib on gene expression of markers of differentiated SMC phenotype during late phase of AAA development. mRNA expression of **(A)** α-actin, **(B)** desmin, **(C)** SM22α, **(D)** calponin, **(E)** Myh11 and **(F)** smoothelin was determined in the abdominal aortas of mice infused with AngII for 28 days, and treated with control or celecoxib diet from day 5 to day 28 of AngII infusion. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 8; "*" represent *p value < 0.05*; "**" represent *p value < 0.01*; unpaired t-test.

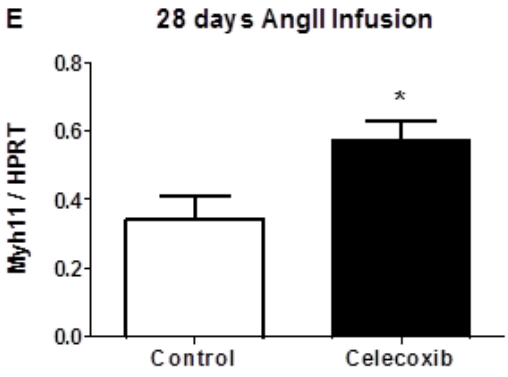
28 days AngII Infusion

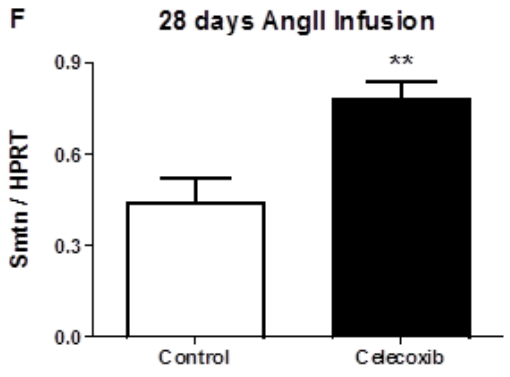








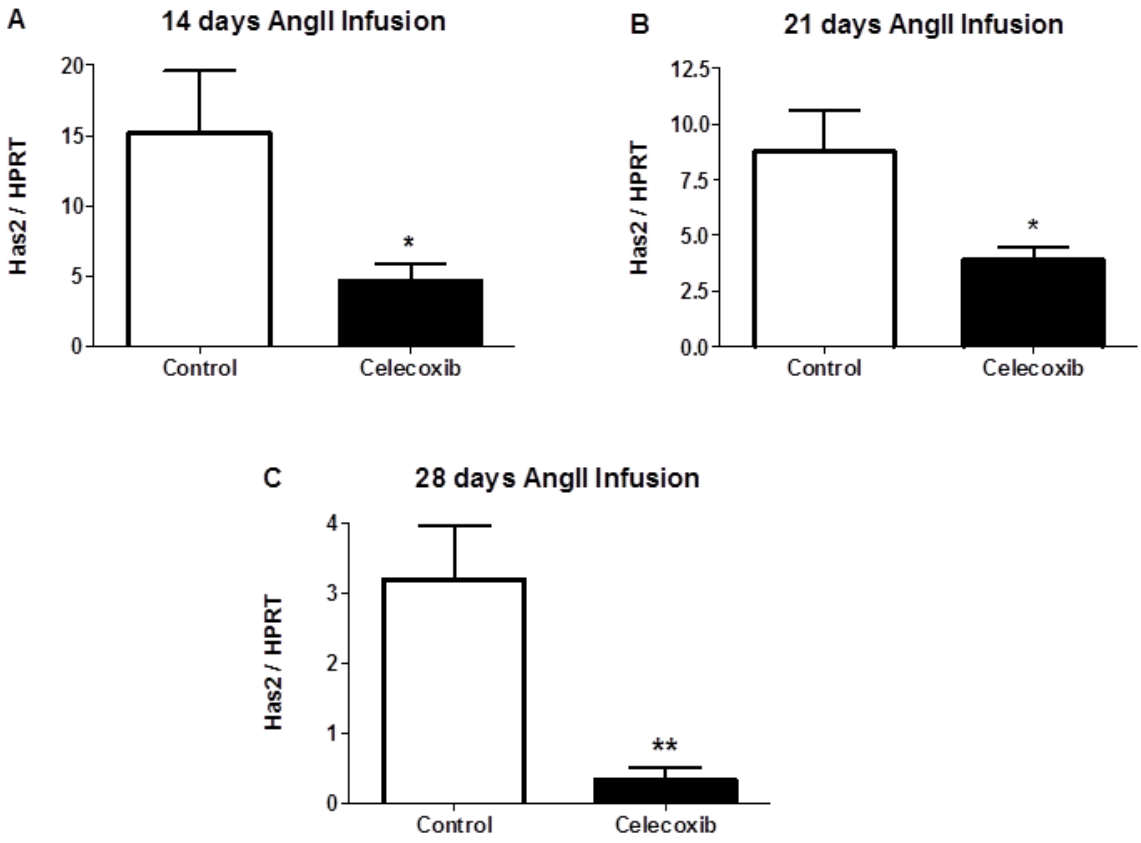




6.1.2. COX-2 inhibition decreases mRNA expression of a marker of de-differentiated SMC phenotype throughout AngII infusion

To further examine the effect of COX-2 inhibition on SMC phenotypic change during AngII-induced AAA development, mice were infused with AngII for 14, 21 or 28 days, and treated with either a control diet, or diet containing celecoxib, as described above. The abdominal aortas of these mice were then analyzed for gene expression changes of Has2, a marker of a synthetic SMC phenotype. Celecoxib treatment from day 5 to day 14 of AngII infusion was found to significantly decrease the mRNA expression of Has2 as compared to the mice on control diet (Figure 6.4A). Thus, COX-2 inhibition was found to be effective in reducing a marker of a de-differentiated SMC phenotype even at an early stage of AAA development. Similar results were obtained at the intermediate time-point of AngII infusion, when, as compared to mice on control diet, celecoxib treatment from day 5 to day 21 of AngII infusion significantly reduced the mRNA expression of Has2 (Figure 6.4B). Finally, celecoxib was found to maintain its effectiveness till the latest stage of disease development, when Has2 mRNA levels in the celecoxib-treated group was significantly lower than the group on control diet after 28 days of AngII infusion (Figure 6.4C). These results suggest that COX-2 inhibition with celecoxib effectively decreases the gene expression of a marker of de-differentiated SMC phenotype throughout the early, intermediate and late stages of AngII-induced AAA development in mice.

Figure 6.4: Effect of celecoxib on gene expression of a marker of de-differentiated SMC phenotype. mRNA expression of Has2 was determined in the abdominal aortas of mice infused with AngII for (A) 14 days, (B) 21 days and (C) 28 days, and treated with control or celecoxib diet beginning 5 days after AngII infusion. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 8; "*" represent *p value < 0.05*; "**" represent *p value < 0.01*; unpaired t-test.

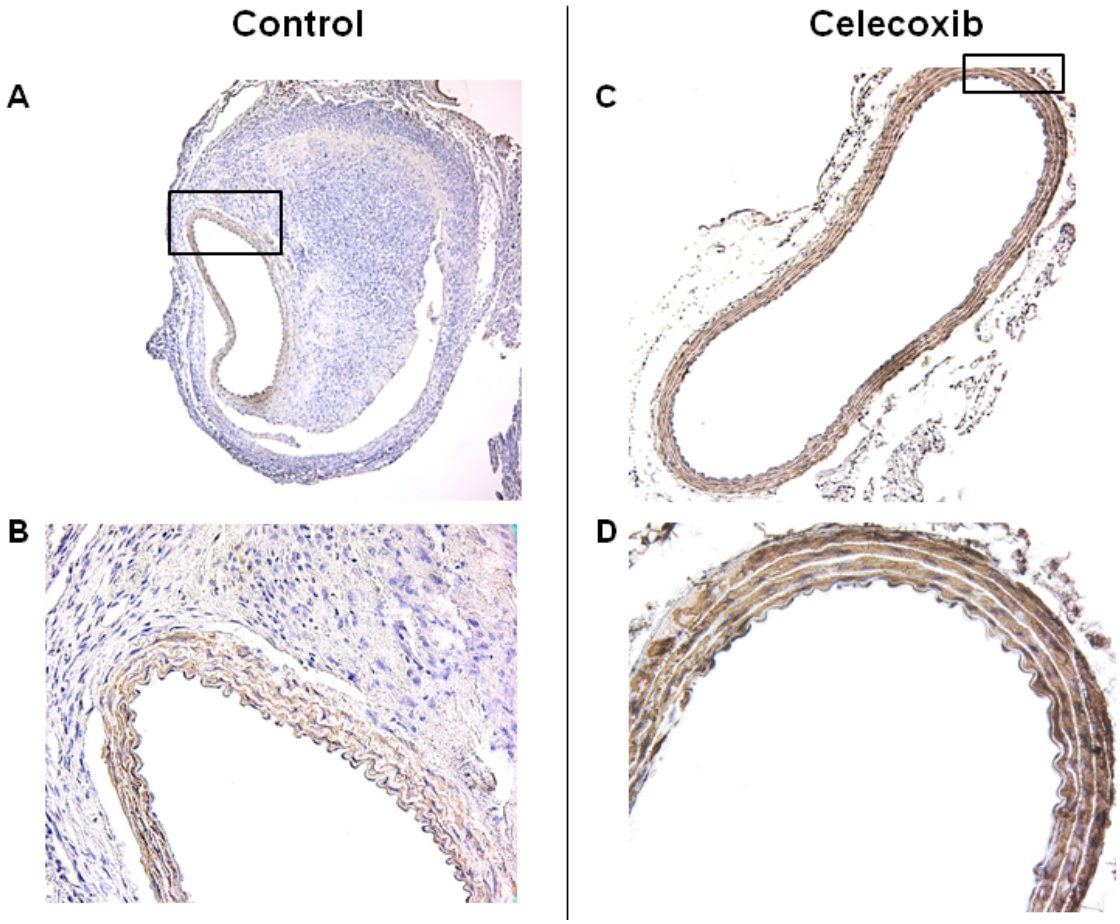


6.1.3. COX-2 inhibition increases protein expression of a marker of differentiated SMC phenotype

To evaluate the changes in protein expression of markers of SMC phenotype during AngII-induced AAA formation, mice were infused with AngII, as described above. 5 days after the AngII infusion, the mice were divided into two groups receiving control diet, or diet containing celecoxib. The mice were then sacrificed after 28 days of AngII infusion, their aortas collected and analyzed for immunohistochemical expression of α-actin, the specific marker for a differentiated SMC. Figure 6.5A shows a large aneurysm of the abdominal aorta of the control group. The aneurysmal region was characterized by a loss of the elastic lamina and α-actin protein expression was undetectable within the cells of this lesion indicating that they are not differentiated SMCs. However, the cells in the medial layer were found to express α-actin, as evident by positive brown staining (Figures 6.5A-B). In contrast to the control group, abundant α-actin expression was observed throughout the abdominal segment of the aorta of mice on the celecoxib diet (Figures 6.5C-D). These findings indicate that following AngII treatment, α-actin protein expression is readily detected in the SMCs of celecoxib-treated mice aorta but is not observed in cells of the aneurysmal lesion in mice treated with a control diet, suggesting that COX-2 inhibition can maintain SMCs in a more differentiated phenotype during AngII-induced AAA development.

Figure 6.5: α-actin protein expression in the abdominal aorta

Immunohistochemical analysis of α-actin protein in the abdominal aorta of mice treated with control diet with **(A)** 100X magnification, and **(B)** 300X magnification, and in the abdominal aorta of mice treated with a celecoxib diet with **(C)** 100X magnification, and **(D)** 300X magnification. Brown staining indicates detection of α-actin protein.



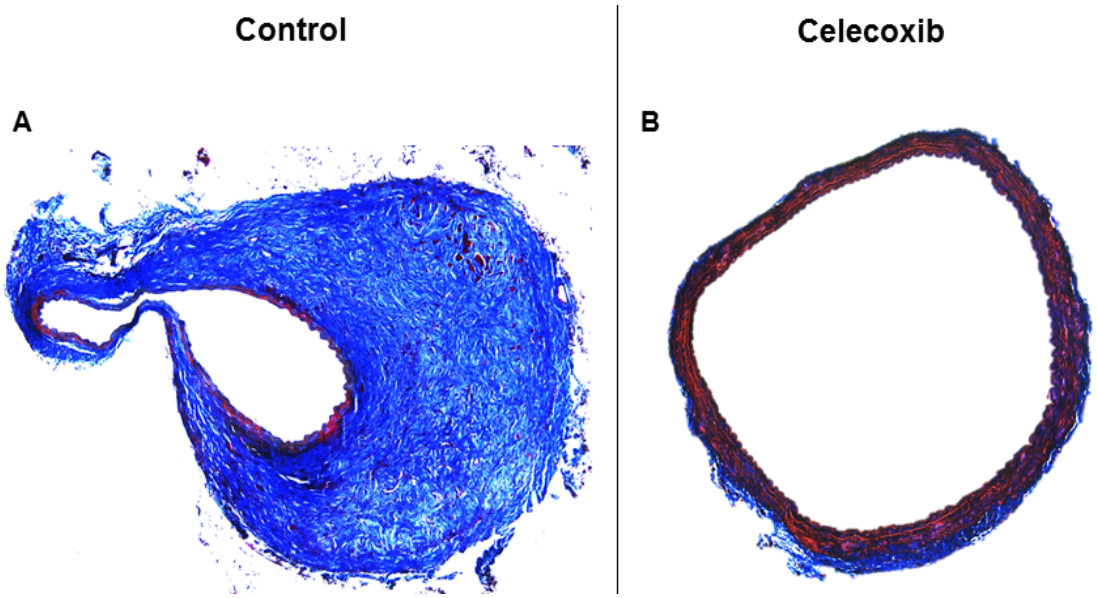
6.1.4. COX-2 inhibition decreases protein expression of markers of de-differentiated SMC phenotype

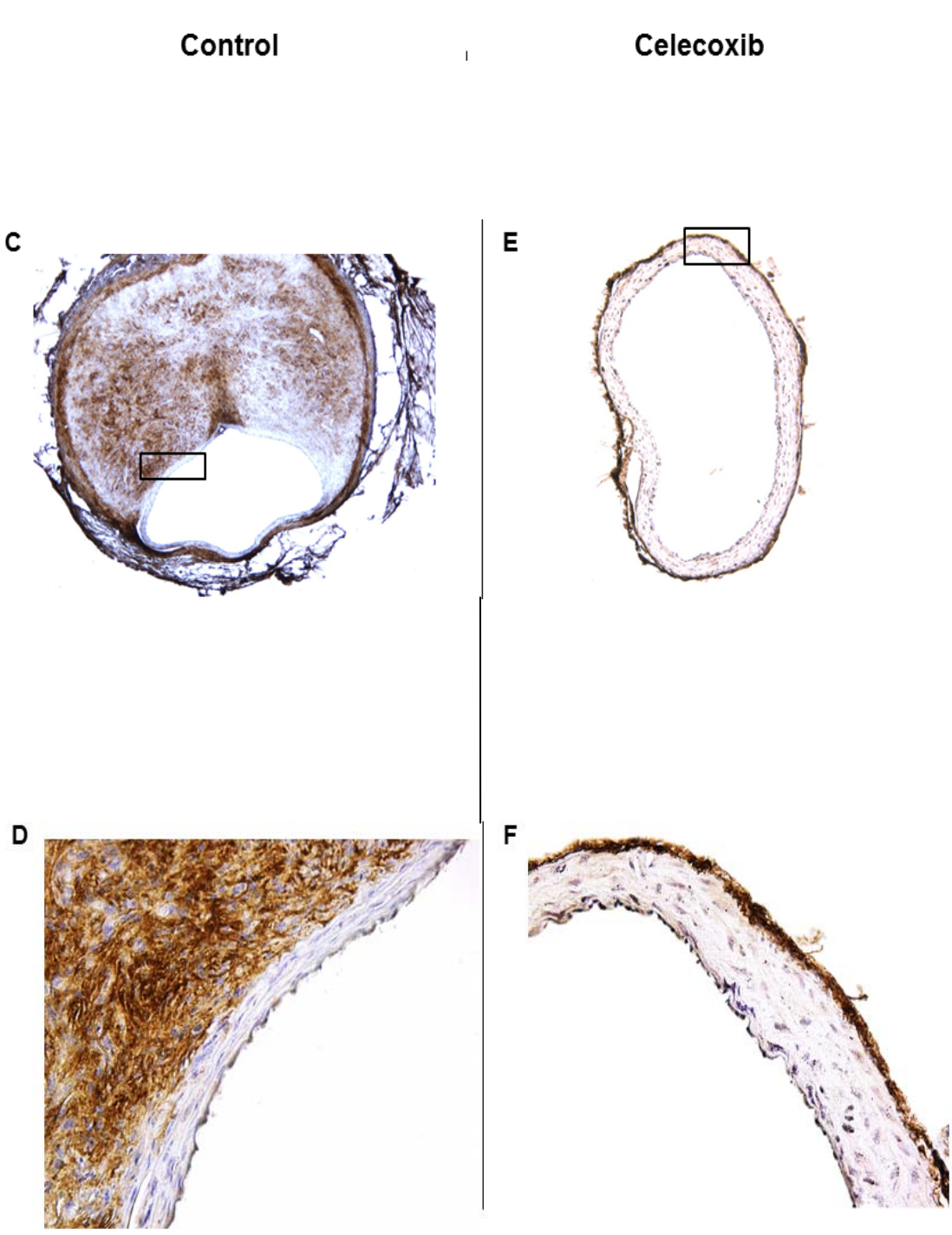
Mice infused with AngII were treated with control or celecoxib diet beginning 5 days after AngII infusion. 28 days after AngII infusion, the mice were sacrificed, and the abdominal aortas were collected to analyze for histological expression of extracellular matrix components collagen and hyaluronic acid (HA), as markers expressed by SMCs of synthetic phenotype. Abundant collagen expression was observed in the aneurysmal lesion in mice treated with control diet, as indicated by the blue stain (Figure 6.6A). However, except the thin layer of adventitia, collagen expression was largely undetected in the aortas of mice treated with celecoxib, with the cells staining red, indicating the presence of a SMC layer (Figure 6.6B). Analysis of the aortas with a HA binding protein revealed that significant levels of HA are synthesized by the cells within the aneurysmal lesion of mice treated with a control diet (Figures 6.6C-D). In contrast, a lower level of HA was detected in the aortas of mice treated with celecoxib (Figures 6.6E-F). Thus, these results suggest that COX-2 inhibition with celecoxib prevents SMCs of the aorta to change to a synthetic phenotype, as determined by reduced production of collagen and HA, during AngII-induced AAA development in mice.

Figure 6.6: Extracellular matrix production in the abdominal aorta

6.6A-B: Histological analysis of collagen in the abdominal aorta in **(A)** control-treated, and **(B)** celecoxib-treated mice after 28 days of AngII infusion, at 100X magnification. Blue staining indicates detection of collagen, and red staining indicates detection of smooth muscle.

6.6C-F: Immunohistochemical analysis of HA in the abdominal aorta of mice treated with control diet at **(C)** 100X magnification, and **(D)** 400X magnification, and in the abdominal aorta of mice treated with a celecoxib diet at **(E)** 100X magnification, and **(F)** 400X magnification. Brown staining indicates detection of HA protein.

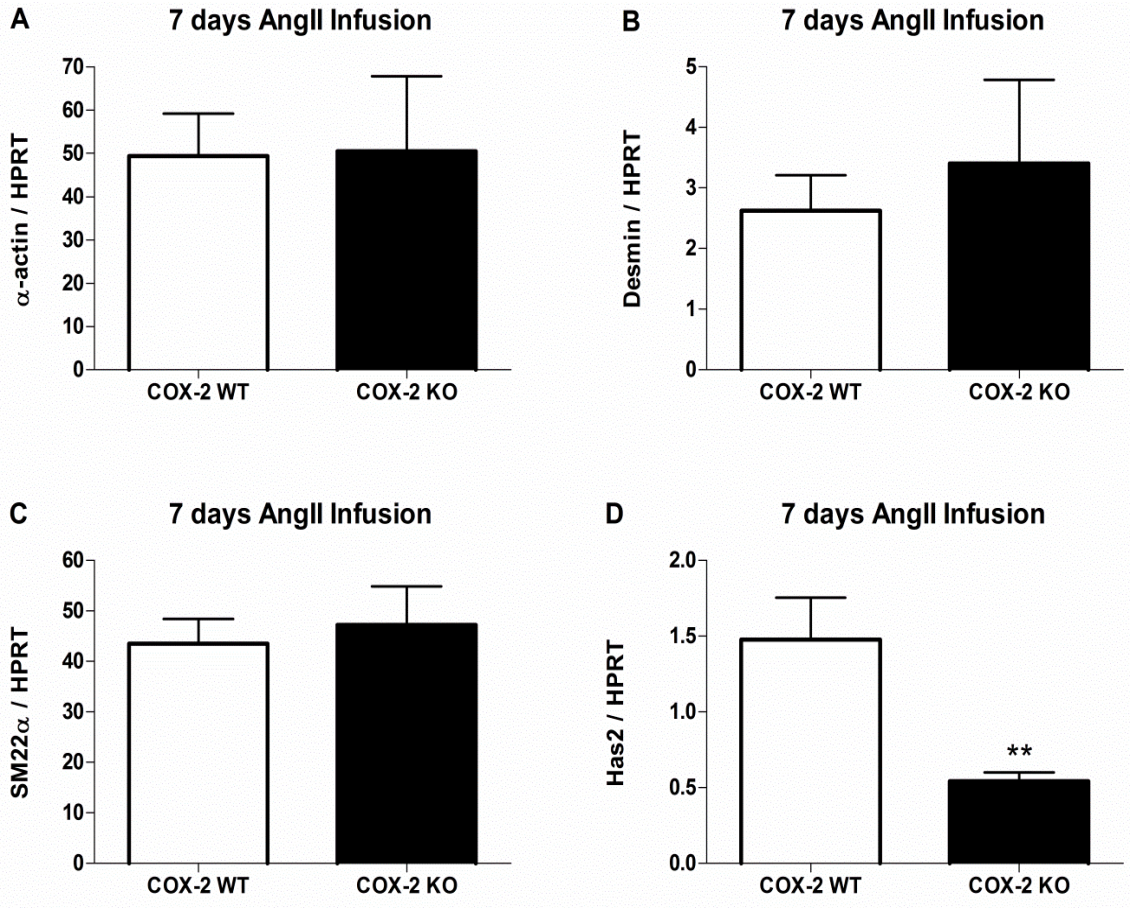



Control
Celecoxib
C
E
D
F

6.2. COX-2 deficiency reduces a marker of de-differentiated SMC phenotype during early aneurysmal development

To examine the effect of deficiency of COX-2 on aortic SMC phenotype during the early stage of AAA development, non-hyperlipidemic COX-2 wild-type (WT) or COX-2 knock-out (KO) mice were infused with AngII at a dose of 1000 ng/kg/min and sacrificed after 7 days of AngII infusion. The abdominal aortas were then collected and analyzed for gene expression of markers of the SMC phenotypes by real-time PCR. COX-2 deficiency did not significantly alter the mRNA levels of α-actin, a marker of differentiated SMC phenotype, as compared to the COX-2 WT mice after 7 days of AngII infusion (Figure 6.7A). Similar results were obtained for desmin (Figure 6.7B) and SM22α (Figure 6.7C), where mRNA expression of these differentiated SMC markers were not found to be significantly different between COX-2 WT and KO mice. In contrast, mRNA levels of Has2, which is specifically expressed by a de-differentiated SMC, was significantly lower in COX-2 KO mice as compared to the corresponding WT mice, in response to AngII infusion (Figure 6.7D). These results indicate that although there is no detectable change in the contractile markers, COX-2 deficiency resulted in a decrease in the gene expression of a synthetic SMC marker during AngII-induced early aneurysmal development.

Figure 6.7: Effect of COX-2 deficiency on gene expression of markers of SMC phenotypes. mRNA expression of **(A)** α-actin, **(B)** desmin, **(C)** SM22α and **(D)** Has2 was determined in the abdominal aortas of COX-2 WT or COX-2 KO mice infused with AngII for 7 days. The housekeeping gene HPRT was used as an internal control for normalizing mRNA levels. Data represented as mean ± SEM; n ≥ 9; "**" represent *p value < 0.01*; Unpaired t-test.



Discussion

Phenotypic modulation of SMCs is critical in regulating vascular function in various cardiovascular disease conditions.[128] Our results, as described in Chapter 4, describe for the first time that, in response to AngII treatment, aortic SMCs change to a de-differentiated phenotype during AAA development in mice. We have also shown that genetic or pharmacological inhibition of COX-2 attenuates AngII-induced AAA formation in mice.[55] The current studies were carried out to elucidate a potential mechanism by which COX-2 regulates the development of these AAAs in this AngII infusion mouse model.

PGs are known to be key regulators of SMC phenotype. Bricaud et al. were the first to report the association between PGs and SMC phenotype, when they showed that phenotypically modified SMCs synthesize more prostacyclin than contractile SMCs.[215] This was followed by a study where exogenously added PGE_1, which is thought to have biological properties similar to prostacyclin by binding to the PGI_2 receptors, IP, stimulated SMCs to change from a contratile to a synthetic phenotype.[216] However, the results derived in this study were based only on morpholological characterization of the cells in culture, without analysis of any of the proteins known to characterize the different phenotypes of SMCs. In addition, apart from binding to the IP receptors, PGE_1 is known to act as a ligand for all the PGE_2 receptors, EP1, EP2, EP3 and EP4 as well, suggesting that this effect of PGE_1 on SMC phenotype can be different from those observed with prostacyclin, due to activation of the EP receptors.[99] More recently, the prostacyclin analog iloprost was found to induce differentiation of human

vascular SMCs by increasing the mRNA and protein expression of smooth muscle specific differentiation markers including α-actin, calponin, h-caldesmon and smooth muscle specific myosin heavy chain, as well as changing the morphology of the cells.[208] Thus, localized release of PGI_2 from the endothelial cells has been suggested to exhibit its cardioprotective effect by a paracrine stimulation of vascular SMCs to a contractile phenotype.[217, 218] In contrast, another vasodilatory prostaglandin, PGE_2 was reported to enhance the de-differentiation of vascular SMCs by increasing PLA_2 secretion, and disorganization of α-actin fibers.[209] While PLA_2 was utilized in this study to characterize de-differentiated SMCs, it has not been shown previously to be a marker of synthetic SMC phenotype. Furthermore, in this report, no other established markers of the differentiated phenotype were shown to be down-regulated to analyze the role of PGE_2 in SMC de-differentiation. Therefore, the role of PGs in regulating SMC differentiation is not well defined.

HA is an extracellular matrix component typically expressed by SMCs of the synthetic phenotype. Studies with human vascular SMCs show that PGE_2 contributes to HA synthesis by inducing the HA synthases Has1 and Has2.[210-212] The induction of HA has been shown to be COX-2 dependent, as treatment of the cells with a high dose of a COX-2 specific inhibitor, etoricoxib was found to inhibit the expression of Has2.[211] In contrast to the previous reports describing the induction of a differentiated phenotype of SMCs by prostacyclin, the prostacyclin analogue, iloprost, is also found to induce the expression of both Has1 and Has2 in human vascular SMCs.[208, 210-212] Thus, although the above studies suggest that the phenotypic modulation of cultured SMCs can

be altered by addition of exogenous PGs, the role of endogenous PGs in regulating SMC phenotype is not clear.

In mouse models of atherosclerosis and neointimal hyperplasia following vascular injury, Fischer et al., recently reported the effectiveness of indomethacin and rofecoxib in decreasing HA accumulation by regulating Has1 and Has2 mRNA expression.[213] This is probably the only in vivo study which has been published describing the role of cyclooxygenases in regulating the expression of HA and its synthases. However, although HA has been associated with a synthetic phenotype, the role of the COX isoforms in regulating other markers of SMC differentiation was not examined. Moreover, while indomethacin is a non-selective inhibitor of both COX-1 and COX-2, the cardiovascular risk of COX-2-selective inhibitor, rofecoxib is a major concern. However, these findings suggest a role of COX-2-dependent regulation of SMC phenotype in vascular remodeling including neointimal hyperplasia and atherosclerosis. Thus, it emphasizes the necessity to identify additional animal models of cardiovascular disease for further examination of the role of COX-2 in regulating SMC phenotype in vivo.

In the current studies, we utilized the AngII induced AAA model to investigate the role of COX-2 in regulating SMC phenotyope at different stages of the disease. To elucidate the role of COX-2 in regulating SMC phenotype during the early stage of AAA development, we treated COX-2 WT and COX-2 KO mice with AngII for 7 days and analyzed the abdominal aortas for changes in gene expression of markers of both contractile and synthetic SMC phenotypes. Compared to WT mice, COX-2 deficiency was found to significantly decrease the mRNA expression of Has2 which characterizes

de-differentiated SMC phenotype. However, the mRNA levels of the differentiation markers such as α-actin, desmin and SM22α were not significantly altered between the aortas of COX-2 WT and KO mice. We also examined the effect of COX-2-selective inhibitor celecoxib on the gene expression of the SMC phenotypic markers at the early stage of AAA development. Similar to the knockout data, after 14 days of AngII infusion, mice, treated with a diet containing COX-2-selective inhibitor celecoxib initiated 5 days after the infusion, displayed no significant change in mRNA expression of the contractile markers, as compared to mice on control diet. In contrast, Has2 mRNA expression in the abdominal aorta was significantly decreased by treatment of mice with celecoxib from day 5 to day 14 of AngII infusion, compared to control treated mice. Thus, the current results indicate that genetic or pharmacological inhibition of COX-2 decrease the expression of a marker of de-differentiated SMC phenotype, but have no effect on the smooth muscle specific differentiation markers during the early phase of AngII-induced AAA development in mice.

We also analyzed the regulation of COX-2 of SMC phenotype during the later stages of AAA progression. In these studies, mice were infused with AngII, and treated with control or celecoxib diet beginning 5 days after AngII infusion. The mice were then sacrificed after an intermediate time-point of 21 days, or a late time-point of 28 days of AngII infusion, and the abdominal aortas were analyzed for mRNA expression of contractile and synthetic markers of SMC phenotype. After both 21 and 28 days of AngII infusion, COX-2 inhibition was found to significantly increase the mRNA expression of the SMC specific contractile markers in the abdominal aorta, as compared to those of control treated mice. In contrast, at both of these time-points, celecoxib treatment

prevented modification to a de-differentiated SMC phenotype by significantly reducing the mRNA levels of Has2, compared to the control mice. Thses results indicate that COX-2 inhibition at both the intermediate (day 21) as well as the late stage (day 28) of AAA development is effective in increasing the gene expression of the contractile markers, while reducing that of the synthetic phenotype.

Additional analysis of the abdominal aortas by immunohistochemistry after 28 days of AngII infusion revealed that abundant α-actin protein was detected in the medial layer of the celecoxib-treated mice, but not in the aneurysmal lesions of the control group. The medial layer in the aortas of the control mice however, continued to express α-actin. In particular, higher expression of α-actin was observed in the medial layer on the distal side of the vessel, opposite to the lesion, while the medial cells adjacent to the lesion were found to express a lower level of the protein, as indiacted by a lighter staining. This could be potentially explained by the gradual loss of the medial SMC layer, as evident by a decrease in expression of contractile markers, to assume a more de-differentiated phenotype. A recent study has described the correlation between medial elastin dissection and aortic motion resulting in the asymmetric expansion of AngII-induced AAAs in mice.[219] Thus, in the current studies, the reduced detection of medial α-actin on the side of the aorta expanding towards the lesion might suggest an inherent difference between the medial cells on either side of the aorta. The accumulation of extracellular matrix components including collagen and hyaluronic acid was also decreased in the aorta of mice that showed reduced AAA development from celecoxib treatment. Thus, these results suggest that COX-2 inhibition is effective in hindering

phenotypic alteration, and thereby maintaining the aortic SMCs in a differentiated phenotype during AngII-induced AAA progression in mice.

Our current results indicate that during the early stage of AngII-induced AAA progression, COX-2 inhibition modified only the de-differentiation marker, Has2, while both the contractile as well as the synthetic markers were significantly altered during the intermediate as well as the late stage of the disease. Although no studies have been reported on the temporal expression of the contractile versus the synthetic markers in vascular SMCs, these results suggest that this COX-2-dependent change in phenotype of SMCs of the aorta during AAA progression may be associated with an induction of cell de-differentiation prior to loss of the contratile phenotype.

The above findings suggest that COX-2 inhibition attenuates AngII-induced AAA progression in mice by maintaining aortic SMCs in a differentiated phenotype. The genetic deficiency of microsomal prostaglandin E synthase-1 (mPGES-1), which contributes to PGE_2 synthesis downstream of COX-2, has been reported to reduce AngII-induced AAA formation.[163] However, although PGE_2 has been suggested to alter SMCs to a synthetic phenotype, the role of mPGES-1 in regulating SMC phenotype during AngII-induced AAA formation has not been reported.[209] The phenotypically modified SMCs are known to synthesize a large amount of HA in atherosclerotic lesions and neointima development following vascular injury.[220] In addition, our current findings, as shown previously in Chapter 5, indicate increased production of HA following AAA development in mice. Has2 is the major isoform among HA synthases which is induced by PGE_2 in vascular SMCs.[151, 211] We also show in our current results that the induction of Has2 during AAA formation is regulated by COX-2. These

findings suggest that mPGES-1-derived PGE_2 could play an important role in regulating SMC phenotype during AngII-induced AAA development in mice.

CHAPTER 7

GENERAL DISCUSSION

7.1. Summary and conclusions

The primary objectives of the current studies were: (1) to determine if COX-2 inhibition with celecoxib can attenuate the progression of AngII-induced AAAs in mice, when treatment is begun after initiation of the disease, (2) to explain the reasons contributing to the increased susceptibility of the abdominal region of the aorta towards AngII-induced aneurysm formation in mice, and (3) to elucidate the mechanisms by which COX-2 regulates the development of AngII-induced AAAs in mice.

The first studies were carried out to investigate the role of COX-2 in AngII-induced AAA development, as described in Chapter 2. We infused mice with AngII, and after 5 days of AngII infusion, treated them with a control diet, or a diet containing the COX-2-selective inhibitor celecoxib. The AngII-induced AAA model is characterized by a rapid development of aneurysmal pathology within 3 days of AngII infusion.[54, 56] Thus, introducing the control and celecoxib diets after 5 days of AngII infusion ensured that COX-2 inhibition was begun after the initial development of the disease. The mice were subsequently sacrificed at different time-points during the AngII infusion to determine the effect of celecoxib on AAA progression. Celecoxib treatment from day 5 to day 14 of AngII infusion was found to produce a trend towards decreased incidence of AAAs, as compared to mice treated with the control diet. However, as the COX-2 inhibitor treatment was extended through the 21 and 28 day time-points of AngII infusion, celecoxib treatment significantly reduced the incidence of AAAs, compared to

the control mice. COX-2 inhibition was also found to effectively reduce the severity of the AAAs formed after both 21 and 28 days of AngII infusion. Of particular importance was the fact that celecoxib treatment from day 5 to day 28 of AngII infusion effectively protected the mice from aortic rupture and death. In summary, COX-2 inhibition with celecoxib, begun after the disease initiation, was effective in improving survival and attenuating the progression of AngII-induced AAAs in mice.

The following studies were carried out to determine mechanisms contributing to the increased susceptibility of the abdominal region of the aorta towards AngII-induced aneurysm development, as described in Chapter 3. Mice were infused with saline, as control, and AngII, following which gene expression changes in the arch, thoracic and abdominal segments of the aorta were analyzed at different time-points after AngII infusion. AngII treatment resulted in a prolonged induction of COX-2 mRNA in the abdominal aorta throughout the infusion period, in contrast to the transient induction observed in the arch and thoracic segments. A direct comparison revealed that COX-2 gene expression in the abdominal segment was significantly greater than the thoracic region during the majority of AngII infusion. Moreover, in contrast to the thoracic region, COX-2 protein was readily detected in the smooth muscle cells of the abdominal aorta. Furthermore, compared to the thoracic region, mRNA expression of the AngII receptor AT1a was found to be significantly greater in the abdominal segment throughout the AngII infusion. In summary, a greater and prolonged induction of COX-2, coupled to an increased expression of the AT1a receptors, was found to contribute to the increased susceptibility of the abdominal region of the aorta towards AngII-induced aneurysm formation.

The final studies were carried out to elucidate the mechanisms by which COX-2 regulated the development of AngII-induced AAAs in mice. We first set out to characterize the phenotype of aortic smooth muscle cells (SMCs) during AngII-induced AAA development, as described in Chapters 3. Mice were infused with saline, as control, and AngII, to investigate the role of AngII in regulating SMC phenotype during AAA formation. Increased proliferation of the SMCs was not detected during AngII-induced AAA development. However, AngII infusion was found to significantly decrease the mRNA expression of the markers of a contractile phenotype, while increasing a marker of the synthetic phenotype. In addition, cells of the aneurysmal lesion were found to undergo a loss of their differentiated phenotype, as evident by decreased α-actin protein expression, and synthesized large amounts of extracellular matrix components, characteristic of a de-differentiated phenotype. Further studies were carried out to determine if this change in SMC phenotype during AngII-induced AAA development was regulated by COX-2, as described in Chapter 4. COX-2 inhibition with celecoxib, begun 5 days after the AngII infusion, was found to significantly increase the mRNA and protein expression of the markers of contractile SMC phenotype during the intermediate and late stages of AAA development, while reducing the markers of the synthetic phenotype. In summary, COX-2 inhibition was found to effectively hinder phenotypic alteration, and thereby maintain the aortic SMCs in a differentiated phenotype during AngII-induced AAA progression in mice.

In conclusion, our findings suggest that COX-2 plays an important role in AAA development in mice, and inhibition of COX-2 with celecoxib attenuates AAA progression by maintaining a differentiated phenotype in aortic smooth muscle cells.

7.2. Clinical relevance and future directions

Abdominal aortic aneurysm (AAA) is a significant health problem, and is currently the 10th leading cause of death in men aged over 55 years.[1, 2] Small AAAs are known to progress at a relatively constant rate over years, with the majority achieving clinically significant severity that results from increased susceptibility of rupture later in life.[4, 13] Currently, there are no pharmacological treatments available for AAAs, with open or endovascular surgical repair being the only options to prevent an impending aortic rupture. However, surgery for AAAs is associated with its own significant risk of mortality.[6] With recent advances in non-invasive imaging methods that are used for screening against a variety of conditions, a significant number of patients with AAAs are being identified. Almost 90% of these detected AAAs are small AAAs, and surgical repair is not recommended until they attain a diameter of 5.5 cm.[221] The early detection of small AAAs would allow for therapeutic intervention to prevent AAA expansion should an effective pharmacological treatment be identified.

One of the primary goals of the current study was to identify a therapeutic strategy which could potentially limit the expansion of AAAs after they are detected. Our studies show that treatment with the COX-2-selective inhibitor celecoxib, begun after the disease initiation, effectively attenuates AngII-induced AAA progression in mice. We have previously reported that pre-treatment of the mice with celecoxib, prior to disease initiation, reduces AAA formation.[55] Moreover, AngII-induced AAAs were not detected in mice genetically deficient in COX-2.[56] Although the above studies were the first to emphasize the importance of COX-2 in AngII-induced AAA development, the role of COX-2 in contributing to AAA progression was not examined. Our current results

are more relevant to the identification of future treatments for AAAs because they indicate that celecoxib is efficacious in limiting the expansion of the disease after it is initiated.

In addition to the inhibition of COX-2, the AngII infusion model in mice has been utilized to investigate a variety of methods with the potential to reduce AAAs. However, in majority of these studies, the efficacy of the agents was shown to occur with a pre-treatment regimen where administration of the agent was first started before initiation of the disease.[66, 164] In addition, some of the agents which were shown to be beneficial following this pre-treatment regimen were not found to produce similar effectiveness in reducing AAAs when treatment was started after disease initiation.[165] Thus, the above reports further emphasize the importance of our current studies in the identification of future AAA treatments, because they describe for the first time the effectiveness of COX-2 inhibition in attenuating AAA progression, when treatment is initiated after early development of the disease.

The AngII infusion model of AAAs displays multiple characteristics of human AAAs, and thus has been widely utilized to characterize the mechanisms contributing to the progression of the disease.[54] One of the advantages of the AngII model has been its association with aortic rupture and death, a characteristic that is similar to the human disease.[54, 163, 166] In our current studies we show that COX-2 inhibition with celecoxib, begun after disease initiation, significantly reduces the incidence as well as the rate of aortic rupture in mice. The improvement in survival that results from the inhibition of COX-2 in the AngII-induced AAA model is a significant outcome and would be a requirement of future pharmacological treatment for AAAs.

Although AAA has been traditionally considered to be a consequence of atherosclerosis, recent evidences suggest that the atherosclerotic burden of a patient is not associated with increases in AAA growth.[13, 18] Thus, our finding that celecoxib displays its effectiveness in reducing AngII-induced AAAs in a nonhyperlipidemic mouse model may be clinically important, as it indicates that celecoxib directly influences AAA formation in the absence of hyperlipidemia, and suggests the potential effectiveness of COX-2 inhibition independent of atherosclerosis development.

Results from our current studies show that an increased expression of COX-2, associated with higher AT1a receptor expression, contributes to the increased susceptibility of the abdominal aorta towards aneurysm formation. The AT1 receptors are known to play an important role during AngII-induced AAA development in mice. Antagonism of the AT1 receptor with losartan has been reported to reduce AAA formation in mice.[87] More recently, the endothelial cell-specific deficiency of the AT1a receptors, has been found to decrease the incidence of AngII-induced ascending aortic aneurysms in hyperlipidemic mice.[89] However, the cell type responsible for AngII receptor expression contributing to AAA development has not been reported. This suggests that determining the specific cell type of the aorta that is responsible for the effects of COX-2 mediated AAA formation could be potentially important in further characterizing the mechanisms contributing to COX-2-dependent AAA formation in mice. Thus, future studies could be directed at utilizing the *Cre-lox* technology to generate mice deficient in COX-2 in specific cell types, such as endothelial cells or SMCs. These conditional COX-2 knockout mice could then be utilized to further define

the cell-specific role of COX-2, as well as the downstream PGs derived from COX-2, in AngII-induced AAA development.

Our current studies suggest that COX-2 plays an important role in regulating the SMC phenotype during AngII-induced AAA development. However, the specific PGs responsible for modulating the SMC phenotype during AAA formation have thus far not been described. With *in vitro* studies suggesting conflicting evidences about the varied effects of different PGs on SMC phenotype, further studies are required to define the role of PGs in regulating SMC differentiation in vitro.[208, 209, 216]

The expression of COX-2 and resultant PGE_2 production has been shown to be induced in aneurysmal tissue, as compared to the normal aorta.[4] Genetic deficiency of microsomal prostaglandin E synthase-1 (mPGES-1), which contributes to PGE_2 synthesis downstream of COX-2, has also been reported to reduce AngII-induced AAA formation in mice.[163] However, although PGE_2 has been shown to induce a de-differentiated SMC phenotype, the role of mPGES-1 in regulating SMC phenotype has not been described. Thus, future studies could be directed at utilizing pharmacological agents which inhibit mPGES-1 to determine the role of COX-2 derived PGE_2 in regulating vascular SMC phenotype during AngII-induced AAA development in mice.

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VITA

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PUBLICATIONS

Kamalika Mukherjee, *Jonathan Gitlin and Charles D. Loftin*

Cyclooxygenase-2 Inhibition Improves Survival and Limits Expansion of Abdominal Aortic Aneurysms in Mice

Manuscript submitted for publication

Kamalika Mukherjee *and Charles D. Loftin,*

Attenuated Progression of Abdominal Aortic Aneurysms in Mice by Celecoxib is Associated with a Differentiated Phenotype of Aortic Smooth Muscle Cells

Manuscript to be submitted

ABSTRACTS AND POSTER PRESENTATIONS

"Cyclooxygenase-2 inhibition attenuates progression of abdominal aortic aneurysms in mice by regulating the smooth muscle cell phenotype." **Kamalika Mukherjee** and Charles D. Loftin. *ASPET Annual meeting at Experimental Biology 2011,* Washington D.C. in April 2011 *and Rho Chi Research Day, College of Pharmacy, University of Kentucky,* Lexington in April 2011

"Celecoxib Attenuates Progression of Abdominal Aortic Aneurysms in Mice by Regulating Smooth Muscle Cell Phenotype." **Kamalika Mukherjee** and Charles D. Loftin. *Symposium on Drug Discovery and Development,* University of Kentucky, Lexington *and Gill Heart Institute Cardiovascular Research Day in* October 2010

"Attenuated Progression of Abdominal Aortic Aneurysms in Mice by Celecoxib is Associated with a Differentiated Phenotype of Aortic Smooth Muscle Cells." **Kamalika Mukherjee** and Charles D. Loftin. *Keystone Symposium on Molecular and Cellular Biology: Bioactive Lipids: Biochemistry and Diseases (D2),* Kyoto, Japan in June 2010

"COX-2 Inhibition Improves Survival and Limits Expansion of Abdominal Aortic Aneurysms in Mice." **Kamalika Mukherjee** and Charles D. Loftin. *Atherosclerosis, Thrombosis and Vascular Biology*, San Francisco in April 2010

"Aortic Aneurysm Development in Mice Localizes with Increased Cyclooxygenase-2 Expression." **Kamalika Mukherjee**, Jonathan Gitlin and Charles D. Loftin. *Center for Clinical and Translational Science*, University of Kentucky, Lexington in February 2010

"COX-2-selective inhibition with celecoxib reduces abdominal aortic aneurysm progression in mice." Kamalika Mukherjee, Jonathan M Gitlin, Charles D. Loftin. *College of Pharmacy, University of Kentucky*, Lexington in March 2008

AWARDS

2nd place Award for Best Poster at Rho Chi Research Day, *College of Pharmacy, University of Kentucky*, Lexington, April 2011

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