MECHANISMS OF CYCLOOXYGENASE-2-DEPENDENT HUMAN AORTIC SMOOTH MUSCLE CELL PHENOTYPIC MODULATION

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MECHANISMS OF CYCLOOXYGENASE-2-DEPENDENT HUMAN AORTIC SMOOTH MUSCLE CELL PHENOTYPIC MODULATION

____________________________________________________

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

Oreoluwa Olufunmilayo Adedoyin

Director: Dr. Charles D. Loftin, Associate Professor of Pharmacy

Lexington, Kentucky

2014

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ABSTRACT OF DISSERTATION

MECHANISMS OF CYCLOOXYGENASE-2-DEPENDENT HUMAN AORTIC SMOOTH MUSCLE CELL PHENOTYPIC MODULATION

Abdominal aortic aneurysm (AAA) is a disease of the aorta characterized by pathological remodeling and progressive weakening of the vessel resulting in the increased risk of rupture and sudden death. In a mouse model of the disease induced by chronic Angiotensin II (AngII) infusion, progression of AAAs is associated with reduced differentiation of smooth muscle cells (SMCs) at the site of lesion development. In the mouse model, the effectiveness of cyclooxygenase-2 (COX-2) inhibition for attenuating AAA progression is associated with maintenance of a differentiated SMC phenotype. However, the safety of COX-2 inhibitors is currently in question due to the increased risk of adverse cardiovascular events. Thus, it is crucial to identify mediators downstream of COX-2 that may provide new targets for treatment of this disease.

Recent studies in humans and mouse models have suggested that the microsomal prostaglandin E synthase (mPGES-1) enzyme, which acts downstream of COX-2, may also be involved in the pathogenesis of the disease. We hypothesized that increased prostaglandin E₂ (PGE₂) synthesis resulting from the induction of both COX-2 and mPGES-1 may result in reduced differentiation of SMCs, and that disruption of this pathway would preserve the differentiated phenotype. To test this hypothesis, human aortic smooth muscle cells (hASMCs) were utilized to examine the effects of a variety of agents involved in AAA development and the COX-2 pathway.

My findings suggest that one of the effects of exposing hASMCs to AngII involves a specific induction of mPGES-1 expression. Furthermore, although different COX-2-derived products may have opposing effects, mPGES-1-derived PGE₂ may be the primary prostanoid synthesized by SMCs which functions to attenuate differentiation. Therefore, mPGES-1 inhibition may provide inhibition of
PGE$_2$ that is more specific than COX-2 inhibitor treatment and may serve as a therapeutic target for attenuating AAA progression by maintaining a differentiated SMC phenotype.

KEYWORDS: vascular smooth muscle cell phenotype, abdominal aortic aneurysm, Angiotensin II, prostaglandin E$_2$, microsomal prostaglandin E synthase

Oreoluwa Olufunmilayo Adedoyin

May 28, 2014
MECHANISMS OF CYCLOOXYGENASE-2-DEPENDENT HUMAN AORTIC SMOOTH MUSCLE CELL PHENOTYPIC MODULATION

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May 28, 2014
DEDICATION

This work is dedicated to:

… the everlasting God, the Lord, the Creator of the ends of the earth …

Isaiah 40:28, NKJV
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This dissertation, while an individual work would not have been accomplished without the input of various people who contributed to my success at different stages of my educational career here at the University of Kentucky. Truly, it does take a village to raise a child!!!

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CHAPTER 1: INTRODUCTION

1.0 Physiology of mammalian blood vessels

During embryogenesis, blood vessels are among the first organs to develop as these vessels are important for further organogenesis as well as for sufficient nutrition of the embryo [1]. Mature blood vessels are comprised of three distinct layers: the tunica intima, tunica media, and tunica adventitia. The tunica intima is composed of endothelial cells lying on a basement membrane of type IV collagen, laminin and heparin sulfate proteoglycans. Between the intima and the media is a layer of internal elastic lamina composed of mainly elastin and hyaluronic acid. The tunica media is primarily composed of elastic fibers and smooth muscle cells (SMCs) arranged circumferentially. The adventitia, which is the outermost layer, consists mainly of collagen fibers, loose connective tissue, fibroblasts, and fat cells, with an external elastic lamina separating this layer from the outer media [2].

1.1 Phenotypic modulation of VSMCs

The SMCs are responsible for maintaining artery wall tension via contraction-relaxation, vascular remodeling, repair, as well as growth [2-6]. Normal smooth muscle cells have a distinct smooth appearance and the differentiated phenotype is specifically marked by the expression of SMC lineage markers and the acquisition of contractile function [7]. Cell differentiation involves
transition from an initial multipotential state to a specialized form thus assuming a different morphology as well as function [6].

Phenotypic modulation is a unique characteristic of vascular SMCs which is demonstrated by their ability to switch their phenotype between the contractile (differentiated) and synthetic (de-differentiated) phenotypes in response to various environmental/external cues such as growth factors/inhibitors, inflammatory mediators, cytokines, and matrix metalloproteinases [8-18]. This characteristic property of vascular SMCs is important in regulation of vascular function in health and disease [19]. The contractile and synthetic phenotypic states are two extremes of the spectrum of SMC phenotypic expression and cells expressing features of both phenotypes have been identified [20]. Furthermore, vascular SMCs do not de-differentiate terminally but retain sufficient plasticity to change back and forth between the differentiated and de-differentiated phenotypes [21]. A variety of transcription factors including myocardin, myocardin related transcription factors (MRTFs), and members of the Krüppel-like zinc finger family (KLF) have been suggested to act as molecular switches involved in the regulation of vascular SMC differentiation [22]. Increased binding of the serum response factor (SRF) and its coactivator myocardin and then to the CArG motif of differentiation marker genes, induces transcription and increased expression of these differentiation markers [23].
1.1.1 The contractile differentiated phenotype

The differentiated/contractile phenotype is demonstrated by expression of several SMC contractile proteins that are used as differentiation markers (e.g. smooth muscle α-actin, SMC myosin heavy chain, β-tropomyosin, desmin, h-caldesmon, calponin, smoothelin, and SM22α) which are involved in maintenance and regulation of contractile function [7, 12, 24-29]. Cells in this phenotype contract in response to chemical and mechanical stimuli and thus function in regulating blood pressure and flow [26].

1.1.2 The synthetic de-differentiated phenotype

Cells in the de-differentiated or synthetic phenotype downregulate the expression of differentiation markers and express high levels of synthetic phenotype markers (e.g. hyaluronic acid, MMP-2, l-caldesmon, vimentin, and non-muscle myosin heavy chain B). Furthermore, these de-differentiated cells exhibit large, rhomboid, flattened, fibroblast-like morphology, and demonstrate an increase in proliferative and migration rates compared with the contractile phenotype [12, 15, 16, 26, 28, 30-34]. Previously, the proliferation and differentiation processes were assumed to be coupled, with reduction in differentiation being a prerequisite for proliferation. However, these processes are now understood to be independent wherein growth factors may induce proliferation independent of reduced differentiation marker expression and vice-versa [35].
1.2 Micro RNAs and the vascular SMC phenotype

Micro RNAs (miRs) are endogenous, small single-stranded, non-coding RNAs about 22 nucleotides in length which base pair with the 3'-untranslated region (UTR) of target RNAs thereby modulating gene expression and protein synthesis [36, 37]. Depending on the degree of complementarity between a mature mRNA and its target mRNA UTR, microRNAs may regulate gene expression by mediating translational repression, activation or degradation of the target mRNA [37]. In vascular SMCs, a variety of miRNAs have been reported to regulate the phenotype of these cells in response to vascular injury thus indicating that manipulation of these targets/mediators may be utilized as potential new therapeutic targets [36].

Numerous reports have indicated that miR-145 which is the most abundant miR in normal vascular walls, has been demonstrated to regulate the phenotype of vascular SMCs. miR-145 is expressed in a gene cluster with miR-143 and deficiency of this cluster in mice has been demonstrated to result in the reduced ability of miR-143/145-deficient mice to acquire the contractile phenotype due to their accumulation of synthetic vascular SMCs and specific defects in GPCR receptor-mediated signaling [38]. Overexpression of these miRs in a rat model of acute vascular injury was demonstrated to decrease neointimal formation while loss of this cluster resulted in altered differentiation of the SMCs and structural modification of the aorta [39]. Furthermore, miR-145 has been identified as a phenotypic marker for the differentiated phenotype in cultured vascular SMCs and it also induces expression of vascular SMC differentiation marker genes [40].
In addition, the miR143/145 cluster has been demonstrated to promote differentiation and repress proliferation in vascular SMCs by inhibiting suppressors of myocardin/serum response factor activity such as Kruppel-like factor 4 (KL4), and ELK1 (member of ETS oncogene family) [22, 41].

Other miRs apart from the miR143/145 cluster have been involved in the regulation of the vascular SMC phenotype. Recent research has identified miR-663 as a novel regulator of phenotypic switch and function in human aortic vascular SMCs. Overexpression of this miR promotes the contractile phenotype and suppresses formation of neointimal lesions in a mouse model of vascular injury by suppression of JUNB expression [42]. Furthermore, increased expression of miR-21 induced by TGFβ and BMP signaling mediates the induction of the contractile phenotype in vascular SMCs [43]. On the other hand, miR-31 has been shown to bind to its target gene cellular repressor of E1A-stimulated genes (CREG), thus negatively regulating vascular SMC phenotypic modulation by repressing expression of SMC differentiation markers [44]. Similarly, miR-221 which is upregulated by PDGF, reduces the expression of vascular SMC differentiation markers by inhibition of c-Kit, which is a positive regulator of myocardin expression [36, 45].
1.3 Phenotypic modulation of VSMCs in the development of vascular disease

Modulation of the vascular SMC phenotype plays a key role in contribution to vascular pathology. The pathogenesis of most cardiovascular diseases usually involves dysfunction of the vascular smooth muscle cells of the tunica media. Fully differentiated vascular SMCs proliferate extremely slowly while expressing low levels of extracellular matrix proteins. However, these processes are reversed and in fact accelerated during vessel remodeling following vessel injury or atherogenesis [46]. In certain vascular disorders such as atherosclerosis [47-49], restenosis due to vascular injury [9, 50-52], hypertension, and abdominal aortic aneurysms, the decrease in protein and mRNA expression of SMC differentiation markers has been observed within the vascular wall [46, 53]. Also, increased expression of de-differentiation markers by SMCs has been associated with these diseases [15, 31, 53-61].

In in vitro vascular SMC culture models, the synthetic phenotype shows changes in SMC morphology from large, flattened, fibroblast-like morphology with increased SMC proliferative and migration rates, as compared to the elongated, spindle-shape in the contractile phenotype [12, 15, 16, 26, 28, 30-33] [34]. The identification of novel pathways altered during the progression of vascular SMC phenotype modulation is important in the identification of mechanisms contributing to cardiovascular disease.
1.3.1 Atherosclerosis

Atherosclerosis which has been recognized as the leading cause of death in the developed world, is a common vascular disorder characterized by arterial hardening due to deposition of lipids, cholesterol and other substances within the arterial walls forming plaques [62]. Rupture of these plaques may result in thrombus formation and vessel occlusion, thus resulting in a myriad of cardiovascular diseases including coronary, cerebrovascular and peripheral artery disease [63]. During progression of atherosclerosis, the resulting endothelial dysfunction due to a combination of changes in shear stress, oxidized LDL and inflammatory cytokines, results in the increased expression of endothelial adhesion molecules and loss of endothelium-derived factors such as prostacyclin and nitric oxide [33, 63-65]. Furthermore, inflammatory cells that have infiltrated into the vessel wall release mediators including growth factors and cytokines that produce SMC phenotypic changes from the contractile to the synthetic state resulting in increased migratory ability and deposition of extracellular matrix (ECM) [62]. As the SMCs migrate into the intima, they release proinflammatory factors that contribute to atherosclerotic lesion formation [19]. These atherosclerotic lesions are characterized by the accumulation of layers of de-differentiated SMCs, as well as lipid-filled macrophages [25]. Thus, alteration of the vascular SMC phenotype affects the ability of the lesion to respond to different agonists [66]. Recently, Yu et al (2012) reported vascular SMC phenotypic modulation with acceleration of atherogenesis in COX-2 deleted hyperlipidemic ApoE-deficient mice. This was demonstrated by the loss of SMC
α-actin and upregulation of vascular cell adhesion molecule-1 (VCAM-1) in the leukocyte infiltrated lesions [67].

1.3.2 Restenosis due to vascular injury

De-differentiation of vascular SMCs is often observed in the vessel wall during progression of restenosis following to vascular wall injury caused by percutaneous transluminal coronary angioplasty [9, 50-52]. Restenosis is an initial repair process induced following vessel wall injury that occurs in response to endothelial denudation or direct vascular trauma produced by the intravascular procedure [52]. These de-differentiated cells subsequently migrate into the subintimal space and increase proliferation rates. The de-differentiated SMCs also synthesize large amounts of extracellular matrix and form lesions which contribute to the restenosis and subsequent failure of the vascular intervention [52].

1.3.3 Abdominal aortic aneurysm (AAA)

Abdominal aortic aneurysm is a disease of the aorta demonstrated by arterial enlargement with predisposition to rupture which is associated with a high mortality rate [18, 24, 68]. Although AAA is an often asymptomatic progressive disease, the aorta may continue to enlarge progressively over months or years increasing the risk of rupture [18, 68]. Ruptured AAAs are the 13th leading cause of death in the United States and the 10th leading cause of death in Caucasian
men 65 – 74 years of age [18, 24, 69, 70]. The primary risk factors include male
gender, advanced age and smoking [24]. In AAAs, the tissue lesion site is
commonly characterized by elastin fragmentation, increased inflammation, and
SMC depletion [24, 69, 71]. In addition, during the progression of AAAs, there is
observable vascular SMC medial degeneration characterized by SMC apoptosis
and reduction in medial SMC density compared with normal aortas [72]. Ailawadi
et al (2009) reported reduced expression of the differentiation markers SM22α
and SMα-actin in aneurysmal tissue obtained from animal and human studies,
when compared with control aorta [53]. Furthermore, Jiao et al (2010) reported
the decrease in the levels of SM22α, SM α-actin, and desmin in the vessel media
during the development and progression of carotid artery aneurysms in rabbits
when compared with those from control rabbits [73]. Current research in our lab
using a mouse model of AAAs induced by chronic angiotensin II (AngII) infusion,
demonstrated that progression of AAAs is also associated with reduced
differentiation of SMCs in the abdominal aorta. However, the treatment of these
mice with celecoxib, an inhibitor of the inducible cyclooxygenase-2 (COX-2)
enzyme, was effective at attenuating AngII-induced AAA progression, and this
was associated with maintenance of a differentiated SMC phenotype in the aorta
[74, 75]. Even though the process of AAA formation and development involves
complex pathological processes, a key factor in development of AAAs is the
phenotypic modulation of vascular smooth muscle cells (VSMCs).

Currently, there are no pharmacological agents for treating AAAs leaving
surgery (open or endovascular repair) as the only option [70, 76]. Surgery to
repair AAA however, is not without its associated risks of morbidity and mortality, and thus, it is important to discover and develop effective non-surgical therapies for prevention and treatment of this disease.

1.4 Prostanoids and cyclooxygenases

Prostanoids are a family of cyclopentane derivatives of polyunsaturated fatty acids formed from arachidonic acid via the cyclooxygenase pathway and are important mediators of allergic reactions and inflammation [26, 77]. The cyclooxygenase (COX) enzymes catalyze the first step in the production of prostanoids. Two isoforms of the COX enzyme exist: COX-1, which is the constitutive enzyme, and COX-2, which is the inducible form. The amino acid sequences for both isoforms have about 75% homology, and the catalytic residues of these enzymes are highly conserved [78]. Both enzymes have different subcellular localizations as well as different tissue expression profiles with the COX-1 enzyme primarily associated with “housekeeping” functions in normal tissues such as hemostatic, gastric protection, and renal water balance [63, 79]. However, in response to stress, cytokines, growth factor stimulation, and inflammatory stimuli, COX-2 which is extremely low in most normal tissues (except in the kidney, testis, tracheal epithelium, and central nervous system) expression is increased. Also, macrophages are a major source of prostaglandins within the vessel wall. In addition, COX-2 expression is increased
in certain pathological conditions such as tissue injury, malignant transformation of gastrointestinal and mammary epithelium [79].

The COX enzymes convert arachidonic acid into prostaglandin G₂ (PGG₂) via oxygenation, and then prostaglandin H₂ (PGH₂) which is synthesized from PGG₂ via peroxidase activity is subsequently converted by tissue specific isomerases/synthases into different prostanoids including prostacyclin (PGI₂), prostaglandin E₂ (PGE₂), prostaglandin D₂ (PGD₂), thromboxane (TXA₂), and prostaglandin F₂α (PGF₂α) [80, 81]. Different cells exhibit differential expression of these synthases, thus determining the expression levels and production profiles of individual prostanoids under basal as well as inflammatory conditions. The cytoplasmic prostaglandin E₂ synthase (cPGES), prostaglandin D₂ synthase (PGDS), prostaglandin F reductase, and thromboxane A₂ synthase (TXAS) are said to be preferentially coupled with COX-1, whereas the microsomal prostaglandin E₂ synthase (mPGES-1), and prostacyclin synthase (PGIS) are often coupled and co-induced with COX-2 in response to acute and chronic inflammatory stimuli [82]. These prostanoids exert their effects through their specific receptors, thus prostanoid synthase inhibitors as well as receptor antagonists have been explored as potential therapeutic agents in the management of several diseases [26]. The role of various prostanoids in the pathogenesis of cardiovascular diseases has been extensively reviewed by Yuhki et al (2010) [83].
1.4.1 Modulation of prostanoid activity

The biological actions of these prostanoids may be modulated at the level of the cyclooxygenase enzymes, individual synthases or the prostanoid receptors [84]. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-1 and/or COX-2, thus limiting the production of prostanoids and the resulting inflammation produced by the action of these lipid mediators [82]. Deletion of COX-2 has been shown to result in re-diversion of arachidonic acid from the prostanoid synthesis pathway to increased synthesis of products of the 5-lipoxygenase pathway [67]. Furthermore, abnormal regulation of the COX-2 enzyme has been implicated in the pathology of numerous diseases including cancer, fever, Alzheimer’s disease, and cardiovascular diseases [85, 86].

1.4.2 Role of Prostanoids in vascular SMC phenotypic modulation

1.4.2.1 Prostacyclin (PGI₂)

Prostacyclin (PGI₂) was discovered in the 1970’s by John Vane and colleagues and observed to relax arteries and inhibit platelet aggregation [87]. Several studies have indicated that a balance between prostacyclin and thromboxane A₂ (proaggregatory) contributes to maintaining the endothelial integrity and thrombus formation and overall vascular homeostasis [64, 88]. PGI₂ is a potent vasodilator, antithrombotic, and inhibitor of vascular SMC proliferation, leukocyte adhesion and platelet aggregation, and contributes to protection and maintenance of vascular homeostasis. PGI₂ is the main prostaglandin product of arachidonic acid metabolism in the endothelial cells and is primarily synthesized
by the action of COX-2 [33, 63, 65, 89]. PGI2 is not stored, but exerts its effects locally and is converted rapidly non-enzymatically to its inactive metabolite, 6-keto prostaglandin F1α (PG F1α) [89]. In certain cardiovascular disorders such as atherosclerosis, myocardial infarction, thrombosis, and pulmonary hypertension, reduced prostacyclin activity has been implicated [33].

**PGI2 signaling**

Prostacyclin activity is mediated via its tissue-specific (predominantly platelets and vascular SMCs) G-protein coupled prostacyclin receptor (IP). Activation of the IP receptor results in Gs (G-protein) coupling resulting in activation of adenylate cyclase (AC), formation of cAMP, and subsequent activation of PKA [33] [64]. PGI2 activation of the IP receptor has also been reported to couple to Gq and mobilize Ca2+, whereas high concentrations of prostacyclin agonists have been reported to preferentially activate PPAR-α or PPAR-δ [64].

It has been previously reported that prostacyclin and prostacyclin analogs induce differentiation in vascular SMC cultures [33, 52, 90]. Iloprost (prostacyclin analog) has been shown to induce SMC-specific differentiation markers and contractile morphology in cultured human vascular SMCs [33, 91, 92]. Recent studies by Tsai et al (2009) demonstrated that PGI2 induces SMC contractile phenotype, suggesting that a deficiency in PGI2 may result in SMC phenotypic alteration to the synthetic state [19]. Furthermore, treatment of vascular SMCs in the synthetic phenotype with varying concentrations of iloprost resulted in
induction of expression of SMC differentiation markers such as SMα-actin, SM2-MHC, h-caldesmon and SM-MHC via activation of the downstream Gs/AC/cAMP/PKA pathway [33]. In addition, iloprost has been shown to induce expression of COX-2, upregulation of 6-keto PGF1α, in addition to inducing prostacyclin release in human vascular SMCs via independently-related prostacyclin signaling pathways (cAMP/PKA and ERK1/2 activation), and the inhibition of Akt-1 signaling [92].

Similarly, PGI2 has been implicated in reducing rates of proliferation and migration in VSMCs in vitro and in vivo [64]. Similar to COX-2 inhibition, defective PGI2 signaling via the IP receptor has been shown to be associated with increase adverse cardiovascular events [93]. Furthermore, Arehart et al (2008) demonstrated that defective human IP receptor increased the rate of atherothrombosis in patients attributable to decreased cAMP signaling in the platelets and VSMCs, which may contribute to reduced ability to inhibit thrombosis as well as VSMC proliferation and de-differentiation respectively [93].

1.4.2.2 Prostaglandin D2 (PGD2)

PGD2 is a prostaglandin which has been shown to inhibit platelet aggregation and nitric oxide release, as well as act as an inductor of sleep and vasodilation [94]. Though there is paucity of information regarding the effect of PGD2 in modifying the phenotype of vSMCs, 15d-PGJ2 which is a stable naturally occurring metabolite of PGD2 has been demonstrated to increase mRNA
expression levels of differentiation markers, thus promoting smooth muscle cell 
differentiation [95-99]. This effect of 15d-PGJ₂ may be mediated via activation of 
the peroxisome proliferator-activated receptor gamma (PPARγ) which is also 
expressed in VSMCs [100-102]. Furthermore, treatment of cultured human 
vascular SMCs with 15d-PGJ₂ has been shown to promote differentiation [95-97].

**PGD₂ signaling**

There exist two forms of PGD₂ synthase, lipocalin-type (L-PGDS) and 
hematopoietic-type (H-PGDS). L-PGDS is mainly responsible for PGD₂ synthesis 
[94]. Also, two receptors, DP1 and DP2, are associated with PGD₂ signaling. 
DP1 receptor activation due to ligand binding results in an increase in 
intracellular cAMP while DP2 receptor activation inhibits cAMP synthesis and 
increases intracellular calcium [82].

### 1.4.2.3 Thromboxane A₂ (TXA₂)

Thromboxane A₂ (TXA₂) is produced in platelets as well as in monocytes 
and VSMCs, and is involved in promotion of platelet aggregation and 
vasoconstriction [63, 77]. TXA₂ has been shown to produce opposing actions to 
PGI₂ in that TXA₂ is a potent vasoconstrictor and platelet activator working in 
concert with PGI₂ (vasodilator) to maintain vascular homeostasis [89]. An 
imbalance in the existence of both prostanoids may contribute to cardiovascular 
dysfunction observed in most vascular diseases [83].
TXA₂ signaling

TPα and TPβ are the two isoforms of the TP receptor, activation of which results in calcium ion mobilization [82]. These TP isoforms may homodimerize (e.g. TPβ/TPβ) or heterodimerize with each other (TPα/TPβ), or with the IP receptor. The TP receptor may couple with Gq to activate PLC or with G13 to activate RhoGEF thus promoting platelet activation, aggregation and vasoconstriction [63].

1.4.2.4 Prostaglandin F₂α (PGF₂α)

PGF₂α is formed from the reduction of PGH₂ due to the action of the PGF endoperoxide reductase/synthase (PGFS). However, this prostanoid may also be formed from PGE₂ through 9-keto reductases or from PGD₂ through 11-keto reductases [84]. Two forms of PGFS have been identified, PGFS-1 and PGFS-2, and two human splice variants of the FP (FPₐ, FP₈) exist [82]. Activation of the FP receptor has been reported to trigger Gq protein signaling which may be linked to cellular proliferation or activation of Rho and may result in changes in cell cytoskeleton and morphology [84].

1.4.2.5 Prostaglandin E₁ (PGE₁)

PGE₁ has been shown to induce and increase proliferation as well as the rate of phenotypic modulation of rat aortic SMCs from the contractile to the synthetic
state early in primary culture. However, later on in the culture, PGE₁ inhibited DNA synthesis and cellular proliferation [77].

1.4.2.6 Prostaglandin E₂ (PGE₂)

PGE₂ is the principal prostanoid secreted during inflammation and is involved in production of proinflammatory cytokines resulting in alteration of vascular SMC contractile function, increased vascular permeability, dilation and pain [4, 52, 85, 103-105]. In addition, PGE₂ has been reported to play a key role in epithelial physiology especially in gastrointestinal tissues [79]. PGE₂ is synthesized by the action of the Prostaglandin E₂ synthase (PGES) enzyme which exists in 3 isoforms: cytosolic (c-PGES), type 1 membrane-associated (mPGES-1) and type-2 membrane associated (mPGES-2) [106, 107]. cPGES and mPGES-2 are expressed constitutively, with cPGES coupling predominantly with the constitutive COX-1 enzyme while mPGES-2 couples with both COX-1 and COX-2 [85, 105, 108, 109]. cPGES is a cytosolic protein constitutively expressed in a variety of tissues and is generally resistant to stimulation by proinflammatory stimuli [110, 111]. mPGES-2 is mainly expressed in the heart and brain, however, its biological function is not well described [111]. mPGES-1, (also known as microsomal prostaglandin E₂ synthase) belongs to the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily [112]. This is the inducible isoform which is associated with the perinuclear membrane, and is generally found to be co-expressed and
functionally coupled with COX-2 in response to a range of extracellular and intracellular stimuli and downregulated by anti-inflammatory glucocorticoids [17, 85, 106, 108, 111, 113-120]. In addition, in most cells, the most prominent PG produced during the delayed response is PGE\(_2\) associated with COX-2 expression, and COX-2 inhibitors reduce PGE\(_2\) production more profoundly than the other prostanoids [85]. The majority of the PGE\(_2\) formed in response to COX-2 induction is dependent on mPGES-1 [121-123]. Recent research has also demonstrated that PGE\(_2\) can also increase COX-2 expression. For example, PGE\(_2\) has been found to increase COX-2 and cPLA2 expression through activation of MAPK and NF\(\kappa\)B pathways [124, 125]. In contrast however, Chandrasekharan et al (2005) reported that PGE\(_2\) production in the murine mammary gland depends on functional coupling of the COX-1 enzyme with mPGES-1[79].

**PGE\(_2\) signaling**

PGE\(_2\) activity is modulated via activation of its G-protein-coupled receptors: EP1, EP2, EP3 and EP4 which are structurally and functionally distinct [105, 126]. These receptors have unique tissue expression profiles and their effects are mediated through different downstream signaling pathways [79, 127-129]. EP2 and EP4 are coupled to Gs and activation of these receptors cause increases in intracellular cAMP levels. Furthermore, EP4 signaling pathway may involve phosphatidyl inositol 3-kinase (PI3K), resulting in protein kinase B (Akt) and extracellular signal-related kinases (ERK) activation [83]. The EP3 receptor which has 8 different splice variants is coupled to both G1 and Gq; and activation
may result in increased intracellular Ca\textsuperscript{2+} levels, reduce, or increase cAMP production depending on the expression of the EP3 splice variant(s) in the cell [79, 82].

In vascular SMCs, the effects of PGE\textsubscript{2} in regulating differentiation will depend on the EP receptor(s) expressed in the cell type. Studies have shown differential expression of the different EP receptors in various disease states and cell culture models [5, 104, 130, 131]. For example, a variety of the EP receptors including EP2, EP3, and EP4 have been shown to be expressed in cultured SMCs obtained from AAA tissues, however only EP2 and EP3 receptors were found in cultured human aortic smooth muscle cells [126]. Expression of these receptors has also been shown to be dependent on vascular SMC culture conditions, with EP4 showing greatest expression and EP1 and EP2 being undetectable under growth-promoting conditions [132]. EP4 receptor protein has been shown to be present in AAA lesions of both mice and human samples and inhibition of this receptor in mice results in reduction of the incidence and severity of AAAs [133, 134].

PGE\textsubscript{2} has been demonstrated to alter actin organization in cultured rat vascular SMCs, which may indicate an effect on de-differentiation [104]. In addition, hyaluronan synthase and MMP activity, which are characteristics of the de-differentiated phenotype, have been shown to be stimulated by PGE\textsubscript{2} in several cell culture and vascular disease models [34, 135-141]. In other cell types, EP receptor expression has been shown to change under different states of differentiation. In chondrocytic cells for example, increased COX-2 expression
was shown to correlate with increased expression of EP2 and EP3a1 mRNA, whereas no change was observed with the EP1 and EP4 receptors [142]. In addition, reduced differentiation of renal glomerular cells induced by PGE$_2$ was suggested to occur via EP4 upregulation and activation [143]. Furthermore, expression of EP4 in the ductus arteriosus, has been shown to increase with advancing gestational age [144, 145]. Thus, these reports suggest that there are changes in EP receptor expression under different states of cell differentiation.

Even though individual prostanoids and their analogs demonstrate a high degree of cognate receptor selectivity, the level of specificity depends on the ligand concentration, as prostaglandin analogues selective for one receptor at a given concentration may lose selectivity at higher concentrations and exhibit cross-reactivity with multiple prostanoid receptors [146]. The potential for nonselective effects of prostanoids or analogues increases the complexity of identifying novel functions of these mediators. The complexity of analysis is further enhanced given that prostanoid action involves the enzymatic activity of phospholipases, cyclooxygenases, and prostanoid synthases, together with specific receptors activating unique intracellular events. Therefore, the complete characterization of a prostanoid-dependent biological response may require the understanding of coordinated processes involving numerous enzymatic and signaling pathways.

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Abdominal aortic aneurysm (AAA) is a cardiovascular disease affecting 1 in every 10 adults over 55 years of age. Ruptured AAAs are the 13\textsuperscript{th} leading cause of death in the United States and the 10\textsuperscript{th} leading cause of death in Caucasian men 65 – 74 years of age in the United States. It is characterized by progressive dilation of the aorta within the abdominal region which may result in rupture and sudden death if left. Unfortunately, there are no medications approved for the prevention or treatment of this disease leaving surgery is the only option. Surgery to repair AAA however, is associated with significant risk of morbidity and mortality, and is therefore reserved for the most severe cases of the disease. For patients with smaller aneurysms who do not qualify for surgical repair however, the medical approach recommended is “watchful waiting” [18, 24, 69, 70, 76].

Recent findings have demonstrated that during the development of this disease, one of the characteristics observed during the pathological remodeling observed within the aorta is the alteration of the differentiation state of the vascular smooth muscle cells (SMCs) within the aneurysmal lesion. The cells in the contractile differentiated state undergo a process of de-differentiation into the synthetic de-differentiated state which is characterized by a loss in the expression of differentiation markers and increase expression of markers of the synthetic phenotype. In human biopsies obtained from patients undergoing aneurysmal repair, Ailawadi et al observed increased expression of markers of the de-differentiated phenotype and decreased expression of markers of the differentiated phenotype compared with normal aorta [53]. Furthermore, using a
mouse model of AAAs induced by chronic angiotensin II (AngII), the progression of AAAs has been shown to be associated with reduced differentiation of SMCs in the abdominal aorta. The treatment of these mice with celecoxib, an inhibitor of the inducible cyclooxygenase-2 (COX-2) enzyme, was effective at attenuating AngII-induced AAA progression, and this was associated with maintenance of a differentiated SMC phenotype in the aorta [74]. These findings suggest that COX-2 may play a role in altering the differentiation state of SMCs within the aorta during development of this disease.

Together with COX-2, other components of the prostanoid synthesis pathway have been implicated in the pathogenesis of AAA. In aneurysmal tissue, the increased expression of COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1), and the increased synthesis of prostaglandin E2 (PGE2) have been observed, as compared to uninvolved aorta [71, 126, 147-151]. Genetic inactivation or pharmacological inhibition of COX-2 in mouse AAA models significantly reduces AAA incidence and severity compared with wild-type mice [71, 148]. Even at the site of ruptured AAAs in humans, the COX-2 gene was found to be overexpressed in aneurysmal lesions compared to normal aortic tissues [151]. Wang et al (2008) demonstrated that deletion of mPGES-1 (an enzyme that acts downstream of COX-2) in AngII-induced AAA mouse models significantly attenuated the incidence and severity of AAAs [128]. Similarly, pharmacological or genetic inhibition of the prostaglandin E subtype 4 receptor (EP4) also attenuates the incidence and severity of AAAs in both human and mouse models of this disease [133, 134]. Despite these promising findings
however, because certain COX-2 inhibitors have been associated with increased risk of adverse side effects in humans, COX-2 inhibitors have not been approved for treatment of this disease in patients. It is therefore important to identify mediators downstream of this enzyme which will provide new targets for the treatment of this disease.

With previous findings of the effectiveness of COX-2 inhibition in attenuating the development and progression of AAAs in a mouse model being associated with maintenance of a differentiated SMC phenotype, the primary objective of the current research is to identify mechanisms by which COX-2 affects SMC differentiation using an in vitro cell culture model of human aortic smooth muscle cells (hASMCs). In order to examine this, the following hypothesis was proposed: Increased prostaglandin E2 (PGE2) synthesis in the hASMCs due to the induction of COX-2 and mPGES-1 may result in reduced differentiation of SMCs, and that disruption of this pathway would preserve the differentiated phenotype. hASMCs were utilized to assess the role of COX-2 and mPGES-1-dependent production of prostaglandin E2 (PGE2) in contributing to reduced SMC differentiation. This project has 3 specific aims:

(1) Examine the role of COX-2 in hASMC phenotypic modulation
   a. Correlate COX-2 levels with changes in the hASMC phenotype in vitro
   b. Investigate the effect of pharmacological inhibition of COX-2 on the hASMC phenotype
(2) Investigate the importance of AngII in hASMC phenotypic modulation

a. Examine the effect of AngII treatment on the hASMC differentiation state
b. Correlate AngII-induced phenotypic changes with the expression of COX-2 and mPGES-1

(3) Determine the role of PGE2 synthesis by COX-2 and mPGES-1 in the induction of hASMC de-differentiation

a. Examine the effect of exogenous PGE2 treatment on hASMC phenotypic modulation.
b. Determine the effect of pharmacological inhibition of mPGES-1 on preventing hASMC de-differentiation
c. Using siRNA mediated knock-down of mPGES-1, determine the effect of reduced mPGES-1 expression and PGE2 synthesis on preventing de-differentiation

A better understanding of the role of the COX-2/mPGES-1/PGE2 pathway in hASMC de-differentiation will be an important step forward in the discovery and development of therapeutics for manipulation of the SMC phenotype, where altered SMC differentiation may be a significant contributor to disease progression.
CHAPTER 3: MATERIALS AND METHODS

3.1 Cell culture

Human aortic smooth muscle cells (hASMCs) were purchased from Cascade Biologics (Invitrogen) and cultured according to manufacturer instructions using recommended media (Medium 231) with supplements to induce either growth or differentiation. The basal culture medium used (Medium 231) was made up of essential and non-essential amino acids, vitamins, other organic compounds, trace minerals, and inorganic salts [152]. Growth promoting media was supplemented with 4.9% fetal bovine serum (FBS), heparin (5 ng/ml), human basic fibroblast and epidermal growth factors (2 ng/ml and 0.5 ng/ml respectively), and recombinant human insulin-like growth factor (2 µg/ml). Differentiation-promoting media was made up of Medium 231 supplemented with 1% FBS, and heparin (30 µg/ml). Both growth-promoting and differentiation-promoting media also contained 1% penicillin and streptomycin. Cells used in all experiments were from passages 2 – 8 and were cultured in a humidified 5% CO₂ atmosphere at 37°C. Cells were cultured under either growth-promoting conditions with growth-promoting media, or under differentiation-promoting conditions with differentiation-promoting media. Varying concentrations of drugs or prostanoid receptor agonists/antagonists were used to treat cells prior to lysis and harvest after specific time periods.

In order to induce differentiation in cells cultured under growth-promoting conditions, the cell media was replaced with serum-deprived media and
maintained under these conditions over time. In order to induce expression of COX-2, or mPGES-1, growing cells were serum-starved overnight and then re-stimulated with serum-containing growth-promoting media.

3.1 Cell lysis and protein collection

After cell culture and treatment, cell culture media were either collected for further analyses or discarded at pre-determined time points and adherent cells were rinsed briefly two times with 1XPBS. Cells were lysed with equal volumes of RIPA lysis buffer made up of 200 mM Tris with pH 7.6, glycerol, 10% SDS, 10% deoxycholate, Triton X-100, 200 mM EDTA containing protease and phosphatase inhibitors (PMSF and leupeptin). Cells lysates were subsequently scraped and pipetted into 1.5 ml Eppendorf tubes for storage at -20°C until further use.

Prior to protein separation via SDS PAGE, protein content in each sample was analyzed using the Bicinchoninic Acid (BCA) method and specific volumes corresponding to equal amounts of protein was added to fixed volumes of loading buffer and heated at 100°C for 10 minutes. The loading buffer was made up of 0.5 M Tris (pH 6.8), glycerol, 10% SDS, 0.5% bromophenol and 5% β-mercaptoethanol. After heating, the tubes were vortexed briefly, spun-down using a microcentrifuge and then loaded unto polyacrylamide gels for protein separation via western blotting.
3.3 Western Blot Analysis

Equal amounts of cell lysates were loaded unto 12% polyacrylamide gels, separated via SDS-PAGE and transferred unto nitrocellulose membranes (GE Healthcare). Membranes were blocked for 1 hour with 5% bovine serum albumin in Tris-buffered saline containing 1% Tween 20. Blots were then incubated overnight at 4°C with specific primary antibodies. Antibodies against SMα-actin (Sigma Aldrich), and SM22α (Abcam Inc.), were used as markers of differentiation while de-differentiation was measured by a corresponding decrease in these protein markers. Either α-tubulin (Cell signaling) or calnexin (Cell signaling) were used as loading controls. Monoclonal α-actin primary antibody was fluorophore conjugated with Cy3, while biotinylated anti-rabbit secondary antibodies together with fluorophore-conjugated streptavidin complexes were used for detection of SM22α, α-tubulin and calnexin. For COX-2 (Vector laboratories) and mPGES-1 (Oxford biomedical research) detection, horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG were used as secondary antibodies. Protein detection of fluorophore-conjugated antibodies was done via fluorescence imaging using the Typhoon laser scanner (GE Healthcare), while HRP-conjugated antibodies were detected by enhanced chemiluminescence. Images were analyzed and quantified via densitometry using the Imagequant analysis software (GE Healthcare).
3.4 mRNA isolation

mRNA was isolated from the hASMCs using the RNeasy kit (Qiagen Inc., CA, USA) according to manufacturer’s instructions. The procedure combines selective binding properties of a silica-based membrane with the speed of microspin technology [153]. Briefly, cultured cells were lysed and homogenized with QIAshredder spin columns in the presence of a highly denaturing guanidine-thiocyanate-containing buffer which inactivates RNases, thus ensuring purification of intact RNA. Next, one volume of 70% ethanol was added to the homogenized lysate, thus providing appropriate binding conditions and then transferred to an RNeasy spin columns thus ensuring that total RNA binds to the membrane and contaminants are washed away. Subsequent washes of the spin columns with RNase-free water yielded high quality RNA elutes for further analysis.

3.5 cDNA preparation via reverse transcription

Total RNA was reverse transcribed in a two-step process adapted from an Invitrogen™ protocol [154]. The first step consisted of adding mRNA to a mix containing random hexamers (1: 60 dilution), deoxynucleotidetriphosphate mix (dNTPs), and DEPC water in Eppendorf tubes. This mixture was heated for 5 minutes at 65°C on a thermal cycler (Eppendorf). To this was added a second mix consisting of 5X reverse transcription buffer, 0.1 M DTT (dithiothreitol), RNAse inhibitor, and Superscript II reverse transcriptase. Eppendorf tubes
containing the final mixture were placed back on the thermal cycler and then heated at 25°C for 10 minutes and then increased to 42°C for 50 minutes. After these heating cycles, the reaction was terminated by heating at 70°C for 15 minutes and the tubes were stored at -20°C prior to being used for gene expression analysis.

3.6 Quantitation of gene expression via RT-PCR

mRNA expression and quantitation of gene expression was performed via the two-step Real-Time Polymerase Chain Reaction (RT-PCR) procedure. Here, the PCR step using Superscript II (Invitrogen) was coupled with fluorogenic 5’ nuclease chemistry (Taqman) using primer/probe assays for smooth muscle α-actin, SM22α, COX-2, microsomal PGE2 synthase (mPGES-1), matrix metalloproteinase -2 (MMP-2), smoothelin, myosin heavy chain 2 (MyhII), and hyaluronan synthase 2 (Has2) (Applied Biosystems (Foster City, CA). mRNA expression of the constitutively expressed housekeeping gene hypoxanthine phospho-ribosyl transferase (HPRT) was quantitated as an internal normalizing control gene. Relative gene expression levels were calculated by comparing cycle times for each target PCR using the the $\Delta\Delta$ct method. To do this, the target PCR Ct values were normalized by subtracting the HPRT Ct value, to give the $\Delta$Ct value. The relative expression levels between treatments were then calculated using the equation: relative gene expression = $2^{-(\Delta\text{Ct}_{\text{sample}}-\Delta\text{Ct}_{\text{control}})}$
3.7 PGE$_2$ Enzyme Immunoassay

PGE$_2$ concentrations in cell culture media were determined using a PGE$_2$ enzyme immunoassay (EIA) monoclonal kit according to the manufacturer’s protocol (Cayman chemical company). This is a competitive assay between the PGE$_2$ present in the cell culture media and a PGE$_2$-acetylcholinesterase conjugate (PGE$_2$ tracer) for a limited amount of PGE$_2$ monoclonal antibody [155]. The wells in the plate are pre-coated with goat polyclonal anti-mouse IgG and blocked with a proprietary formulation of proteins. Each well is incubated with a fixed amount of the PGE$_2$ tracer, cell culture media containing free PGE$_2$ and specific PGE$_2$ monoclonal antibody for approximately 18 hours and stored at 4$^\circ$C. Since the amount of PGE$_2$ in the media varies, the amount of PGE$_2$ tracer that is able to bind to the PGE$_2$ monoclonal antibody will be inversely proportional to the concentration of PGE$_2$ in the well. The antibody-tracer complex binds to the goat polyclonal anti-mouse IgG used to pre-coat the wells. After incubation, the mixture is discarded from the wells and after a series of wash steps, Ellman’s reagent which contains a substrate to the acetylcholinesterase is added to the wells and the plate is placed on an orbital shaker and kept in the dark for 75 minutes to develop. An enzymatic reaction occurs between the acetylcholinesterase and the substrate producing a yellow color which absorbs strongly at 412 nm. The absorbance of the solution based on the intensity of this color is determined spectrophotometrically using a plate reader set to 405 nm. The amount of free PGE$_2$ present in the well is inversely proportional to the amount of PGE$_2$ tracer bound to the well during the incubation.
3.8 Immunocytochemistry

hASMCs were plated onto 12 or 24 well plates, fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking with 1% bovine serum albumin (BSA) for 1 hour, cells were incubated overnight in either Cy3-conjugated primary antibody for SMα-actin-Cy3 antibody (in 1%BSA) at 4°C. Alternatively other primary antibodies were detected with Cy3-conjugated secondary antibodies. Cell nuclei were visualized with SYBR green (AB systems) after incubation for 45 minutes at room temperature. Cells were washed with 1XPBS and plates were scanned and images were captured by the GE Healthcare Typhoon fluorescence imager after each incubation. Using the ImageJ analytical software, quantitation of fluorescent intensity/densitometry was obtained and the ratio of α-actin to SYBR green was expressed as the extent of differentiation observed in these cells.

3.9 mPGES-1 RNA interference via siRNA transfection

hASMCs were transfected with mPGES-1 siRNA using the Lipofectamine® RNAiMAX transfection reagent (Invitrogen, NY USA), and following the reverse transfection protocol as outlined by the company. 82.5 nM of predesigned annealed siRNA targeted to mPGES-1 (siRNA ID 15540, Life Technologies) and 3 uL of transfection agent were pre-plated onto empty 12 well plates and then cell suspension in growth media was added to the wells. After two days, the growth media was replaced with serum-free media for either 1 or 2
days, and cell media was collected for PGE$_2$ EIA analysis. Both protein and mRNA samples were isolated from replicate treatments of these cells and analyzed for phenotypic changes via western blotting or real time PCR respectively.

3.10 Statistical analyses

Each experiment was repeated at least three times and statistical analyses were carried out using the GraphPad Prism software version 5.04 (GraphPad Software Inc., San Diego, CA Prism 5.04). The mean and SEM were calculated for each parameter with a single treatment considered as $n$ of 1. hASMC phenotype comparisons between differentiated and de-differentiated cells were analyzed by two-way repeated measures ANOVA. Bonferroni post tests were used where indicated. To compare the significance of the difference between 3 or more treatment groups, one-way ANOVA test, followed by the Dunnett’s multiple comparison post-hoc tests was utilized. Unpaired Student’s t test was used to compare the effects observed between two groups. Statistical significance was defined as $P < 0.05$. 

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CHAPTER 4: CHARACTERIZATION OF THE HUMAN AORTIC SMOOTH MUSCLE CELL PHENOTYPE

Introduction

Phenotypic modulation is a unique characteristic of vascular SMCs demonstrated by their ability to switch their phenotype between the contractile or differentiated phenotype to the synthetic or de-differentiated phenotype in response to various external cues such as growth factors, inflammatory mediators, and cytokines [8-18]. These cells do not differentiate terminally but retain sufficient plasticity to switch back and forth along a continuum between the differentiated and de-differentiated phenotypes. This unique characteristic of vascular SMCs in phenotypic modulation is important in the regulation of vascular function in health and disease. Both phenotypic states are simply two extremes of the spectrum of vascular SMC phenotypic expression since cells expressing features of both phenotypes do exist [19-21].

The principal function of vascular SMCs within the vasculature is contraction, and this is mediated by the action of contractile proteins which are expressed at increased levels in mature differentiated SMCs. The action of SMC contraction and relaxation results in the alteration of the luminal diameter of the blood vessels and contributes to the regulation of blood pressure and blood flow [12, 46, 52]. Normal mature vascular SMCs exist in a quiescent non-proliferative state and demonstrate a well-differentiated contractile phenotype. Cells in this differentiated phenotype express greater levels of SMC contractile proteins such as smooth muscle α-actin, SM22α, SM myosin heavy chain, and smoothelin
compared with cells in the synthetic phenotype [7, 12, 24-29]. Furthermore, cells in the contractile phenotype proliferate slowly while expressing low levels of extracellular matrix proteins. On the contrary, cells in the de-differentiated phenotype express increased levels of synthetic markers such as hyaluronan synthase, collagens, and matrix-metalloproteinases, and have increased proliferation and migration rates [12, 16, 26, 28, 30-34].

Alteration of the differentiated vascular SMC phenotype plays a key role in contributing to vascular pathology. In certain vascular disorders such as atherosclerosis, restenosis following vascular injury, hypertension, and abdominal aortic aneurysms, a decrease in protein and mRNA expression of SMC differentiation markers has been observed. This results in a change from the differentiated to the de-differentiated phenotype within the SMCs of the affected regions within the vascular wall. An important characteristic of this change from the differentiated to the de-differentiated phenotype involves the increased synthesis of extracellular matrix components, such as collagen, hyaluronic acid and elastin [9, 30, 46-53]. The de-differentiation process, is also associated with the re-entry of the SMCs into the cell cycle which is accompanied by increased proliferation and migration rates [52]. This phenotypic shift to a less-differentiated phenotype, characterized by the reduction in differentiation markers and increase in markers of the synthetic phenotype may be a crucial component of the repair process [46]. When the vascular injury is resolved, the environmental cues within the vessel change, causing the SMCs to reacquire their normal contractile phenotype [9].
The addition of heparin to cell culture media and/or reduction of serum concentration are methods that have been shown to induce and maintain differentiation in smooth muscle cells. Heparin is a natural glycosaminoglycan demonstrated to possess potent anti-proliferative and anti-migratory activity in vascular smooth muscle cell in vitro and in vivo [156, 157]. Various molecular mechanisms have been proposed for these actions in vascular SMCs. Heparin has been shown to induce cell cycle arrest [158, 159], inhibit production of matrix degrading enzymes [156, 160], regulate expression of extracellular matrix proteins [161] induce growth-arrest specific genes [162, 163] and to inhibit the activation of mitogen-activated genes [164]. In addition, heparin has been shown to also promote the contractile phenotype in vascular SMCs by increasing differentiation marker expression [165-168]. This characteristic of heparin has been demonstrated to be mediated in part by the increase in TGFβ secretion, a factor known to enhance the differentiation of SMCs [9, 169-171].

In addition to induction of vascular SMC differentiation by heparin, the complete removal of serum albumin (serum deprivation) from cell culture media has been demonstrated to promote the differentiated phenotype in vascular SMCs [23, 44, 172, 173]. When SMCs are cultured under serum-free conditions over time, these cells are induced to differentiate with acquisition of the requisite morphology, contractile ability and increased expression of SMC differentiation markers at comparable levels to that of differentiated cells [23]. However, this induction of differentiation has been shown to be reversed upon administration of growth factors, or serum-containing media [172, 174].
Although a variety of different growth factors have been shown to induce the synthetic phenotype, the identification of factors responsible for inducing the contractile phenotype has been limited. One such endogenous factor that has been proposed to maintain a differentiated phenotype under conditions of serum-deprivation is the cellular repressor of E1A-stimulated genes (CREG). CREG is upregulated under conditions of serum-deprivation and has been shown to promote differentiation by increasing expression of contractile markers, inhibiting cell proliferation, and inhibiting the synthetic phenotype of vascular SMCs under these conditions [175].

In contrast to agents that promote differentiation, the presence of a variety of growth factors, and/or increased serum concentrations in the cell culture medium have been demonstrated to promote cell proliferation, migration, and phenotypic modulation from the contractile to the synthetic state in vascular SMCs [6, 166, 167, 174, 176, 177]. Peyton et al (2002) demonstrated that increased serum (10%) promotes vascular SMC proliferation by the induction of heme oxygenase (HO-1) mRNA and protein expression. This serum-induced increase in DNA synthesis was observed both in the presence and absence of growth factors [178]. Similarly, increased serum and/or growth factor stimulation in vascular SMCs have been observed to promote synthesis of proteoglycans thereby promoting the synthetic SMC phenotype [179]. Furthermore, platelet-derived growth factor (PDGF) has been shown to destabilize the cytosolic α-actin mRNA pool at the transcriptional level resulting in significant reduction in actin
mRNA and protein, thus promoting the de-differentiated SMC phenotype [180, 181].

To characterize the phenotype of our in vitro cell culture model, human aortic smooth muscle cells (hASMCs) were cultured under different media conditions and the effect of culture media on the SMC phenotype was examined by measuring mRNA expression and protein levels of different markers of both the contractile and synthetic phenotypes.

Results

4.1 Induction of phenotypic modulation by cell culture media

4.1.1 α-actin and SM22α are expressed at greater levels in the differentiated phenotype compared with the de-differentiated phenotype

To induce differentiation in hASMCs, cells were cultured in differentiation-promoting media while de-differentiation-promoting media was used to promote the synthetic phenotype. Compared to media promoting the de-differentiated phenotype, differentiation-promoting media was made up of increased amounts of heparin (6000-fold) and reduced amounts of fetal bovine serum (1%). On the other hand, de-differentiation-promoting media contained increased serum (5-fold), reduced heparin, and a variety of growth factors.

Cells were cultured under both conditions over time and after each time point, cells were lysed and then either harvested for analysis via western blotting
or alternatively, mRNA was collected for real-time PCR analysis. Significant increase in α-actin and SM22α were used as the major markers of differentiation.

The level of α-actin expression was compared between differentiated (DC) and de-differentiated conditions (d-DC) at each individual time-point. Significantly increased α-actin mRNA expression was observed in cells cultured under differentiation-promoting conditions compared with cells cultured under de-differentiation-promoting conditions at each time point (Figure 4.1A). This finding was also observed in the protein levels of α-actin with significantly greater levels observed in cells cultured under differentiation-promoting conditions compared with cells cultured under conditions promoting de-differentiation. In addition, over the 5-day time period there was a significant increase in α-actin protein levels in cells cultured under conditions promoting differentiation but this was not observed under de-differentiation promoting conditions (Figure 4.1B). With SM22α, significantly increased protein levels was observed in cells cultured under differentiation promoting conditions but this increase ceased to be significant beyond the 3rd day (Figure 4.1C).

In addition, even though protein levels of both α-actin and SM22α were significantly increased under conditions promoting differentiation compared with growth, there was greater increase in the expression levels of α-actin compared with SM22α. There was a 12-fold increase in α-actin levels at the 5 day time-point when compared with 1 day in cells cultured under differentiation-promoting conditions. For SM22α however, the increase observed was only 5-fold. Furthermore, the fold increase in protein levels when comparing cells in
differentiation media to growth at every time point was greater with α-actin than SM22α (Figure 4.1.1B & C).

**Figure 4.1.1** α-actin and SM22α are expressed at greater levels in the differentiated phenotype compared with the de-differentiated phenotype

(A) mRNA expression of α-actin in hASMCs cultured for 1 to 4 days under conditions promoting differentiation or growth. Densitometry and representative western blot showing protein levels of (B) α-actin and (C) SM22α, in hASMCs cultured for 1 to 5 days under conditions promoting differentiation (DC) or de-differentiation (d-DC). Data represents mean ± SEM (n≥3), * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001; two-way ANOVA used to determine differences due to culture media and time. Bonferroni multiple comparisons examining α-actin expression due to culture media were conducted within each time point. α-actin expression after 1 day in differentiation-promoting media was used as control.
Figure 4.1.1 (continued)

A. \( \alpha \)-actin mRNA

\[ \frac{\alpha \text{-actin}}{\alpha \text{-tubulin}} : \text{HPRT} \]

Time course

B. \( \alpha \)-actin

Percent of control
\( \frac{\alpha \text{-actin/}\alpha \text{-tubulin}}{\text{control/}\alpha \text{-tubulin}} \)

Time course
4.1.2 Expression of hyaluronan synthase 2 is increased in the synthetic phenotype

Hyaluronan synthase 2 (HAS2) is a marker of the synthetic phenotype associated with the pathological remodeling of the vascular wall during development of various vascular diseases [74,182,183]. Cells were cultured under conditions promoting either de-differentiation or differentiation for 2 days. Cells were lysed and harvested both at the 1 day and 2 day time points. mRNA
was isolated and analyzed for HAS2 expression via real time PCR. An increase in HAS2 was used to indicate de-differentiation in these cells.

In contrast to α-actin and SM22α expression patterns, cells cultured under differentiation-promoting conditions expressed significantly lower levels of HAS2 at each time point compared with cell cultured under conditions promoting de-differentiation. Furthermore, under both conditions over time, HAS2 expression was significantly reduced evidenced by greater expression of HAS2 after 1 day compared with 2 days.

Figure 4.1.2 HAS2 expression is increased in the synthetic phenotype

mRNA expression of HAS2 in hASMCs cultured for 1 or 2 days under conditions promoting either differentiation or de-differentiation. Data represents mean + SEM (n≥3), **** = p<0.0001; two-way ANOVA to determine differences in HAS2 expression due to culture media and time. Bonferroni multiple comparisons examining HAS2 mRNA expression due to culture media at each time point.

HAS2 mRNA

![Graph showing HAS2 mRNA expression over time](image)

<table>
<thead>
<tr>
<th>Time course</th>
<th>DC</th>
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<tr>
<td>d1</td>
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<td>d2</td>
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**** = p<0.0001
4.2 Induction of differentiation by serum-deprivation

As seen in Figure 4.1, cells cultured over time in differentiation-promoting media are induced to express increased levels of differentiation markers with a significant increase in levels of α-actin and SM22α right from the first day of culture. As an alternative method of inducing differentiation and examining the effects of various treatments under conditions without the potential for interaction with serum-containing components, cells were cultured in de-differentiation-promoting media for 2 days and then deprived of serum over time. Cells were lysed and harvested daily over a 5-day time period and subsequently analyzed for protein levels of differentiation markers (α-actin and SM22α) via western blotting. Cells cultured for 2 days under conditions promoting de-differentiation which were not exposed to the serum-deprived medium were utilized as controls.

Control cells expressed nearly undetectable levels of α-actin and SM22α and these levels remain reduced even after introducing serum-free medium to these cells for 1 day. However, after 2 days in serum-deprived media, the cells began to express increased levels of α-actin, and this trend was significant after 3 to 5 days of serum-deprivation compared to controls. With SM22α protein levels, significant increase was observed from the 3rd day in serum deprivation media up to the 5-day time point. Compared to controls, the increase observed in α-actin protein levels over time was much greater than with SM22α protein levels. At the 3 day time point, α-actin levels were about 20-fold greater while about a 1.5-fold increase was observed with SM22α. By the 5th day in serum-deprivation media, over a 50-fold increase was observed with α-actin, whereas
only about a 3-fold increase was observed with SM22α.

**Figure 4.2 Serum deprivation over time induces differentiation in hASMCs**

Densitometry and representative western blot showing protein levels of (A) α-actin and (B) SM22α, in hASMCs cultured under conditions of serum-deprivation for 5 days. Control cells were not exposed to serum-free media. Data represents mean ± SEM (n≥3), * = p < 0.05, *** = p<0.001, **** = p < 0.0001; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.
Figure 4.2 (continued).

Discussion

Normal mature blood vessels under physiological conditions possess vascular smooth muscle cells that exist in the contractile, differentiated, and quiescent state. However, during the progression of cardiovascular diseases such as atherosclerosis, restenosis due to vascular injury, and aortic aneurysms, pathological remodeling of the vessel wall is observed. During these disease processes, a phenotypic switch from the contractile to the synthetic phenotype with decreased mRNA and protein expression of SMC markers of differentiation has been reported [9, 46-53, 175].
Both α-actin and SM22α are proteins which have been shown in the literature to be markers of the differentiated state [184-186]. Greater levels of α-actin are expressed in mature, differentiated vascular SMCs and this actin isoform is the predominant marker of differentiation in these cells. Furthermore, α-actin is the single most abundant protein in the cells, accounting for approximately 40% of the cell protein population [46]. As shown in Figure 4.1.1A & B, α-actin mRNA and protein levels were significantly increased at each time point in hASMCs cultured under conditions promoting differentiation, as compared to cells under de-differentiation-promoting conditions. This significant increase was consistent over multiple days of culture, thus suggesting that the quantitation of α-actin protein expression is an effective method of identifying the differentiated state in these hASMCs. In addition, the interaction between both the cell culture media as well as the duration of cell culture both have a significant effect on the increased expression of α-actin in hASMCs cultured under conditions promoting differentiation.

SM22α is a 22 kDa protein which serves as an actin-association protein involved in organization of the vascular SMC cytoskeleton and the regulation of vascular function [187]. This protein is also a well-established marker of the differentiated smooth muscle phenotype, as increased levels have been observed with vascular SMC differentiation [184-186, 188]. In these hASMCs, a significant increase in SM22α protein levels with differentiation-promoting conditions was observed, as compared to cells cultured under conditions promoting de-differentiation. However, this effect was seen only during the first 3
days of culture (Figure 4.1.1C). Compared with the differentiation-promoting media, the de-differentiation promoting media, which contained an increased concentration of serum together with the presence of additional growth factors, effectively induced de-differentiation of these hASMCs. In contrast, the contractile phenotype produced by the differentiation media resulted from the absence of high serum and additional growth factors, together with the presence of increased levels of heparin [6, 165-168, 174, 176, 177].

Increased confluence of vascular smooth muscle cells \textit{in-vitro} has been demonstrated to increase expression of markers of differentiation. There was no significant difference in SM22α levels between cells cultured under differentiation- or growth-promoting conditions at the 4 or 5 day time-points. Furthermore, in cells cultured under conditions promoting de-differentiation significant increase in the levels of α-actin and SM22α at the 5-day time point, was observed compared to 1 day. The tendency of differentiation marker expression to increase in cells cultured under de-differentiation-promoting conditions for prolonged time may be as a result of increased cell density and confluence, thus resulting in the induction of differentiation under these conditions [189].

Hyaluronic acid (HA) is linear polysaccharide possessing a loose and highly hydrated matrix which functions in tissue homeostasis and affects the cellular phenotype. Even though it may be synthesized by 3 main synthases (HAS1, HAS2 and HAS3), HAS2 is the predominant isoform detected in cultured human aortic SMCs [31, 190]. HAS2 mRNA expression was measured because
this isoform is known to be highly expressed in vivo during SMC phenotypic changes that occur during the development of cardiovascular pathologies including atherosclerosis, neointimal hyperplasia and abdominal aneurysm development [74, 182, 183]. At each time point of analysis (Figure 4.1.2), there was significant increase in HAS2 mRNA expression in cells cultured under de-differentiation-promoting conditions compared with differentiation-promoting conditions. Also, an inverse correlation between HAS2 and α-actin mRNA expression was observed (Figure 4.1.1), where the increase in α-actin was associated with a decrease in HAS2 expression. Thus, in addition to reduction in differentiation marker expression, HAS2 was utilized as the primary marker of de-differentiation in these cells.

As an alternative method of inducing differentiation, cells were cultured in de-differentiation-promoting media for 2 days and then serum-deprived over time. Under these conditions of serum-deprivation, a trend toward increased expression of α-actin and SM22α was observed, and these levels were significant from the 3-day time point up to the 5-day time point. Even though levels of both markers increased daily with serum-deprivation, an exponential increase in α-actin levels was observed over time, with up to a 50-fold increase after 5-days in serum deprivation media, compared to about a 3-fold increase in SM22α at the same time point. Therefore, similar to the cells cultured over time in differentiation-promoting media containing high levels of heparin, the serum deprivation method of inducing differentiation also determined that the
quantitation of α-actin expression was more effective than SM22α for identifying the differentiated phenotype.

In summary, for subsequent experiments, hASMCs were cultured either in differentiation-promoting media, or under conditions of serum-deprivation to induce the differentiated phenotype in these cells. In addition, de-differentiation-promoting media was used to induce the de-differentiated phenotype in cultured hASMCs. The observed increase in these markers of differentiation (α-actin and/or SM22α) in cultured cells was used to assess differentiation while decrease in these markers denoted reduced differentiation or de-differentiation. Furthermore, the significant increase in HAS2 expression was a characteristic of the synthetic phenotype in vitro. Even though the pattern of differentiation induction was similar between both differentiation markers, the fold increase in α-actin protein expression over time was greater than that observed with SM22α. Since α-actin expression was consistently expressed in cells cultured under conditions promoting differentiation, and was exponentially greater than SM22α expression, α-actin was selected as the primary marker of differentiation in these hASMCs.
CHAPTER 5: ANGIOTENSIN II TREATMENT CONTRIBUTES TO REDUCED DIFFERENTIATION IN HUMAN AORTIC SMOOTH MUSCLE CELLS

Introduction

Vascular smooth muscle cells (SMCs) are crucial components of the vessel wall which are responsible for maintenance of structural and functional characteristics such as contraction and relaxation, remodeling and repair, growth and development within the blood vessel [191]. These functions may be regulated by local and systemic factors resulting in the promotion of physiological processes, or mediating pathological mechanisms in the development of vascular diseases. One of such factors is angiotensin II (AngII), which is a potent, vasoactive, multifunctional peptide synthesized from its precursor (angiotensin I) by the angiotensin converting enzyme (ACE).

AngII is a pro-inflammatory mitogenic agent which has been demonstrated to regulate cell proliferation and apoptosis, influence cell migration and deposition of extracellular matrix within the vessel wall [191]. In in vitro vascular SMC culture models, AngII has been demonstrated to induce cell proliferation and migration, in part, by mediating an increase in mRNA and protein expression of the inducible cyclooxygenase enzyme (COX-2) [192, 193]. Alternatively, AngII treatment in vascular SMCs may also contribute to cell proliferation and survival by promoting release of proinflammatory cytokines such as IL-1β [194], inducing expression of growth-promoting factors such as IGFR-1 [195, 196] and PDGF-D [197] or by increasing gene expression of ROS and ET-1 in these cells [198]. All
these effects have been shown to be mediated by activation of numerous signaling pathways downstream of the Angiotensin II receptors [191].

Angiotensin II has also been reported to regulate the vascular SMC phenotype and contribute to vascular remodeling by regulating the expression of markers of both the contractile and synthetic phenotype, as well as altering the composition of extracellular matrix components. AT1 receptor activation by AngII has been demonstrated to induce mRNA expression of α-actin in vascular SMCs mediated partly by increased myocardin expression and SRF binding to CArG elements within the SM α-actin promoter [199]. Furthermore, AngII treatment in quiescent cells increased mRNA and protein expression of calponin also mediated by AT1 receptor activation [200, 201]. On the other hand, increase in osteopontin (synthetic marker) has been reported with AngII treatment in aortic SMCs [202]. Also, enhanced AngII signaling due to increased expression of ACE in miR-143/145 mutant mice is associated with a phenotypic shift in vascular SMCs from the contractile to the synthetic state [38]. These contrasting findings indicate that AngII stimulation is able to induce both differentiation and de-differentiation in vascular SMCs cultured under different conditions by activating different downstream signaling pathways.

In animal models of vascular disease, AngII treatment has been observed to contribute to disease pathology by mediating a phenotypic shift from the contractile to the synthetic phenotype in vascular SMCs. Medial hypertrophy of the aorta and coronary arteries in a model of AngII-induced hypertension in rats has been shown to be associated with increased expression of vascular SMC
synthetic markers, thus indicating a phenotypic shift to the immature de-differentiated phenotype [203, 204]. Research findings in our lab utilizing an AngII-infused mouse model of abdominal aortic aneurysms (AAAs) have demonstrated that increased expression of COX-2 in vascular smooth muscle cells within the aneurysmal lesion may contribute to pathogenesis of this disease [71, 148]. Furthermore, COX-2 may contribute to the formation of AngII-induced AAAs by altering the differentiated phenotype, because COX-2 inhibition with celecoxib preserves the differentiated phenotype and limits progression of AAAs in mice [74]. In addition to COX-2 induction, the inducible microsomal synthase enzyme (mPGES-1) and prostaglandin E2 have been associated with the pathogenesis of this disease in the AngII-induced mouse model [128, 205]. These findings suggest that AngII may promote the pathogenesis of AAAs by the induction of specific products of the prostanoid synthetic pathway.

In humans, aortic SMCs located within the aneurysmal lesion have been shown to exhibit reduced expression of SMC markers of differentiation compared to normal aorta [53]. In addition, there is increased expression of COX-2 and microsomal prostaglandin E2 synthase (mPGES-1), and explant tissue obtained from human AAA biopsies produce increased levels of prostaglandin E2 (PGE2) [147, 150, 151]. Due to the potential contribution of AngII to the disease pathology in humans, clinical studies have proposed that inhibition of AngII production resulting from treatment with ACE inhibitors may be beneficial in treating AAAs in humans [206-208].
Therefore, based on results showing increased expression of COX-2 and mPGES-1 during development of AAAs [147, 150, 151, 209, 210] as well as reports associating AngII with alteration of the vascular SMC phenotype [199-201], the hypothesis that treatment of vascular SMCs with AngII \textit{in vitro} will induce expression of COX-2 and mPGES-1, while reducing differentiation in these cells was proposed. In order to test this, human aortic smooth muscle cells (hASMCs) were cultured under different conditions and the cells were treated with varying concentrations of AngII over time. The effect of AngII treatment on the cell phenotype was studied by measuring protein levels of the markers of differentiation, $\alpha$-actin and/or SM22$\alpha$. In addition, the expression of COX-2 and mPGES-1 were also measured in order to examine the potential effect of AngII treatment on the expression of these.

\section*{Results}

\subsection*{5.1 Effect on $\alpha$-actin expression from treatment of differentiated hASMCs with angiotensin II under conditions promoting differentiation.}

Cells cultured under conditions promoting differentiation are induced to differentiate evidenced by the expression of higher levels $\alpha$-actin over time (see Figure 4.1.1). I hypothesized that treatment of hASMCs with AngII under differentiation-promoting conditions would reverse differentiation by significantly reducing the expression levels of markers of differentiation in these cells. To investigate the role of AngII in the modulation of the differentiated SMC
phenotype, hASMCs were cultured under conditions promoting differentiation for two days and thus induced to differentiate. The cells were subsequently treated with 0.1, 0.5, or 1 μM concentrations of AngII for either 1 or 2 days under these same conditions. Control cells were treated with 0.1% PBS for the same time period. Cells were lysed, harvested at each time point, and then analyzed for protein levels of α-actin via western blotting.

Treatment with AngII for 1 day did not result in reduction in levels of α-actin (Figure 5.1A). However, at the 2-day time point, a trend toward reduction in differentiation was observed, demonstrated by lower levels of α-actin in AngII treated cells compared with controls (Figure 5.1B).
Figure 5.1 Effect on α-actin expression from treatment of differentiated hASMCs with angiotensin II under conditions promoting differentiation.

Densitometry and representative western blots showing protein levels of α-actin in differentiated hASMCs treated with AngII for (A) 1 day or (B) 2 days under conditions promoting differentiation. Data represents mean ± SEM (n=3), percent change in α-actin protein levels in AngII treated groups compared with controls.
5.2 Treatment of differentiated hASMCs with Angiotensin II under de-differentiation-promoting conditions reduces α-actin protein levels

Since there was no significant reduction in α-actin levels in differentiated cells treated with AngII under differentiation-promoting conditions, the effect of treating the cells with AngII under de-differentiation-promoting conditions was subsequently examined. Vascular SMCs in the contractile differentiated state may be induced to de-differentiate and proliferate in the presence of media containing growth factors [9, 46]. Therefore, I hypothesized that AngII treatment of differentiated cells under growth-promoting conditions will accentuate or
promote the reduction in α-actin levels in AngII-treated cells compared with vehicle-treated controls.

Cells were plated for 2 days in differentiation promoting media and then treated with either 0.1 or 0.5 µM of AngII in growth-promoting media for 1 or 2 days. Control cells were treated with vehicle for the same time period. Cells were lysed, harvested at each time point, and analyzed for protein levels of α-actin via western blotting.

The lower concentration of AngII (0.1 µM) trended towards increasing α-actin expression whereas the higher concentration (0.5 µM) consistently reduced α-actin expression in these cells at each time point (Figure 5.2 A & B). Treatment with 0.1 µM AngII trended toward increase in α-actin levels while 0.5 µM showed reduction compared with vehicle-treated controls.

Since there was a trend toward reduction in α-actin expression with 0.5 µM AngII treatment, differentiated cells were treated daily with either 0.5 µM or 1.0 µM AngII over a longer time period (1 to 4 days) under de-differentiation-promoting conditions, and the effect on α-actin protein levels was examined via immunocytochemistry. Treatment for either 1 or 2 days did not result in significant reduction in α-actin expression (Figure 5.2 C&D). However, by the 3rd day, significant reduction in α-actin levels was observed with 1.0 µM AngII treatment. Furthermore, both 0.5 and 1.0 µM AngII treatments resulted in significant reduction in α-actin protein levels compared to vehicle-treated cells by the 4th day of treatment (Figure 5.2 E&F).
Figure 5.2 Treatment of differentiated hASMCs with Angiotensin II under de-differentiation-promoting conditions reduces α-actin protein levels.

Densitometry and representative western blots showing protein levels of α-actin in differentiated hASMCs treated with AngII for (A) 1 day or (B) 2 days under conditions promoting de-differentiation. Immunocytochemistry with fluorescent imaging quantitation showing α–actin expression in differentiated cells treated with AngII under de-differentiation-promoting conditions for (C) 1, (D) 2, (E) 3, or (F) 4 days. Data represents mean + SEM (n≥3); percent change in α-actin protein levels with AngII treatment compared with non-drug control.
Figure 5.2 (continued).

A. \( \alpha \)-actin protein

\[
\begin{array}{ccc}
\text{Percent of control} & \text{AngII (\( \mu \text{M} \))} \\
\text{(\( \alpha \)-actin/\( \alpha \)-tubulin)} & 0 & 0.1 & 0.5 \\
\end{array}
\]

B. \( \alpha \)-actin and calnexin

C. \( \alpha \)-actin protein

\[
\begin{array}{ccc}
\text{Percent of control} & \text{AngII (\( \mu \text{M} \))} \\
\text{(\( \alpha \)-actin/sybr green)} & 0 & 0.5 & 1.0 \\
\end{array}
\]
Figure 5.2 (continued)

D. Percent of control ($\alpha$-actin/sybr green)

E. Percent of control ($\alpha$-actin/sybr green)

F. Percent of control ($\alpha$-actin/sybr green)
5.3 Reduced α-actin levels in differentiated hASMCs treated with AngII under de-differentiation-promoting conditions is associated with increased mPGES-1 expression but not COX-2 expression

With the observation of a trend toward reduction in α-actin protein levels when differentiated cells were treated with 0.5 µM AngII under conditions promoting de-differentiation, protein expression levels of mPGES-1 and COX-2 at this concentration of AngII were subsequently examined. Cells were cultured under differentiation-promoting conditions and then treated with 0.5 µM AngII under conditions promoting de-differentiation for either 1 or 2 days. Control cells were treated with the vehicle (0.1% PBS) for the same time period. At each time point, cells were lysed and harvested and protein levels of COX-2 and mPGES-1 were examined via western blotting.

Treatment with 0.5 µM AngII did not affect COX-2 expression at either the 1 or 2 day time points. However, at the 2-day time point, there was significant increase in mPGES-1 protein levels.

In addition, protein levels of α-actin in these cells pursuant to mPGES-1 induction by AngII treatment, was subsequently examined. To do this, cells were differentiated as above and then treated with 0.5 µM AngII under de-differentiation-promoting conditions for 3 days. At the 3-day time point, cells were fixed and analyzed for α-actin protein levels via immunocytochemistry. Significant decrease in α-actin protein levels was observed in 0.5 µM AngII treated cells compared with vehicle-treated controls.
Figure 5.3 Reduced α-actin levels in differentiated hASMCs treated with AngII under de-differentiation-promoting conditions is associated with increased mPGES-1 expression but not COX-2 expression

Densitometry and representative western blots showing protein levels of (A) COX-2 or (B) mPGES-1 in differentiated hASMCs treated for 1 or 2 days with 0.5 μM AngII under conditions promoting de-differentiation. Data represents mean + SEM (n≥3), percent change in α-actin protein levels with AngII treatment compared with non-drug control. (C) Immunocytochemistry with fluorescent imaging quantitation showing α–actin expression in differentiated cells treated with 0.5 μM AngII for 3 days under conditions promoting de-differentiation. Data represents mean + SEM (n≥3), ** = p < 0.01; unpaired Student’s t test.
Figure 5.3 (continued)

mPGES-1 protein

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AngII (0.5μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 day</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**5.4 Angiotensin II treatment does not reduce α-actin protein levels in serum-deprived hASMCs**

In order to examine the effect of AngII in media without the potential for interference by protein-binding components of serum, serum-deprived hASMCs were treated with AngII at different time points. Serum-deprivation has also been shown to induce differentiation in vascular SMCs [23, 173, 174]. Therefore, based on the results showing that AngII treatment reduces α-actin levels in hASMCs (Figure 5.3), I hypothesized that AngII treatment will inhibit the induction of differentiation in serum-deprived cells. To test this hypothesis, hASMCs were
cultured under growth promoting conditions for 2 days and then the media was changed to serum-free media. AngII treatment was initiated at different time points during the serum-starvation period.

5.4.1 Effect on α-actin expression from the initiation of AngII treatment at the beginning of serum-deprivation

After culturing cells in growth promoting media for 2 days, the cell media was changed to serum-free media. At the 2 day time point in which cell media was changed to serum-deprived media, 0.5 µM or 1 µM AngII in serum-free media was added to these cells and incubated for 4 days. Vehicle-treated controls were incubated in 0.1% PBS in serum-free media. Cells were lysed and harvested each day over the four days and analyzed for α-actin expression via western blotting.

AngII treatment of growing cells under serum-deprivation conditions trended towards increase in α-actin protein levels (Figure 5.4.1). These results indicated that AngII treatment did not reduce α-actin expression in treated cells at either time point. Instead, there was an unexpected trend toward increase in α-actin expression in the AngII treated cells and this was significant at with 0.5 µM AngII treatment at the 2 day time point.

Figure 5.4.1 Effect on α-actin expression from the initiation of AngII treatment at the beginning of serum-deprivation

Densitometry and representative western blots showing protein levels of α-actin in growing cells treated for (A) 1, (B) 2, (C) 3, or (D) 4 days with 0.5 µM or 1.0 µM
AngII under serum-free conditions. Data represents mean + SEM (n=3), * = p < 0.05 one-way ANOVA with post hoc analysis with Dunnett’s Multiple Comparison test.
Figure 5.4.1 (continued)

C.

Percent of control
(α-actin/α-tubulin)

0 50 100 150 200

0 0.5 1.0

Control
AngII (μM)

α-actin

α-tubulin

D.

Percent of control
(α-actin/α-tubulin)

0 50 100 150 200

Control
AngII (μM)

α-actin

α-tubulin
5.4.2 Effect on α-actin expression from the initiation of AngII treatment after 2 days of serum-deprivation

As observed from preliminary data, after 2 days in serum starved media, hASMCs begin to commence the process of differentiation by expressing increasing levels of markers of differentiation (see Figure 4.2). I hypothesized that AngII treatment of cells at this 2 day time point under these conditions over time will result in inhibition of differentiation evidenced by significant reduction in expression levels of markers of differentiation. To test this hypothesis, hASMCs were cultured under growing conditions for 2 days and then the cell media was changed to serum-free media for two days. Next, either 0.5 or 1.0 µM of AngII was added into this cell culture media for between 1 to 3 days. Control wells were treated with vehicle (0.1% PBS). At each time point, cells were lysed and harvested for α-actin and SM22α protein levels via western blotting.

Treatment with AngII did not result in significant reduction in α-actin protein levels at each time point examined. Even though there was a trend toward decrease in SM22α levels after 1 day of treatment, this reduction however was not consistent over the 2 or 3 day time points (Figure 5.5.2).

Figure 5.4.2 Effect on α-actin expression from the initiation of AngII treatment after 2 days of serum-deprivation

Densitometry and representative western blots showing protein levels of (A) α-actin, or (B) SM22α in growing cells treated for 1, 2, or 3 days with 0.5 µM or 1.0
µM AngII under serum-free conditions. Data represents mean ± SEM (n=3), one-way ANOVA with post hoc analysis with Dunnett’s Multiple Comparison test.

1 day

A. \( \alpha \)-actin protein

\[
\text{Percent of control (\( \alpha \)-actin/\( \alpha \)-tubulin)}
\]

\[
\begin{array}{ccc}
0 & 0 & 0.5 \\
100 & 100 & 100
\end{array}
\]

\[
\begin{array}{c}
\alpha \text{-actin} \\
\alpha \text{-tubulin}
\end{array}
\]

B. SM22\( \alpha \) protein

\[
\text{Percent of control (SM22\( \alpha \)/\( \alpha \)-tubulin)}
\]

\[
\begin{array}{ccc}
0 & 0 & 0 \\
100 & 50 & 50
\end{array}
\]

\[
\begin{array}{c}
\text{SM22}\alpha \\
\alpha \text{-tubulin}
\end{array}
\]
Figure 5.4.2 (continued)

2 days

A. \( \alpha \)-actin protein

\[
\begin{array}{c|c|c}
\text{Percent of control (\( \alpha \)-actin/\( \alpha \)-tubulin)} & \text{Control} & \text{AngII (\( \mu \)M)} \\
\hline
0 & \text{---} & \text{---} \\
0.5 & 150 & 200 \\
1.0 & 100 & 150 \\
\end{array}
\]

\( \alpha \)-actin
\( \alpha \)-tubulin

B. SM22\( \alpha \) protein

\[
\begin{array}{c|c|c}
\text{Percent of control (SM22\( \alpha \)/\( \alpha \)-tubulin)} & \text{Control} & \text{AngII (\( \mu \)M)} \\
\hline
0 & \text{---} & \text{---} \\
0.5 & 150 & 200 \\
1.0 & 100 & 150 \\
\end{array}
\]

SM22\( \alpha \)
\( \alpha \)-tubulin
Figure 5.4.2 (continued)

3 days

α-actin protein

A.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Control</th>
<th>AngII (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

α-actin

α-tubulin

SM22α protein

B.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Control</th>
<th>AngII (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

SM22α

α-tubulin
5.4.3 Effect on α-actin expression from the initiation of AngII treatment after 3 days of serum-deprivation

As observed from preliminary data, hASMCs cultured initially under growth-promoting conditions and then switched to serum-free media are induced to differentiate during the serum-deprivation over time (See Fig 4.2). At the 3-day time-point of serum-deprivation, significant increase in the levels of differentiation markers was observed up till the 5-day time point. I hypothesized that treatment of cells with AngII at this 3 day time-point over time will reverse differentiation in these cells evidenced by reduced expression levels of differentiation markers.

To test this hypothesis, hASMCs were cultured under growing conditions for 2 days and then the cell media was changed to serum-free media for 3 days. At the 3-day time point, either 0.5 or 1.0 µM of AngII was added into this cell culture media for 1 or 2 days. Control cells were treated with 0.1% PBS for the same duration.

The results showed that AngII treatment had no significant effect on the reduction of differentiation in treated cells compared with vehicle-treated controls (Figure 5.4.3).
Figure 5.4.3 Effect on α-actin expression from the initiation of AngII treatment after 3 days of serum-deprivation

Densitometry and representative western blots showing protein levels of (A) α-actin, or (B) SM22α in growing cells treated for 1, or 2 days with 0.5 μM or 1.0 μM AngII under serum-free conditions. Data represents mean + SEM (n=3).

A.  α-actin protein

<table>
<thead>
<tr>
<th>Percent of control</th>
<th>Control</th>
<th>AngII (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

α-actin
α-tubulin

1 day
Figure 5.4.3 (continued)

**B.**

**SM22α**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AngII (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>120</td>
<td>150</td>
</tr>
<tr>
<td>1.0</td>
<td>130</td>
<td>170</td>
</tr>
</tbody>
</table>

α-tubulin

2 days

**A.**

**α-actin protein**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AngII (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>110</td>
<td>120</td>
</tr>
<tr>
<td>1.0</td>
<td>120</td>
<td>130</td>
</tr>
</tbody>
</table>

α-actin

α-tubulin
Figure 5.4.3 (continued)

**SM22α protein**

![Bar graph showing percent of control SM22α/α-tubulin with AngII (μM) vs Control]

**Discussion**

AngII is the active biological peptide of the renin-angiotensin system (RAS) known to have potent vasoconstrictor effects as well as involvement in both vascular and cardiac remodeling by acting on its receptors AT$_1$ and AT$_2$ [203, 211]. AngII is synthesized by a sequence of steps involving proteolytic cleavage of angiotensinogen to angiotensin I by renin and then subsequent cleavage of angiotensin I to AngII by the angiotensin converting enzyme (ACE). The AT$_2$ receptor expression is known to be expressed in fetal tissues and declines with maturity, but may begin to increase again during development of
certain cardiovascular diseases [212]. AngII signaling through the AT1 receptor has been shown to contribute to the known physiological effects of AngII in adult humans [213]. Under physiological conditions, ACE expression in the vasculature is low [38]. However, signaling due to aberrant activity of AngII within the vasculature may result in increased cardiac dysfunction and development of diseases [212, 214].

Various animal models have been used to demonstrate pathological effects of abnormal AngII signaling within the cardiovascular system. Chronic AngII infusion in animal models has been shown to increase the luminal diameter of the aorta, and systolic blood pressure, and induce the formation of aortic aneurysms in mice [215, 216]. Therefore, clinical studies have proposed that inhibition of Angiotensin II production by ACE inhibitors may be beneficial in treating AAAs in humans [206-208].

AngII-induced thoracic or abdominal aortic aneurysm formation in animal models has also been shown to be associated with the reduced expression of differentiation markers in the vascular SMCs of the aneurysmal lesion [217]. Research in our lab has also reported increased COX-2 expression, reduced differentiation marker mRNA expression, and increased expression of a marker of de-differentiation in SMCs of aneurysmal lesions in AngII-induced AAAs in mice. The effectiveness of celecoxib (COX-2 inhibitor) in treating this disease was associated with maintenance of a differentiated phenotype in these cells [74]. Based on these findings, together with the research showing that transformation of vascular SMCs from the contractile to the synthetic phenotype...
is associated with an increase in AngII production [218], I proposed that treatment of hASMCs with AngII would increase expression of COX-2 and mPGES-1, and reduce differentiation.

To study the AngII effects on the hASMC phenotype in vitro, cells were induced to differentiate and then treated with AngII at various stages of the differentiation process, as well as under different culture media conditions. Treatment of differentiated hASMCs with AngII under conditions promoting differentiation resulted in a non-significant trend toward a reduction in differentiation evidenced by reduced levels of α-actin over time (Figure 5.1). Treatment with AngII under de-differentiation-promoting conditions also demonstrated a trend toward reduction in differentiation especially at the 0.5 µM concentration. However, 0.1 µM AngII treatment resulted in a trend toward increase in α-actin expression at both 1 and 2 day time point. Next, the effect on α-actin protein levels, of daily treatment of differentiated cells with increased AngII concentrations over a longer time frame under de-differentiation-promoting conditions, was investigated via immunocytochemistry. Though there was no significant difference in α-actin protein levels after the first 2 days of treatment, treatment with either 0.5 µM or 1.0 µM AngII however resulted in significant reduction in α-actin expression both at the 3 and 4 day time points (Figure 5.2 E & F).

Furthermore, the effect of AngII treatment on the expression of mPGES-1 and COX-2 was examined. Treatment with 0.5 µM AngII under conditions promoting de-differentiation resulted in significant induction of mPGES-1 protein
levels after treatment for 2 days. There was however no significant increase in COX-2 expression levels with AngII treatment for either 1 or 2 days AngII (Figure 5.3). These findings were quite unexpected since in vitro SMC culture models indicate that AngII treatment increases COX-2 expression in vascular SMCs.

The induction of COX-2 expression due to AngII stimulation may be an early event in cultured vascular SMCs. AngII treatment in rat vascular SMCs was shown to significantly increase COX-2 mRNA expression after treatment for 1 hour and increase COX-2 protein levels after 4 hours. In this study, at the 24 hour time point, the COX-2 expression had returned to basal states [192]. Also, Frias et al (2003) observed significant increase in COX-2 levels in rat aortic smooth muscle cells following a 0.5 to 4 hour incubation with AngII [219]. Similarly, AngII treatment in vascular SMCs induced COX-2 mRNA accumulation, commenced 30 minutes after stimulation and increased up to 2 hours but had diminished greatly by 6 hours after AngII challenge [78]. Furthermore, these authors observed maximal induction of protein levels of COX-2 after 4 hours of stimulation with AngII after which COX-2 protein levels subsequently reduced over a 48 hour time period. Thus, there may have been no observable increase in COX-2 expression with AngII treatment in our studies due to the fact that these cells were analyzed for COX-2 protein levels after much longer AngII-treatment periods (24 to 48 hours), during which COX-2 expression had probably returned to basal states. It is probable that harvesting AngII-treated cells at earlier time points in this study may have demonstrated significant increase in COX-2 protein in these cells.
Alternatively, the lack of induction of COX-2 protein may be because in our studies, cells were treated with AngII under conditions promoting de-differentiation. This may have resulted in maximal induction of COX-2 such that it was no longer possible to induce COX-2 production with AngII treatment under these conditions. The increased presence of serum and growth factors has been shown to contribute to the expression of COX-2 in cells [220-223]. Since the induction of COX-2 previously observed in various in vitro models was observed in confluent quiescent cells SMCs [78, 192] which were stimulated with AngII in the absence of serum and for shorter periods of time, this major difference in treatment conditions may have further inhibited induction of COX-2 protein.

It is interesting to note also that significant reduction in α-actin levels did not occur in differentiated cells treated with AngII under de-differentiated-promoting conditions for 1 to 3 days (Figure 5.2). Only at the 4-day time point was there significant reduction in α-actin protein levels in these cells. This reduction occurred after the significant induction of mPGES-1 expression at the 2 day time point suggesting that the reduction in differentiation marker expression with AngII treatment may not occur early, but subsequent to the induction of mPGES-1 levels (Figure 5.3).

The effect of AngII treatment on the induction of differentiation via serum-deprivation was subsequently examined. Serum-deprivation promotes the differentiated state in vascular SMCs by inducing the expression of markers of differentiation. Furthermore, serum-deprivation provides a suitable condition in which we can examine the effect of AngII treatment without the potential for
interaction with serum-containing components. Regardless of the time point of AngII administration during the process of serum-deprivation, AngII treatment did not significantly reduce the expression of protein levels of α-actin or SM22α in these cells (Figure 5.4). In fact, there was a trend toward increase in the protein levels of differentiation markers in AngII-treated cells over time. These findings suggest that AngII may not induce reversal of differentiating cells to the synthetic phenotype in these hASMCs.

The absence of a suitable mitogenic trigger for the activation of pathological AngII signaling may be a major factor why there was no significant decrease in α-actin levels in serum-deprived hASMCs treated with AngII under the same conditions. AngII treatment under de-differentiation-promoting conditions introduces a variety of growth factors to the cell media which may provide the suitable milieu for AngII activity and signaling [212]. Thus, AngII treatment under conditions of serum-deprivation conditions may not have significantly reduced the expression of differentiation markers in these cells due to the absence of these mitogenic cues.

Furthermore, even though AngII treatment in confluent quiescent cells under conditions of serum-starvation has been shown to induce the expression of COX-2 in cells, this effect has been associated with the induction of cell proliferation [78, 192], not differentiation. Since COX-2 protein was not measured under these conditions, it is difficult to ascertain if COX-2 levels were upregulated with AngII treatment under these conditions of serum-deprivation. Regardless of the COX-2 expression profile under these conditions, there was a trend toward...
an increase in differentiation with AngII treatment (Figure 5.4.1). AngII-mediated increase in differentiation markers has been reported by other investigators. The increase in α-actin gene transcription, as well as calponin mRNA expression and protein levels, has been observed previously following treatment of cultured vascular SMCs with AngII [199-201].

The ability of AngII to regulate the vascular SMC phenotype would depend on AngII receptor expression in the cells. AT1 receptors are abundantly expressed in cultured vascular SMCs and activation of this receptor by AngII mediates the growth-promoting effects. The AT2 receptor however is expressed in early development and during vascular injury [201]. Reduced expression of in AT1 and AT2 receptor was observed in passage-dependent manner with older passages showing reduced expression of these receptors. Heparin treatment however recovered expression of these receptors in the later passages and increased expression of vascular SMC markers of differentiation [168]. Sabri et al (1997) demonstrated that the reduction of SMC differentiation and elevation of blood pressure in a mouse model of AngII induced hypertension was due to activation of the AT₁ receptor [203]. Conversely, Yamada et al (1999) demonstrated that AngII activation of the AT₂ receptor enhances differentiation of aortic SMCs [224]. In these studies, the receptor expression pattern of the AngII receptor was not examined thus cannot attest to their presence or absence in hASMCs cultured under different conditions. Therefore, the effectiveness of AngII treatment under these conditions may have resulted from increased expression of the AT₁ receptor.
Angiotensin II may also be synthesized endogenously by the vascular
SMCs [225]. Fibronectin is an extracellular matrix glycoprotein which contributes
to vascular SMC migration, growth and phenotypic modulation. Upon phenotypic
switch from the contractile to the synthetic phenotype induced by fibronectin,
vascular and human aortic SMCs have been demonstrated to produce increased
levels of AngII, thus contributing to increased proliferation of SMCs [218, 226].
Furthermore, endogenous AngII synthesis was increased in vascular SMCs
obtained from spontaneously hypertensive rats compared with normotensive
ones, and this increase was associated with increased DNA synthesis,
production of growth factors, and decreased expression of differentiation markers
in these cells [227, 228]. Endogenous concentrations of AngII produced in these
hASMCs cultured under different conditions were not measured, but current
research indicates that endogenous AngII production increases during a shift
from the contractile to the synthetic phenotype in hASMCs [218]. However, the
treatment of differentiated cells with AngII under conditions promoting de-
differentiation may have potentiated the actions of the endogenous AngII or other
factors contributing to reduction in differentiation, thereby resulting in the
observed decrease in α-actin expression (Figure 5.3).

In conclusion, AngII stimulation of differentiated hASMCs in vitro may
promote de-differentiation by the induction of mPGES-1 expression in these
cells. Increased mPGES-1 synthesis results in increased production of
prostaglandin E₂ (PGE₂) and this is the principal prostanoid secreted in pro-
inflammatory conditions. However, there was considerable variability of response
observed with AngII treatment in these cells, with AngII acting to either reduce or increase expression of markers of differentiation under different cell culture and treatment conditions. Only with treatment of differentiated cells with AngII under conditions promoting de-differentiation was there a significant reduction in \( \alpha \)-actin levels. Therefore, in order to further investigate the mechanisms responsible for the reduced differentiation of hASMCs observed with AngII treatment, the contributions of COX-2, mPGES-1, and PGE\(_2\) (products of the prostanoid synthesis pathway) observed to be upregulated in AAA disease models, were subsequently examined.
CHAPTER 6: PHARMACOLOGICAL INHIBITION OF COX-2 by CELECOXIB

PROMOTES DIFFERENTIATION IN HUMAN AORTIC SMOOTH MUSCLE CELLS

Introduction

The cyclooxygenase (COX) enzymes catalyze the rate-limiting step in the biosynthesis of prostanoids. Two isoforms of the COX enzyme exist: COX-1, which is the constitutive enzyme, and COX-2, which is the inducible form. The COX-1 enzyme is primarily associated with “housekeeping” functions in normal tissues such as hemostatic, gastric protection, and renal water balance [63, 79]. However, in response to stress, cytokines, growth factor stimulation, and inflammatory stimuli, COX-2 expression increases [79]. The COX enzymes convert arachidonic acid first into prostaglandin G2 (PGG2) via oxygenation, and then to prostaglandin H2 (PGH2) via peroxidase activity. PGH2 is subsequently converted by tissue specific synthases into different prostanoids including prostacyclin (PGI2), prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), thromboxane (TXA2), and prostaglandin F2a (PGF2a) [80, 81]. These prostanoids exert their actions by activating and signaling through their individual receptors. Thus, the biological actions of these prostanoids may be modulated at the level of the cyclooxygenase enzymes, individual synthases or the prostanoid receptors [84].

Increased expression of the inducible cyclooxygenase enzyme (COX-2) has been implicated in the pathogenesis of numerous diseases including cancer,
atherosclerosis, osteoarthritis, and aneurysms [149, 229-233]. More specifically, during the development of abdominal aortic aneurysms (AAAs), recent research in animal models, as well as human aneurysmal biopsies, has implicated the COX-2 enzyme in the pathogenesis of this disease. In AngII-induced mouse models of AAAs, increased expression of COX-2 is observed in SMCs of the aortic aneurysmal lesions compared with normal aorta [148, 150]. The, genetic deletion of this COX-2 enzyme has been demonstrated to significantly reduce the incidence and severity of this disease in animal models [148]. In addition, pharmacological inhibition of COX-2 with celecoxib is effective in reducing the incidence, severity, and progression of this disease in murine models [75, 205]. Similarly, in human biopsy explants from AAA lesions, the increase in COX-2 expression has been observed when compared to normal, uninvolved aorta [147, 149, 234].

Aside from the involvement of COX-2 during development of this disease, alteration of the vascular SMC phenotype has also been observed in the developing aneurysm in both human biopsies and animal models of AAAs. Ailawadi et al (2009) observed significant downregulation of markers of differentiation in the SMCs of aneurysmal lesions, compared with the uninvolved aorta both in human biopsy specimens, as well as an animal model of this disease [53]. In an AngII-induced animal model of AAAs, reduction in differentiation demonstrated by decreased expression of markers of differentiation and increased expression of markers of de-differentiation are also observed in the aneurysmal lesion, compared with normal uninvolved aorta. In
the AngII-induced AAA mouse model, pharmacological inhibition of the COX-2 enzyme with celecoxib is associated with preservation of the differentiated vascular SMC phenotype [74]. Therefore, the hypothesis that increased expression of COX-2 will promote reduced differentiation in hASMCs and that pharmacological inhibition of COX-2 in vitro will be effective in promoting hASMC differentiation was proposed.

Results

6.1 Characterization of cyclooxygenase-2 (COX-2) expression in hASMCs

6.1.1 Increased hASMC differentiation over time correlates with decreased COX-2 expression.

The expression of COX-2 in cultured hASMCs was examined and correlated with differentiation marker expression both at the mRNA and protein levels. Cells were cultured under either de-differentiation-promoting (d-DC) or differentiation-promoting conditions (DC) over a period of 4 days for analysis of mRNA and for a period of 5 days for protein analysis. Samples were harvested after every 24 hours and analyzed via real-time PCR for mRNA and western blotting for protein.

Significant reduction in COX-2 mRNA expression in cells cultured under differentiation-promoting conditions compared with cells cultured under de-differentiation-promoting conditions at each time point was observed (Figure
A similar result was also observed in the protein levels of COX-2 at each time point, with cells cultured in de-differentiation-promoting media demonstrating greater levels of COX-2 compared with cells in differentiation-promoting media (Figure 6.1.1C). This COX-2 expression was correlated with that of α-actin and an inverse relationship between both their mRNA expression and protein levels was observed. Cells cultured under differentiating-promoting conditions expressed significantly higher levels of α-actin mRNA and protein compared with COX-2 at each time point (Figure 6.1.1B & C)). These results indicate that COX-2 expressing hASMCs show reduced expression of differentiation markers.
Figure 6.1.1 Increased hASMC differentiation over time correlates with decreased COX-2 expression.

mRNA expression of (A) COX-2 and (B) α-actin in hASMCs cultured for 1 to 4 days under conditions promoting differentiation or de-differentiation. (C) Representative western blot showing protein levels of COX-2 and α-actin, and SM22α, with α-tubulin as loading control, in hASMCs cultured for 1 to 5 days under conditions promoting differentiation or growth. Data represents mean + SEM (n≥3), **** = p < 0.0001, unpaired Student’s t test comparing expression levels between differentiation conditions (DC) and growth conditions (d-DC) at each individual time point.

A.

![COX-2 mRNA graph](image)
6.1.2 Serum-stimulation increases while serum-deprivation decreases COX-2 protein levels in hASMCs.

The increased presence of serum and growth factors has been shown to contribute to the expression of COX-2 in cells [220-223]. On the other hand, the reduction or removal of serum has been observed to induce differentiation in cells [23, 173, 174]. Since recent findings (6.1.1) determined that cells cultured
under conditions promoting de-differentiation demonstrated significantly increased COX-2 expression compared with differentiation-promoting media, the hypothesis that serum-deprivation would reduce expression of COX-2 while increasing differentiation in these cells was proposed. The levels of COX-2 protein in serum-deprived cells were examined and then correlated with hASMC differentiation. To do this, cells were cultured under de-differentiation-promoting conditions for 2 days and then the media was changed to serum-free media for 5 days. Cells were lysed and harvested daily and subsequently analyzed for protein levels of COX-2 and the markers of differentiation, α-actin and SM22α, via western blotting.

Cells cultured for 2 days under de-differentiation-promoting conditions expressed increased COX-2 protein levels compared with conditions of serum-deprivation. By the first day of serum-deprivation, the COX-2 protein levels were drastically reduced compared with the control, and the COX-2 protein levels continually reduced over time till it was undetectable (Figure 6.1.2A). However, as observed previously (Figure 4.2), serum deprivation resulted in an increase in α-actin and SM22α protein levels, thus indicating the increased differentiation of the cells.

The induction of COX-2 expression that occurs in response to de-differentiation-promoting media in cells that were previously serum deprived was also examined. The re-stimulation of serum-deprived cells with de-differentiation-promoting media increased COX-2 protein levels for the first 2 days, which then declined by the third and fourth days (Figure 6.1.2B).
**Figure 6.1.2 Serum-stimulation increases while serum-deprivation decreases COX-2 protein levels in hASMCs.**

Representative western blots showing protein levels of COX-2 and α-actin, with α-tubulin as loading control, in hASMCs cultured for (A) 2 days in de-differentiation-promoting media and serum deprived for 5 days, (B) 2 days in de-differentiation promoting media, serum deprived for 1 day and then re-stimulated with de-differentiation-promoting media for 1 to 4 days.

**A.**

<table>
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**B.**

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</table>
6.2 Effect of COX-2 inhibition by celecoxib on hASMC phenotypic modulation.

6.2.1 COX-2 inhibition by celecoxib increases α-actin levels in hASMCs cultured under differentiation-promoting conditions.

The effect of cyclooxygenase 2 (COX-2) inhibition by celecoxib on the hASMC phenotype \textit{in vitro} was investigated. Since hASMC cultured under conditions promoting either de-differentiation or differentiation do express COX-2, albeit at varying levels, hASMCs were treated with celecoxib under both conditions. For treatment with celecoxib under reduced levels of COX-2 expression, cells were cultured under differentiation-promoting conditions. The cells were cultured for 2 days in differentiation-promoting media, and then treated with either 2, 5 or 10 µM celecoxib for 1, 2 or 3 days. Non drug-treated controls were incubated with media containing the vehicle (0.1% DMSO). At each time point, cells were lysed, harvested and analyzed for α-actin protein levels via western blotting.

A trend toward increase in α-actin protein levels in the celecoxib-treated cells compared with untreated controls was observed. Although there was a trend for increased α-actin expression following treatment with 5 µM celecoxib for 1 or 2 days, the change was not statistically significant (Figure 6.2.1). At the 2 and 3 day time points, cells treated with 10 µM celecoxib showed significantly increased α-actin levels compared with vehicle-treated controls.
Figure 6.2.1 COX-2 inhibition by celecoxib increases α-actin levels in hASMCs cultured under differentiation-promoting conditions.

Densitometry and representative western blot showing protein levels of α-actin in celecoxib-treated hASMCs cultured under differentiation-promoting conditions for (A) 1 day, (B) 2 days and (C) 3 days. Data represents mean ± SEM (n≥3), * = p < 0.05, ** = p < 0.01; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.
Figure 6.2.1 (continued)

B.

![Bar graph showing percent of control (α-actin/calnexin) for Celecoxib (μM)]

C.

![Bar graph showing percent of control (α-actin/calnexin) for Celecoxib (μM)]
6.2.2 COX-2 inhibition increases α-actin levels in hASMCs cultured under conditions promoting de-differentiation.

Upon observing increased levels of α-actin in celecoxib-treated cells under conditions promoting differentiation, the effect of celecoxib on cells cultured under de-differentiation-promoting conditions in which higher expression of COX-2 was observed was examined. Cells were cultured under de-differentiation-promoting conditions for 2 days and then treated with 2, 5, or 10 µM celecoxib for either 1 day or 2 days. At each time point, cells were fixed and analyzed for α-actin levels via immunocytochemistry.

Significant increase in α-actin levels in cells treated with 2 µM celecoxib at the 1 day time point was observed (Figure 6.2.2A). However, treatment with either 5 µM or 10 µM of celecoxib at the 1 day time point did not result in a statistically significant increase in α-actin expression compared to vehicle-treated controls. In addition, there were no significant differences in α-actin expression with any of the treatment concentrations after 2 days of treatment (Figure 6.2.2B).
Figure 6.2.2 COX-2 inhibition increases α-actin levels in hASMCs cultured under conditions promoting de-differentiation.

Densitometry quantitation of α-actin expression in differentiated cells treated with celecoxib under de-differentiation-promoting conditions for (A) 1 day or (B) 2 days. Data represents mean + SEM (n=3), * = p < 0.05; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.
6.2.3 Treatment with celecoxib under serum-deprivation conditions inhibits the induction of differentiation in hASMCs.

Previous results had shown that increased differentiation observed in serum-deprived hASMC is associated with reduced expression of COX-2 (Figure 6.1.2A). The increase in differentiation was observed to begin after 2 days of serum-deprivation and the continued increase correlated with the number of days the cells were in serum-free media. Since, serum deprivation induces differentiation in these cells, and reduced COX-2 levels were observed under these conditions, it was hypothesized that treatment of hASMCs with celecoxib under conditions of serum-deprivation would inhibit any remaining COX-2 that was expressed during the progression of differentiation thereby potentiating the induction of differentiation under these conditions. The effect of COX-2 inhibition at the beginning of serum-deprivation on the induction of differentiation marker expression in these cells was examined. Cells were cultured for 2 days in de-differentiation-promoting media and then treated at the beginning of serum-withdrawal with either 2, 5, or 10 µM of celecoxib for between 1, 2, or 3 days. Cells were harvested each day and then analyzed for α-actin and SM22α protein levels via western blotting.

Under conditions of serum deprivation, it was observed that celecoxib treatment did not enhance differentiation in these cells as there was no significant increase in either α-actin or SM22α protein levels at any of the time points examined. In fact, by the third day of celecoxib treatment, there was significant reduction in the protein levels of both markers of differentiation.
Figure 6.2.3 Celecoxib treatment under serum-deprivation conditions inhibits the induction of differentiation in hASMCs.

Representative western blot and densitometry quantitation of (A) α-actin and (B) SM22α in growing cells treated with either 2, 5, or 10 μM of celecoxib under serum-starved conditions for 1, 2, or 3 days. Data represents mean + SEM (n=3), * = p< 0.05, ** = p<0.01; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.

1 day

A. α-actin

<table>
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<th>Control</th>
<th>Celecoxib (μM)</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>2</td>
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<td>10</td>
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α-actin α-tubulin
Figure 6.2.3 (continued)

B. SM22α

<table>
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<th>Percent of control (SM22α/α-tubulin)</th>
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- Control
- Celecoxib (µM)

2 days

α-tubulin

A. α-actin

<table>
<thead>
<tr>
<th>Percent of control (α-actin/α-tubulin)</th>
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<td>150</td>
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- Control
- Celecoxib (µM)

2 days

α-tubulin
Figure 6.2.3 (continued)

B. **SM22α**

- **Control**
- **Celecoxib (μM)**

![Graph showing percent of control (SM22α/α-tubulin) for SM22α expression over 3 days.](image)

![Western blot images for SM22α and α-tubulin.](image)

3 days

A. **α-actin**

- **Control**
- **Celecoxib (μM)**

![Graph showing percent of control (α-actin/α-tubulin) for α-actin expression over 3 days.](image)

![Western blot images for α-actin and α-tubulin.](image)
6.2.4. Inhibition of COX-2 by celecoxib under serum-stimulated conditions reduces hASMC differentiation over time.

Culturing hASMCs under de-differentiation-promoting conditions over time increased the expression of COX-2 in these cells (Figure 6.1.1). Furthermore, induction of COX-2 expression in cells which were serum-deprived overnight and then re-stimulated with de-differentiation-promoting media for 1 or 2 days was observed (Figure 6.1.2). During both experiments, the increase in COX-2 expression correlated with decrease in the expression of markers of differentiation. Therefore, the hypothesis that pharmacological inhibition of COX-
2 by celecoxib under conditions of serum-re-stimulation will induce differentiation in hASMCs was proposed. To test this hypothesis, cells were cultured for 2 days under de-differentiation-promoting conditions, and then quiesced overnight by serum-deprivation. Next, cells were re-stimulated for 1, 2 or 3 days with de-differentiation-promoting media containing vehicle, 2, 5, or 10 µM celecoxib. hASMCs were lysed and harvested at each time point and analyzed for protein levels of α-actin, SM22α and COX-2 via western blotting.

After one day of celecoxib treatment under re-stimulated conditions, there was a slight trend toward an increase in α-actin and SM22α protein levels in celecoxib treated cells, compared with vehicle-treated controls. After 2 days of treatment, the 5µM celecoxib concentration was significant for SM22α but not α-actin expression. COX-2 protein levels however began to trend toward reduction after 1 day treatment and this decrease was significant at all concentrations by the 2 day time point. In contrast, treatment with 2, 5, or 10 µM celecoxib for 3 days under conditions of re-stimulation resulted in significant reduction of α-actin and COX-2 expression at all 3 celecoxib concentrations, and a significant reduction of SM22α at the 10 µM celecoxib treatment (Figure 6.2.4).

**Figure 6.2.4. Inhibition of COX-2 by celecoxib under serum-stimulated conditions reduces hASMC differentiation over time.**

Representative western blot and densitometry quantitation of (A) α-actin and (B) SM22α in growing cells treated with either 2, 5, or 10 µM of celecoxib under serum-stimulated conditions for 1 day, 2 days, or 3 days. Data represents mean
+ SEM (n=3), * = P< 0.05, ** = p<0.01, *** = p< 0.001, and **** = p<0.0001; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.

1 day

A. α-actin

B. SM22α

0 2 5 10

Percent of control
(α-actin/α-tubulin)

Control
Celecoxib (μM)

0 2 5 10

Percent of control
(SM22α/α-tubulin)

Control
Celecoxib (μM)
Figure 6.2.4 (continued)

C. **COX-2**

Percent of control
(COX-2/α-tubulin)

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2 days

A. **α-actin**

Percent of control
(α-actin/α-tubulin)

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Figure 6.2.4 (continued)

B. **SM22α**

![SM22α BAR CHART]

C. **COX-2**

![COX-2 BAR CHART]
Figure 6.2.4 (continued)

3 days

A. α-actin

![Graph showing the percent of control (α-actin/α-tubulin) for α-actin with control and Celecoxib (μM) conditions.](image)

B. SM22α

![Graph showing the percent of control (SM22α/α-tubulin) for SM22α with control and Celecoxib (μM) conditions.](image)
Discussion

There have been a limited number of reports examining the role of COX-2 in the regulation of vascular SMC differentiation. One previous report showed conflicting findings on the effects of celecoxib treatment in a rat model of aortic remodeling induced by fluid shear stress. In this previous report, celecoxib treatment did not significantly increase expression of α-actin or myosin heavy chain-II, but did show an increase in desmin expression [235]. Furthermore, the effectiveness of celecoxib in reducing the incidence and severity of aneurysms in AngII-induced mouse models of abdominal aortic aneurysms is associated with
preservation of the differentiated SMC phenotype [74]. In order to characterize the potential role of COX-2 in regulating vascular SMC differentiation, the expression of the COX-2 enzyme under different cell culture conditions was examined and correlated with the expression of markers of hASMC differentiation (α-actin and/or SM22α). Subsequently, the effects of pharmacological inhibition of COX-2 by celecoxib on the hASMC phenotype under conditions of increased or decreased COX-2 expression were investigated.

An inverse correlation between COX-2 and differentiation marker expression was observed in these cultured hASMCs. Increase in hASMC differentiation induced either by differentiation-promoting media (Figure 6.1.1) or serum-deprivation (Figure 6.1.2A) was associated with reduced COX-2 expression. However, COX-2 expression was significantly increased in cells cultured under growth-promoting conditions, compared to cells cultured under differentiation-promoting conditions, at each time point (Figure 6.1.1). In addition, re-stimulation of serum-deprived cells induced expression of COX-2 after serum deprivation for 1 or 2 days (Figure 6.1.2B). Thus, incubation of hASMCs in media containing growth-promoting agents including increased serum and growth factors is associated with the induction of COX-2 mRNA and protein expression. In addition, based on the differentiation state of these cells, differing levels of COX-2 expression in these hASMCs was observed regardless of the cell culture conditions.

Cell stimulation by media containing serum and/or growth factors has been demonstrated to induce the expression of COX-2 in a variety of cell types.
including vascular SMCs [220-223]. Under growth-promoting conditions, cells are cultured in media containing increased serum (about 5% FBS) and a variety of growth factors, thus resulting in increased transcription and translation of COX-2 in these cells compared with cells cultured under differentiation-promoting conditions. Removal of these stimulants (serum and growth factors) during serum-deprivation however, resulted in the significant reduction in COX-2 expression, with protein levels measured under this condition becoming greatly reduced over time. Furthermore, serum and growth factors have been shown to trigger activation of vascular SMCs to the synthetic phenotype [174]. Conversely, with the induction of differentiation in serum-deprived cells, significant reduction in COX-2 protein levels over time was observed.

To further understand the mechanisms by which COX-2 may regulate the cells phenotype the effects of pharmacological inhibition of COX-2 by celecoxib on cell differentiation under conditions of increased or decreased COX-2 expression was examined. Celecoxib treatment of differentiating cells further promoted differentiation in both a time and dose-dependent manner (Figure 6.2.1), whereas with cells cultured under conditions promoting de-differentiation, celecoxib induced differentiation only at the lowest concentration (Figure 6.2.1). Since culturing cells under de-differentiation-promoting conditions greatly induces COX-2 expression, it was expected that a higher treatment concentration of celecoxib of 10 µM would be required to inhibit COX-2 sufficiently to promote differentiation, similar to that observed in the differentiated cells. However, it was surprising to observe that the lowest celecoxib concentration used (2 µM)
promoted a significant increase in α-actin after treatment for 1 day, whereas, the highest concentration of 10 µM trended toward a reduction in α-actin levels over time. The 2 µM celecoxib treatment was proposed to provide more specific inhibition of COX-2 than either the 5 or 10 µM concentrations under these conditions thereby resulting in the observed increase in α-actin expression in these cells.

Previous reports have shown that serum-deprivation over time induces the expression of differentiation markers in vascular SMCs [23, 173, 174] and our current findings show that this condition is associated with reduced expression of COX-2, the levels of which become undetectable over time (Figure 6.1.2). Based on this finding, it was proposed that since reduced expression of COX-2 is associated with increased expression of differentiation markers, then inhibition of COX-2 by celecoxib under these conditions would promote the early induction of differentiation. At the initiation of serum-deprivation, the expression of COX-2 in these cells is significantly reduced but there was no significant increase in α-actin expression until the 3rd day of serum-deprivation (Figure 6.1.2A). In order to investigate if inhibition of COX-2 will induce differentiation in treated cells before the 3-day time point, hASMCs were treated with 2, 5 or 10 µM celecoxib under conditions of serum-deprivation over time. Celecoxib treatment however, did not induce differentiation marker expression in these cells (Figure 6.2.3). On the contrary, COX-2 inhibition by celecoxib under conditions of serum-deprivation over time was observed to reduce expression of α-actin and SM22α and this reduction was significant by the 3rd day of celecoxib treatment.
In general, it is expected that cell treatment with pharmacological agents in conditions of serum-free media provides the opportunity to explore effects of these agents on hASMC differentiation without the potential for interaction with constituents in the serum. The observation of de-differentiation in cells treated with celecoxib under conditions of serum-deprivation is potentially due to multiple factors. First, celecoxib is a molecule which significantly binds to albumin. Studies analyzing protein binding in serum have shown that approximately 97% of celecoxib is bound to albumin [236]. The high level of protein binding in serum-containing media would be expected to limit the activity of the compound. Thus, in serum-free media, the activity of celecoxib would be expected to be increased, thereby lowering the concentration required to effectively inhibit COX-2. Concentrations of celecoxib that are approximately 10-fold higher than those required for inhibiting COX-2 are known to inhibit other intracellular targets, the best characterized being the Akt signaling pathway. In the current studies utilizing celecoxib treatments in serum-free media, the lack of protein binding may have allowed sufficient inhibition of targets other than COX-2 and the observed reduction in expression of differentiation markers. A second potential mechanism includes the inhibition of COX-1. Similar to the inhibition of Akt, concentrations of celecoxib that are approximately 10-fold higher than those required for inhibiting COX-2 result in the inhibition of COX-1. Because individual prostanoid synthases are coupled differently to either COX-1 or COX-2, the inhibition of COX-1 by celecoxib could result in reduced production of different prostanoids than that which results from inhibiting COX-2. It is known that
prostacyclin affects cellular differentiation in ways that are opposite of the effects of PGE2. Therefore, effects of celecoxib observed in the current studies may have also resulted from the inhibition COX-1 resulting in a decrease of prostacyclin synthesis [33].

Serum-stimulation on the other hand increases COX-2 expression in vascular SMCs. An increase in COX-2 protein levels in the cells that had been deprived of serum for overnight and then re-stimulated with serum-containing growth-promoting media was observed. Even though the increase was sustained during the first 2 days after re-stimulation, the COX-2 levels gradually began to reduce from the 3rd day onwards (Figure 6.1.2B). It was proposed that celecoxib treatment under conditions of increased COX-2 expression would inhibit COX-2 and induce hASMC differentiation in a dose-dependent manner. Though there was a slight trend toward increase in SM22α protein levels in treated cells compared with controls after the 2nd day of treatment, this effect was abolished by the 3rd day with significant reduction in both α-actin and SM22α protein levels in celecoxib-treated cells. Furthermore, there was significant reduction in the protein levels of COX-2 at both the 2nd and 3rd day time points in celecoxib-treated cells compared with controls (Figure 6.2.4).

The observation of significant reduction in COX-2 levels with celecoxib treatment under serum re-stimulated conditions was quite an interesting finding (Figure 6.2.4). Celecoxib is a COX-2 inhibitor which acts by inhibiting COX-2 and thus preventing it from converting arachidonic acid to the crucial substrate (PGH2) which is required for the formation of downstream prostanoids. Even
though there are numerous research findings demonstrating the COX-2 inhibitory actions of celecoxib, there is a dearth of research showing selective reduction in COX-2 expression levels in vascular SMCs by celecoxib treatment. Xu et al (1999) reported inhibition of COX-2 transcription with reduction in COX-2 mRNA and protein levels by aspirin and sodium salicylate in a cell-cycle specific manner [237]. As observed in Figure 6.1.2B, COX-2 protein levels increase when cells are serum-stimulated following a 24 hour cell quiescence. By the 3rd day after re-stimulation, levels of COX-2 begin to decrease. The reason for this reduction in COX-2 levels with celecoxib treatment is not known. One potential mechanism may result from celecoxib inhibiting the production of a prostanoid that acts to increase expression of COX-2. One such prostanoid that has been shown to increase expression of COX-2 is PGE$_2$. Our current studies show that PGE$_2$ synthesis is significantly increased following serum stimulation and this increase is significantly inhibited by celecoxib treatment. Therefore, the observed reduction in COX-2 expression following celecoxib treatment may have resulted from the inhibition of PGE$_2$, thereby limiting the effect of this prostanoid on inducing COX-2 expression.

A limitation of these findings is the fact that even though there is an inverse relationship between COX-2 expression and the expression of differentiation markers, significant increase in differentiation with celecoxib treatment could not be consistently demonstrated under all the cell culture conditions studied. In addition, one cannot conclusively attribute the correlation observed to causation. It is important to note that COX-2 inhibition just like COX-
2 deletion, inhibits the production of prostaglandins formed downstream of this enzyme. Tang et al (2014) observed that COX-2 deletion in vascular cells in animal model of atherosclerosis has been shown to reduce levels of prostaglandins while increasing levels of α-actin in these cells [238]. Therefore, the increase in differentiation observed with COX-2 inhibition by celecoxib may be attributable to the inhibition of specific prostanoids downstream of COX-2 that may be important in decreasing differentiation in these cells.

Moreover, despite the promising findings regarding the effectiveness of COX-2 inhibition in treatment of AAAs in animal models, as well as the potential for celecoxib to promote differentiation or reverse de-differentiation in hASMCs, treatment of patients suffering from AAA disease with a COX-2 inhibitor may not be a feasible therapeutic option for treating this disease in humans. This is because of the increased risk of adverse cardiovascular events associated with long-term use of certain COX-2 inhibitors [239, 240]. Therefore, to further understand the mechanisms of COX-2 induced SMC phenotypic modulation, key mediators of the prostaglandin synthesis pathway downstream of COX-2 which have been demonstrated to contribute to AAA development were examined as this could potentially provide new targets for treatment of this disease.
CHAPTER 7: INHIBITION OF MICROsomAL PROSTAGlandin E
SYNTHASE AND PROSTAGlandin E₂ PROMOTES DIFFERENTIATION IN
HUMAN AORTIC SMOOTH MUSCLE CELLS

Introduction

Prostaglandin E₂ (PGE₂) is the principal prostanoid secreted during inflammation and is involved in production of pro-inflammatory cytokines in a variety of cells [4, 17, 52, 103-105]. The increased expression of inflammatory mediators induced by PGE₂ may contribute to alteration of vascular SMC contractile function, increased vascular permeability, dilation and pain [4, 52, 85, 103-105]. PGE₂ may be synthesized by conversion of arachidonic acid to the substrate PGH₂ by the rate-limiting cyclooxygenase enzyme (COX) and subsequent action of the Prostaglandin E₂ synthase (PGES). PGES exists in 3 main isoforms: cytosolic (c-PGES), type 1 membrane-associated/microsomal (mPGES-1) and type-2 membrane associated (mPGES-2) [106, 107]. However, the majority of the PGE₂ formed in response to COX-2 induction is dependent on mPGES-1 [121, 122].

In cardiovascular diseases such as abdominal aortic aneurysms (AAAs), certain products of the prostanoid synthetic pathway such as COX-2, mPGES-1 and PGE₂ have been implicated in the development of this disease in humans as well as in animal models of the disease. Recent studies have shown increased expression of COX-2, mPGES-1, and PGE₂ synthesis in AAAs [126,147,149, 150]. Similarly, Wang et al (2008) demonstrated that mPGES-1 deletion in AngII-
induced AAA mouse models suppressed PGE$_2$ production, and significantly attenuated the incidence and severity of AAAs [128]. Also, genetic deletion of COX-2 or pharmacological inhibition of this enzyme in an animal model of this disease has been shown to reduce the incidence and severity of this disease [148, 205].

Apart from the involvement of mediators of the prostanoid synthetic pathway in this disease, reduced differentiation of vascular smooth muscle cells within the vessel wall has been observed during the development of AAAs. Recent research from our laboratory has demonstrated an inverse association between the increase in COX-2 expression within the SMCs of the aneurysmal lesions and the reduced expression of differentiation markers. In these studies, selective inhibition of the COX-2 enzyme with celecoxib was observed to preserve the differentiated phenotype of these cells and significantly attenuate AAA progression compared with saline controls [74, 75]. Furthermore, in human aneurysmal lesions, reduced expression of markers of differentiation and induced expression of de-differentiation markers was observed in the human aortic smooth muscle cells [53].

The mechanisms by which COX-2 promotes reduced differentiation of vascular SMCs were investigated by examining prostanoids downstream of the COX-2 enzyme. Based on the previous results showing the induction of mPGES-1 with AngII treatment (Figure 4.3), as well as the promotion of differentiation in cells treated with celecoxib (Figure 5.1), the hypothesis that COX-2 may
contribute to reduced differentiation in vascular SMCs by the action of mPGES-1-derived PGE₂ was proposed.

To understand the mechanisms by which the inducible cyclooxygenase enzyme (COX-2) contributes to reduced differentiation observed during development of abdominal aortic aneurysms (AAA), human aortic smooth muscle cells (hASMCs) as our in vitro cell culture model were utilized to study the effects of various treatments on modulation of the cell phenotype. hASMCs were cultured under different conditions and treated with PGE₂ exogenously over time. Furthermore, the contribution of endogenous mPGES-1-derived PGE₂ on the cell phenotype by inhibiting mPGES-1 both pharmacologically (15-dPGJ₂) and genetically (mPGES-1 siRNA) were examined. The concentration PGE₂ synthesized under various treatment conditions was measured via enzyme immune assay while mRNA expression and protein levels of a wide variety of markers of the differentiated as well as de-differentiated phenotype were examined by real-time PCR and western blotting, respectively.

Results

7.1 Effect of Prostaglandin E₂ on hASMC phenotype

7.1.1 Prostaglandin E₂ treatment under differentiation-promoting conditions reduces differentiation in hASMC

To study the role of PGE₂ on the hASMC phenotype, cells were cultured under differentiation-promoting conditions and treated with PGE₂ under
conditions promoting either differentiation. Cells were plated directly into cell
culture plates in differentiation promoting media for 2 days. At the 2 day time-
point, cells were treated with either 0.2 µM or 1 µM PGE\textsubscript{2} under conditions
promoting differentiation. Vehicle-treated controls were treated with 0.1% ethanol
under the same conditions. Protein levels of α-actin were measured via
immunocytochemistry and normalized to cellular DNA detection as determined
by SYBR green detection at each time point.

Treatment of differentiated cells with PGE\textsubscript{2} under differentiation-promoting
conditions, resulted in a dose-dependent trend toward reduction in α-actin levels
which was significant with 1 µM treatment at the 1 day time point (Figure 7.1.1A)
but not after 2 days (Figure 7.1.1B).
Figure 7.1.1 Prostaglandin E$_2$ treatment under differentiation-promoting conditions reduces α-actin levels in differentiated hASMCs

Immunocytochemistry with fluorescent imaging quantitation showing α–actin expression in differentiated cells treated with PGE$_2$ under differentiation-promoting conditions for (A) 1 day or (B) 2 days. Data represents mean + SEM (n=3), * = p < 0.05; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.

A.

B.
7.1.2 Prostaglandin E\textsubscript{2} treatment under de-differentiation-promoting conditions reduces $\alpha$-actin levels in differentiated hASMCs

Previously, increased synthesis of mPGES-1 (the major enzyme that produces PGE\textsubscript{2}) was observed in differentiated hASMCs treated with AngII under de-differentiation-promoting conditions (Figure 4.3). Therefore, to study the role of PGE\textsubscript{2} on the phenotype of differentiated cells treated with PGE\textsubscript{2} under conditions promoting growth, cells were cultured under differentiating-promoting conditions for 2 days and subsequently treated with either 0.2 $\mu$M or 1 $\mu$M PGE\textsubscript{2} in de-differentiation-promoting media for either 1 or 2 days. Vehicle-treated controls were treated with 0.1% ethanol under the same conditions. At each time point, cells were lysed, harvested, and analyzed for $\alpha$-actin protein levels via western blotting.

The treatment of hASMCs with PGE\textsubscript{2} under growth-promoting conditions for 1 day significantly reduced $\alpha$-actin levels compared with controls (Figure 7.1.2A). Although there was a trend toward a reduction in $\alpha$-actin levels with the 1 $\mu$M PGE\textsubscript{2} concentration after 2 days of treatment, this effect was not statistically significant (Figure 7.1.2B).

**Figure 7.1.2 Prostaglandin E\textsubscript{2} treatment under de-differentiation-promoting conditions reduces $\alpha$-actin levels in differentiated hASMCs**

Densitometry and representative western blot showing protein levels of $\alpha$-actin in hASMCs cultured under differentiation-promoting conditions and treated with PGE\textsubscript{2} for either (A) 1 day, or (B) 2 days under conditions promoting de-
differentiation. Data represents mean + SEM (n≥3), **** = p < 0.0001; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.
7.2 Prostaglandin \( \text{E}_2 \) inhibits the induction of differentiation in hASMCs

To further examine the effects of \( \text{PGE}_2 \) on phenotypic modulation, the role of \( \text{PGE}_2 \) on the induction of differentiation in these cells was explored. As an alternative method of inducing differentiation and examining the effects of various treatments under conditions without the potential for interaction with serum-containing components, cells were cultured in de-differentiation-promoting media for 2 days and then deprived of serum over time. Previous findings with serum-deprivation indicated that after 2 days of serum deprivation, the cells began to differentiate as indicated by a 5-fold increase in SM \( \alpha \)-actin, as compared to non-serum-deprived controls (Figure 4.2). After 3 days in serum-free medium, the cells demonstrated significantly increased levels of both \( \alpha \)-actin and SM22\( \alpha \).

7.2.1 Prostaglandin \( \text{E}_2 \) treatment initiated after 2 days of serum-deprivation reduces \( \alpha \)-actin protein levels in hASMCs

Differentiation was induced hASMCs by the serum-deprivation method and then the cells were treated with either 0.2 \( \mu \text{M} \) or 1 \( \mu \text{M} \) \( \text{PGE}_2 \) in serum-free media at the 2 day time point. Cells were treated with \( \text{PGE}_2 \) for between 1 to 3 days and cell lysates were collected at each time point and analyzed by measuring protein levels of \( \alpha \)-actin via western blotting.

In cells deprived of serum for 2 days, significant reduction \( \alpha \)-actin protein levels after treatment with \( \text{PGE}_2 \) at the 1, 2 and 3 day time points was observed (Figure 7.2.1). Surprisingly, the reduction in \( \alpha \)-actin observed with 0.2 \( \mu \text{M} \) \( \text{PGE}_2 \) was greater than that observed at the higher concentration of 1 \( \mu \text{M} \). In addition,
Despite the duration of treatment, the reduction in α-actin levels observed over time was similar among the 3 time points.

**Figure 7.2.1 Prostaglandin E₂ treatment initiated after 2 days of serum-deprivation reduces α-actin protein levels in hASMCs**

Representative western blots and densitometry quantitation of protein levels of α-actin in hASMCs serum-deprived for 2 days and then treated with PGE₂ for (A) 1 day, (B) 2 days, or (C) 3 days. Data represents the mean ± SEM (n≥3), * = p < 0.05, and ** = p < 0.01.; one-way ANOVA (Dunnett's Multiple comparison test).
Figure 7.2.1 (continued)

B.

![Bar graph showing percent of control (α-actin/α-tubulin) with values for 0, 0.2, and 1.0 μM PGE2.]

C.

![Bar graph showing percent of control (α-actin/α-tubulin) with values for 0, 0.2, and 1.0 μM PGE2.]

**Note:** The graphs illustrate the effect of PGE2 on the expression of α-actin and α-tubulin, with significant differences denoted by asterisks (* and **) compared to the control.
7.2.2 Prostaglandin E₂ treatment initiated after 3 days of serum-deprivation, reduces protein levels of SMC markers of differentiation

In previous experiments, significant induction of markers of differentiation in serum-deprived hASMCs from the 3 day time point onwards had been observed. Thus, in order to study the effect of PGE₂ treatment on differentiation, the treatment was commenced at the 3-day time point of serum-deprivation. Cells were treated with PGE₂ for either 1 or 2 days and cell lysates were collected at each time point and analyzed by measuring protein levels of α-actin and SM22α via western blotting.

Similar to PGE₂ treatment after 2 days of serum-deprivation, treatment after 3 days resulted in significant reduction of both α-actin and SM22α protein levels, as compared with vehicle-treated controls. However, at both time points, there was a greater reduction in SM22α levels than with α-actin levels, indicated by the increased significant difference between treated and control cells in the SM22α group, as compared to the α-actin group.
Figure 7.2.2 Prostaglandin E₂ treatment initiated after 3 days of serum-deprivation reduces protein levels of SMC markers of differentiation

Representative western blots and densitometry quantitation of (A) α-actin, or (B) smooth muscle 22α in hASMCs deprived of serum for 3 days and then treated with PGE₂ for 1 day or 2 days. Data represents the mean ± SEM (n≥3), * = p < 0.05, ** = p < 0.01, *** = p < 0.001; one-way ANOVA (Dunnett’s Multiple comparison test)
Figure 7.2.2 (continued)

**SM22α**

| Percent of control (SM22α/α-tubulin) |
|-----------------|-----------------|-----------------|
| 0               | 0.2             | 1.0             |
| **Control**     | **PGE₂ (μM)**  |

**α-actin**

| Percent of control (α-actin/α-tubulin) |
|-----------------|-----------------|-----------------|
| 0               | 0.2             | 1.0             |
| **Control**     | **PGE₂ (μM)**  |

2 days
Figure 7.2.2 (continued)

**SM22α**

![Bar graph showing SM22α expression levels](image)

B.

<table>
<thead>
<tr>
<th>Percent of control (SM22α/α-tubulin)</th>
<th>Control</th>
<th>PGE2 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>**</td>
</tr>
</tbody>
</table>

7.2.3 Prostaglandin E₂ treatment initiated after 3 days of serum-deprivation reduces mRNA levels of SMC markers of differentiation

Since there was significant reduction in α-actin and SM22α protein levels after PGE₂ treatment under conditions of serum-deprivation, mRNA expression of a variety of differentiation markers as well as markers of de-differentiation, were examined under these conditions. Cells were deprived of serum for 3 days and treated with PGE₂ over a 2-day time period. mRNA was isolated from cell lysates daily, and examined for the expression of the differentiation markers α-actin, SM22α, smoothelin, and Myh11, by real time PCR.

PGE₂ treatment for both 1 day and 2 days significantly reduced mRNA expression of markers of differentiation in serum-deprived cells. The greatest
extent of reduction was observed with mRNA expression of Myosin Heavy Chain II (Myh11) at both time points. Even though significant reduction in α-actin expression was observed with 1 day treatment, the expression ceased to be significant at the 2 day time point, as there was observed an upward trend in α-actin expression in PGE₂ treated cells.
Figure 7.2.3 Prostaglandin E$_2$ treatment initiated after 3 days of serum-deprivation reduces mRNA levels of SMC markers of differentiation

mRNA expression of (A) $\alpha$-actin, (B) Smooth muscle 22$\alpha$ (C) Myosin heavy chain II (D) Smoothelin in hASMCs following incubation in serum-free media for 3 days and then treated with PGE$_2$ in serum-free media for 1 or 2 days. Data represents the mean + SEM (n$\geq$3), * = p < 0.05, *** = p < 0.001; one-way ANOVA (Dunnett’s Multiple comparison test).
Figure 7.2.3 (continued)

**B. SM22α**

- **Control**
- **PGE₂ (μM)**

**C. Myh11**

- **Control**
- **PGE₂ (μM)**
Figure 7.2.3 (continued)

**Smoothelin**

- **Control**
- **PGE₂ (μM)**

2 days

**α-actin**

- **Control**
- **PGE₂ (μM)**

* indicates statistical significance.
Figure 7.2.3 (continued)

B. **SM22α**

- **Control**
- **PGE2 (µM)**

C. **Myh11**

- **Control**
- **PGE2 (µM)**
7.2.4 Prostaglandin E\(_2\) treatment initiated after 3 days of serum-deprivation induces mRNA levels of SMC markers of de-differentiation

Since it was observed that PGE\(_2\) treatment reduces differentiation marker expression in hASMCs, the effect of PGE\(_2\) treatment on the expression of markers of de-differentiation was also examined in these cells. Cells were deprived of serum for 3 days and treated with either 0.2 \(\mu\)M or 1 \(\mu\)M PGE\(_2\) over a 2-day time period. mRNA was isolated from cell lysates at each time point, and examined for the expression of the de-differentiation markers HAS2 and MMP2, by real-time PCR.

Neither HAS2 nor MMP2 mRNA levels were significantly different between cells treated with vehicle or PGE\(_2\) for 1 day. However, at the 2 day time point, a dose-dependent significant increase in mRNA expression for both MMP2 and HAS was observed following PGE\(_2\) treatment.
Figure 7.2.4 Prostaglandin E$_2$ treatment initiated after 3 days of serum-deprivation induces mRNA levels of SMC markers of de-differentiation

mRNA expression of (A) HAS2, and (B) MMP2 in hASMCs serum starved for 3 days and then treated with PGE$_2$ for 1 or 2 days. Data represents the mean ± SEM (n≥3), * = p < 0.05; one-way ANOVA (Dunnett’s Multiple comparison test)

1 day

A. HAS2

B. MMP2
Figure 7.2.4 (continued)

2 days

**HAS2**

A. [Graph showing the expression of HAS2 in control and PGE2 (μM) conditions for 0, 0.2, and 1.0 days].

**MMP2**

B. [Graph showing the expression of MMP2 in control and PGE2 (μM) conditions for 0, 0.2, and 1.0 days].
7.3 Inhibition of mPGES-1 promotes hASMC differentiation

7.3.1 Serum-stimulation increases endogenous concentrations of PGE₂

In order to further examine the role of PGE₂ in hASMC phenotypic modulation, endogenous production of PGE₂ under different cell culture conditions was assessed. The cells were cultured under conditions of serum-deprivation or serum-stimulation and the levels of PGE₂ produced by the cells were determined.

hASMCs were cultured under growth-promoting conditions for 2 days, then the media was changed to serum-free media overnight, and then the cells were re-stimulated with growth promoting media. At the point of re-stimulation, the control cells were treated with fresh serum-free media, thus leaving these cells unstimulated. After 1 day, cell culture media was collected and then analyzed for PGE₂ concentrations via enzyme immunoassay.

Significant reduction in PGE₂ concentrations in media obtained from cells maintained in serum-free media, compared with cells stimulated with serum-containing media was observed (Figure 7.3.1). There was about a 20-fold increase in PGE₂ concentrations produced in cells that were in serum-containing media compared with cells in serum-free media. Therefore, the method of serum-stimulation to increase PGE₂ concentrations endogenously was subsequently used.
Figure 7.3.1 Serum-stimulation increases endogenous concentrations of PGE$_2$

Enzyme immunoassay analysis of PGE$_2$ concentrations in culture media obtained from cells cultured for 1 day under re-stimulated or unstimulated conditions. Data represents the mean + SEM (n≥3) **** = p < 0.0001; unpaired Student’s t-test.

7.3.2 Inhibition of COX-2 or mPGES-1 reduces PGE$_2$ concentrations in hASMCs

Since it was previously demonstrated that that exogenous addition of PGE$_2$ to differentiated or serum-deprived cells reduced hASMC differentiation (see sections 7.1 and 7.2), the effect of endogenously produced PGE$_2$ on the hASMC phenotype was examined. PGE$_2$ production was stimulated endogenously by serum-stimulation as shown above (7.3.1), and then treated the cells with appropriate inhibitors under these conditions.
Previous results (see Figure 6.1.2A) as well as current literature [220-223] had shown that COX-2 is activated during re-stimulation with serum, therefore, the effect of COX-2 inhibition on PGE₂ production was initially examined. The hASMCs were cultured under de-differentiation-promoting conditions for 2 days, the media was changed to serum-free media overnight, and then the cells were re-stimulated with de-differentiation-promoting media containing varying concentrations of celecoxib (0.01 µM, 0.1 µM, and 5 µM) or vehicle. After 1 day, the cell culture media was collected and then analyzed for PGE₂ concentrations via enzyme immunoassay.

There was a dose-response effect observed with celecoxib treatment under conditions of re-stimulation. Compared to vehicle-treated controls, the highest concentration of celecoxib used (5 µM) provided the greatest reduction in PGE₂ observed in these cells (about 800-fold reduction), followed by 0.1 µM (about 400-fold reduction), and then 0.01 µM (about 50-fold reduction) (Figure 7.3.2A).

Current research indicates that mPGES-1 is generally found to be co-expressed with COX-2 in vivo and in vitro [17, 106, 108, 111, 113-119]. Therefore, the effect of pharmacological inhibition of mPGES-1 on PGE₂ concentrations in hASMCs under conditions of re-stimulation was examined. hASMCs were cultured under de-differentiation-promoting conditions for two days and then made quiescent by changing to serum-deprived medium for 1 day. After 1 day of serum-deprivation, cells were treated for 1 day with de-differentiation-promoting media containing either celecoxib, or 15d-PGJ₂.
(mPGES-1 inhibitor). As a positive control, cells were cultured under the same conditions but replaced with serum-deprived media (unstimulated) containing 0.1% ethyl acetate, while negative control cells were re-stimulated with growth-promoting media (re-stimulated) containing vehicle (0.1% ethyl acetate). Cell media was collected for measurement of PGE$_2$ concentrations via enzyme immunoassay analysis (EIA).

Significant increase in PGE$_2$ concentrations in re-stimulated cells was observed compared with unstimulated cells (Figure 7.3.2B). Treatment of cells with celecoxib (COX-2 inhibitor) significantly reduced PGE$_2$ levels beyond the levels observed with unstimulated cells. Compared with the re-stimulated controls, 15d-PGJ$_2$ also reduced significantly PGE$_2$ concentrations in treated cells, though not to the extent observed by celecoxib treatment or by serum-deprivation. Overall, compared with re-stimulated controls, celecoxib treatment had the greatest effect on reducing PGE$_2$ levels with about an 800-fold reduction observed. Treatment with either 0.1 µM or 1 µM 15d-PGJ$_2$ yielded about a 10-fold reduction in PGE$_2$ concentrations compared with re-stimulated controls.
Figure 7.3.2 Inhibition of COX-2 or mPGES-1 reduces PGE$_2$ concentrations in hASMCs

Enzyme immunoassay analysis (EIA) of PGE$_2$ concentrations in hASMCs treated with (A) varying concentrations of celecoxib for 1 day under conditions of re-stimulation. (B) celecoxib, and varying concentrations of 15d-PGJ$_2$ under conditions of re-stimulation. Data represents mean + SEM (n = 4), **** = P < 0.0001 one-way ANOVA (Dunnett’s multiple comparison test).
7.3.3 mPGES-1 inhibition by 15d-PGJ₂ increases α-actin protein levels in hASMCs cultured under serum-deprivation, serum-stimulation, and de-differentiation-promoting conditions

In order to examine the effect of pharmacological inhibition of mPGES-1 and PGE₂ synthesis, cultured cells were treated with an mPGES-1 inhibitor (15d-PGJ₂) under conditions of serum-deprivation and serum-stimulation. Cells were serum-deprived for 1 day and then treated with varying concentrations of 15d-PGJ₂ in either serum-deprived media or de-differentiation-promoting media (serum stimulation). Control cells were treated with vehicle (0.1% ethyl acetate).
After 1 day of treatment, cells were fixed and analyzed for $\alpha$-actin expression via immunocytochemistry.

Results showed that $\alpha$-actin levels increased in cells treated with 15d-PGJ$_2$ under both conditions. The increase observed with treatment under serum-deprivation conditions was significant compared with vehicle-treated controls. However, though there was a trend towards increase in $\alpha$-actin levels with treatment under conditions of re-stimulation, this effect was not significant. Since treatment with either 0.1 or 1.0 $\mu$M of 15d-PGJ$_2$ did not result in significant induction of $\alpha$-actin, differentiated cells were treated with a higher concentration of 15d-PGJ$_2$ (10 $\mu$M), under de-differentiation-promoting conditions for 1 day, and $\alpha$-actin expression was examined via immunocytochemistry.

Significant induction in $\alpha$-actin protein levels in cells treated with 10 $\mu$M of 15d-PGJ$_2$ under de-differentiation-promoting conditions was observed compared with vehicle-treated controls (Figure 7.3.3).

Figure 7.3.3 mPGES-1 inhibition by 15d-PGJ$_2$ increases $\alpha$-actin protein levels in hASMCs cultured under serum-deprivation, serum-stimulation, and de-differentiation-promoting conditions

Immunocytochemistry with fluorescent imaging quantitation showing $\alpha$–actin expression in hASMCs treated with 15d-PGJ$_2$ for 1 day under (A) serum-free conditions and (B) serum-stimulated conditions (C) de-differentiation-promoting
conditions. Data represents mean ± SEM (n = 4), *** = P < 0.001; unpaired Student’s t-test for (A) and (C); one-way ANOVA for (B).
7.3.3.1 Rosiglitazone (PPARγ agonist) does not increase α-actin protein levels in hASMCs

In order to ascertain if the increase in α-actin observed with 15d-PGJ₂ treatment was due to mPGES-1 inhibition and not by the activation of PPARγ, rosiglitazone a PPARγ agonist, was used to treat the cells and the effect on the hASMC phenotype was examined. Cultured hASMCs were treated with varying concentrations of rosiglitazone under conditions promoting differentiation (serum-deprived) or de-differentiation (serum-stimulated) for 1 day and then protein levels of α-actin were subsequently measured by western blotting. Vehicle-treated controls were treated with media containing 0.1% DMSO. After 1 day of incubation, cells treated with rosiglitazone under serum-deprived conditions were fixed and analyzed for α-actin expression via immunocytochemistry whereas,
Rosiglitazone-treated cells under conditions promoting de-differentiation were lysed and harvested for analyses via western blotting.

Results showed that α-actin levels did not increase in cells treated with Rosiglitazone under either condition. Under conditions of serum-deprivation, there was significant reduction in α-actin levels upon treatment with both 1 and 10µM Rosiglitazone (Figure 7.3.4A). In addition, under serum-stimulated conditions, rosiglitazone treatment did not induce any increase in α-actin protein levels in treated cells (Figure 7.3.4B).
Figure 7.3.3.1 Rosiglitazone (PPAR\(\gamma\) agonist) does not increase \(\alpha\)-actin protein levels in hASMCs

(A) Immunocytochemistry with fluorescent imaging quantitation showing \(\alpha\)–actin expression in hASMCs treated with Rosiglitazone for 1 day under serum-deprived conditions and (B) Representative western blot with densitometry quantitation showing \(\alpha\)–actin expression in hASMC treated with Rosiglitazone for 1 day under serum-stimulated conditions. Data represents mean ± SEM (n = 4), *** = P < 0.001; one-way ANOVA with Dunnetts post hoc test for (A) and unpaired Student’s t-test for (B).
7.3.4 Small interfering RNA inhibition of mPGES-1 mRNA expression promotes hASMC differentiation

Having demonstrated that pharmacological inhibition of mPGES-1 with 15d-PGJ₂ results in the increase in α-actin protein levels and significantly reduced PGE₂ concentrations, the effect of modifying the mPGES-1/PGE₂ pathway on the hASMC phenotype via siRNA-mediated mPGES-1 knockdown was subsequently examined. hASMCs were transfected with mPGES-1 siRNA according to the manufacturer’s protocol via the reverse transfection method and plated the cells in growth media for 2 days. Control wells were treated with the transfection agent in the absence of the siRNA. After 2 days of incubation, the
media was replaced with serum-free media for 2 days. After each day of serum deprivation, cell culture media was collected for PGE$_2$ analysis by enzyme immune assay while cells were lysed and analyzed for either mRNA or protein expression.

7.3.4.1 siRNA mediated mPGES-1 knockdown induces mRNA expression of SMC markers of differentiation after 1 day of serum-deprivation

mPGES-1 siRNA-transfected cells were cultured for 2 days under dedifferentiation-promoting conditions and then serum deprived for 1 day. Cells were lysed, harvested, and mRNA was isolated after 1 day in serum-free media. mRNA expression levels of mPGES-1, and a variety of markers of differentiation ($\alpha$-actin, SM22$\alpha$, and smoothelin) was subsequently examined.

Significant reduction in mPGES-1 mRNA expression was observed after 1 day of serum-deprivation and this was associated with a significant increase in the mRNA expression of $\alpha$-actin and smoothelin (Figure 7.3.4.1). Although there was a trend toward an increase in SM22$\alpha$ expression in mPGES-1 siRNA transfected cells, this effect was not significantly different compared with the control cells.
Figure 7.3.4.1 siRNA mediated mPGES-1 knockdown induces mRNA expression of SMC markers of differentiation after 1 day of serum-deprivation

mRNA expression of (A) mPGES-1, (B) α-actin, (C) SM22α (D) Smoothelin in hASMCs transfected with mPGES-1 siRNA for 2 days in growth promoting media and then switched to serum-free media for 1 day. Data represents the mean + SEM (n≥3), * = p < 0.05, **** = p < 0.0001; unpaired Student’s t-test.
Figure 7.3.4.1 (continued)

**B. α-actin**

![Graph showing α-actin expression comparison between Control and mPGES-1 siRNA groups.]

**C. SM22α**

![Graph showing SM22α expression comparison between Control and mPGES1 siRNA groups.]

*Note: The graph for SM22α indicates a significant difference (indicated by *) between the Control and mPGES1 siRNA groups.*
7.3.4.2 siRNA mediated mPGES-1 knockdown induces mRNA expression of SMC markers of differentiation after 2 days of serum-deprivation

mPGES-1 siRNA-transfected cells were cultured for 2 days under de-differentiation-promoting conditions and then serum deprived for 2 days. Cells were lysed, harvested, and mRNA was isolated after 2 days in serum-free media. mRNA expression levels of mPGES-1, and a variety of markers of differentiation (α-actin, SM22α, and smoothelin) was subsequently examined.

There was significant reduction in mPGES-1 mRNA expression after 2 days of serum-deprivation and this was associated with significant increase in the mRNA expression of α-actin and smoothelin (Figure 7.3.4.2). Similar to the 1 day
experiment, the increase in SM22α expression in mPGES-1 siRNA transfected cells was not statistically significant.

**Figure 7.3.4.2** siRNA mediated mPGES-1 knockdown induces mRNA expression of SMC markers of differentiation after 2 days serum-deprivation

mRNA expression of (A) mPGES-1 (B) α-actin, (C) SM22α, and (D) Smoothelin in hASMCs transfected with mPGES-1 siRNA for 2 days in de-differentiation-promoting media and then switched to serum-free media for 2 days. Data represents the mean + SEM (n≥3), (n = 2 for mPGES-1), *= p< 0.05, *** = p < 0.001; unpaired Student’s t-test.
Figure 7.3.4.2 (continued)

B. \(\alpha\)-actin

\[ \begin{align*}
\frac{\alpha\text{-actin}}{\text{HPRT}} & \\
\text{Control} & \quad 0.10 \\
\text{mPGES1 siRNA} & \quad 0.20
\end{align*} \]

C. SM22\(\alpha\)

\[ \begin{align*}
\frac{\text{SM22}\alpha}{\text{HPRT}} & \\
\text{Control} & \quad 2.0 \\
\text{mPGES1 siRNA} & \quad 6.0
\end{align*} \]
7.3.4.3 siRNA mediated mPGES-1 knock-down increases protein levels of SMC markers of differentiation after serum-deprivation for 1 day

Protein levels of mPGES-1 and markers of differentiation in cells transfected with mPGES-1 siRNA and compared these with vehicle-transfected cells were examined. hASMCs were transfected with mPGES-1 siRNA and cultured for 2 days in de-differentiation-promoting media. After 2 days of incubation, the cell media was changed to serum-deprived media and then cells were lysed, harvested and analyzed by western blotting after 1 day of incubation in serum-free media.

The treatment of hASMCs with mPGES-1 siRNA resulted in a significant reduction in mPGES-1 protein levels and this was associated with a significant increase in protein levels of the differentiation markers, α-actin and SM22α.
(Figure 7.3.4.3). The increase in SM22α protein levels was greater than that observed for the increase in α-actin protein levels.

**Figure 7.3.4.3 siRNA mediated mPGES-1 knock-down increases protein levels of SMC markers of differentiation after serum-deprivation for 1 day**

Representative western blots and densitometry quantitation showing protein levels of (A) mPGES-1 (B) α-actin, (C) SM22α in hASMCs transfected with mPGES-1 siRNA for 2 days in de-differentiation-promoting media and then switched to serum-free media for 1 day. Data represents the mean ± SEM (n≥3), * = p < 0.05, **= p < 0.01; unpaired Student’s t-test.
Figure 7.3.4.3 (continued)

**B. α-actin**

![Graph showing α-actin levels](image)

**C. SM22α**

![Graph showing SM22α levels](image)
7.3.4.4 siRNA-mediated mPGES-1 knock-down increases protein levels of SMC markers of differentiation after serum-deprivation for 2 days

Protein levels of mPGES-1 and markers of differentiation in cells transfected with mPGES-1 siRNA were examined and compared with vehicle-transfected cells. hASMCs were transfected with mPGES-1 siRNA and cultured for 2 days in growth promoting media. After 2 days of incubation, the cell media was changed to serum-deprived media and then cells were lysed, harvested and analyzed by western blotting after 2 days of incubation in serum-free media.

A trend toward a reduction in mPGES-1 levels was observed though this was not significant. However, there was a significant increase in protein levels of both α-actin and SM22α in mPGES-1 siRNA transfected cells (Figure 7.3.4.4).
Figure 7.3.4.4 siRNA-mediated mPGES-1 knock-down increases protein levels of SMC markers of differentiation after serum-deprivation for 2 days

Representative western blots and densitometry quantitation showing protein levels of (A) mPGES-1 (B) α-actin, (C) SM22α in hASMCs transfected with mPGES-1 siRNA for 2 days in growth promoting media and then changed to serum-free media for 2 days. Data represents the mean + SEM (n≥3), ** = p < 0.01; unpaired Student’s t-test.
Figure 7.3.4.4 (continued)

B. α-actin

\[
\text{Percent of control (α-actin/α-tubulin)}
\]

\[
\begin{array}{c}
\text{Control} \\
\text{mPGES-1 siRNA}
\end{array}
\]

\begin{align*}
\text{α-actin} & \quad \text{α-tubulin} \\
\end{align*}

C. SM22α

\[
\text{Percent of control (SM22α/α-tubulin)}
\]

\[
\begin{array}{c}
\text{Control} \\
\text{mPGES-1 siRNA}
\end{array}
\]

\begin{align*}
\text{SM22α} & \quad \text{α-tubulin} \\
\end{align*}
7.3.4.5 siRNA-mediated mPGES-1 knockdown reduces mRNA expression of SMC markers of de-differentiation

With the finding that the knockdown of mPGES-1 increased the expression of differentiation markers, the effect of mPGES-1 knockdown on the expression of markers of the de-differentiated phenotype in hASMCs was examined. HAS2 and MMP-2 are both markers of the synthetic phenotype which are associated with decreased differentiation of vascular SMCs in certain cardiovascular diseases [53, 74]. Therefore, mRNA expression of HAS2 and MMP2 were examined in hASMCs transfected with mPGES-1 siRNA and then compared with vehicle-transfected cells.

hASMCs were transfected with mPGES-1 siRNA and cultured for 2 days in growth-promoting media. After 2 days of incubation, the cell media was changed to serum-deprived media and incubated for 2 days. Cells were lysed, harvested, and mRNA was isolated for real-time PCR analysis at each after either 1 or 2 days in serum-deprived media. Significant reduction in both HAS2 and MMP2 mRNA expression levels in the mPGES-1 siRNA-transfected cells was observed, as compared with vehicle-treated controls (Figure 7.3.4.6).

Figure 7.3.4.5 siRNA-mediated mPGES-1 knockdown reduces mRNA expression of SMC markers of de-differentiation.

mRNA expression of (A) HAS2, and (B) MMP2 in hASMCs transfected with mPGES-1 siRNA for 1 or 2 days in growth promoting media and then switched to
serum-free media. Data represents the mean ± SEM (n≥3), ** = p < 0.01, *** = p < 0.001; unpaired Student’s t-test.

1 day

A. HAS2

B. MMP2

161
Figure 7.3.4.5 (continued)

2 days

A. HAS 2

B. MMP2

Control          mPGES-1 siRNA

**
7.4 PGE$_2$ regulates COX-2 expression in hASMCs

7.4.1 PGE$_2$ treatment increases COX-2 expression under serum-deprivation conditions

The presence of a feedback loop between PGE$_2$ and COX-2 in which increased synthesis of PGE$_2$ either promotes induction of COX-2 expression (positive feedback) [124, 125, 241] or reduces COX-2 expression (negative feedback) [242-244] has been demonstrated in a variety of cell types. Due to these different reports regarding the regulation of COX-2 expression by PGE$_2$, the expression levels of COX-2 in these hASMCs both after treatment with PGE$_2$ exogenously, and after mPGES-1 siRNA-mediated knock down were examined. Cells were cultured under de-differentiation-promoting conditions for 2 days and then deprived of serum for 3 days. At this point, 1 µL of either 0.2 mM or 1.0 mM of PGE$_2$ was added to the media for 1 or 2 days after which mRNA was isolated from the cells and then analyzed for COX-2 expression via real-time PCR.

At each time point, there was significant increase in COX-2 mRNA expression with PGE$_2$ treatment compared with untreated controls (Figure 7.4.1). At the 2-day time point, there was about a 5-fold increase in COX-2 mRNA expression levels in cells treated with 1 µM PGE$_2$ compared with the vehicle-treated control.
Figure 7.4.1 PGE₂ treatment increases COX-2 expression under serum-deprivation conditions

mRNA expression of COX-2 in hASMCs serum starved for 3 days and then treated with PGE₂ for (A) 1 day or (B) 2 days. Data represents the mean ± SEM (n=3), ** = p< 0.01; one-way ANOVA (Dunnetts Multiple comparison test)
7.4.2 mPGES-1 mediated siRNA knock-down reduces COX-2 expression under serum-deprivation conditions over time

Since exogenous PGE\(_2\) treatment induced COX-2 mRNA expression in treated cells (7.4.1), the effect of endogenous loss of PGE\(_2\) on the expression of COX-2 in these hASMCs was examined. Cells were cultured directly in dedifferentiation-promoting media containing mPGES-1 siRNA for 2 days and then the culture media was changed to serum-free media for 1 to 2 days. At each time point, cells were harvested and mRNA was isolated for analysis by real-time PCR. In cells treated with mPGES-1 siRNA, significant reduction in COX-2 mRNA expression compared with vehicle-transfected controls was observed at each time point (Figure 7.4.2).
Figure 7.4.2 mPGES-1 mediated siRNA knock-down reduces COX-2 expression under serum-deprivation conditions over time

mRNA expression of COX-2 in hASMCs transfected with mPGES-1 siRNA for 2 days in growth promoting media and then switched to serum-free media for (A) 1 day or (2) 2 days. Data represents the mean + SEM (n≥3), *= p< 0.05, *** = p < 0.001; unpaired Student’s t-test.
7.5 PGE$_2$ treatment reduces mPGES-1 expression in hASMCs

mPGES-1 has been reported to be co-expressed and functionally coupled with COX-2 in response to a range of extracellular and intracellular stimuli [17, 106, 108, 111, 113, 117-120]. However, other investigators have demonstrated that mPGES-1 and COX-2 are neither co-expressed nor functionally coupled [79, 245, 246]. Therefore, upon observing the increase in COX-2 mRNA with PGE$_2$ treatment and the decrease in COX-2 expression in cells transfected with mPGES-1 siRNA, the expression of mPGES-1 in PGE$_2$-treated cells was examined in order to determine if the expression patterns between both enzymes would be similar. Cultured hASMCs were serum-deprived for 3 days and then treated with PGE$_2$ for either 1 or 2 days. Cells were harvested and mRNA was isolated and analyzed for mPGES-1 expression via real-time PCR. PGE$_2$ treated cells did not increase mPGES-1 levels as observed with COX-2 treatment in Figure 7.4.1. On the contrary, significant reduction in mPGES-1 mRNA levels were observed with PGE$_2$ treatment at both time points (Figure 7.5.1).
mRNA expression of mPGES-1 in hASMCs serum deprived for 3 days and then treated with PGE₂ for (A) 1 day and (B) 2 days. Data represents the mean ± SEM (n≥3), **= p< 0.01, ***= p< 0.001; one-way ANOVA (Dunnetts Multiple comparison test).
7.6 Small interfering RNA (siRNA) mediated mPGES-1 knockdown reduces PGE$_2$ production by hASMCs

The effect of mPGES-1 siRNA-mediated knock-down on the production and release of PGE$_2$ into the cell culture media at each time point was examined. hASMCs were transfected with mPGES-1 siRNA according to the manufacturer’s protocol via the reverse transfection method and plated the cells in de-differentiation-promoting media for 2 days. Control wells were treated with the transfection agent in the absence of the siRNA. After 2 days of incubation, the media was replaced with serum-free media and then collected after either 1 or 2 days. Subsequently, the media was assessed for PGE$_2$ concentration via enzyme immunoassay analysis.

Following mPGES-1 siRNA transfection, knockdown of mPGES-1 mRNA resulted in reduced PGE$_2$ concentrations after both days. However, this effect was significant only at the 2-day time point (Figure 7.6).
Figure 7.6 siRNA mediated mPGES-1 knock down reduces PGE$_2$

concentrations in hASMCs

Enzyme immunoassay analysis of PGE$_2$ concentrations in cells transfected with mPGES-1 siRNA for 2 days and then deprived of serum for (A) 1 day, or (B) 2 days. Data represents mean + SEM (n = 4), ***= p < 0.001; unpaired Student’s t-test.
7.7 Effect of EP4 receptor activation by CAY10580 on hASMC differentiation

PGE₂ exerts its actions via a family of G-protein coupled receptors: EP1, EP2, EP3 and EP4 having unique tissue expression profiles and whose effects are mediated through different downstream signaling pathways [79, 127-129]. EP4 receptor protein has been shown to be present in AAA lesions of both mice and human samples, and inhibition of this receptor in mice results in reduction of the incidence and severity of AAAs [133, 134]. On the contrary, the deletion of the EP4 receptor on bone-marrow-derived cells has been shown to enhance the formation of AAAs in AngII-induced mouse models of the disease [247].

Based on the findings implicating EP4 receptor activation in the development of AAAs, the hypothesis stating that activation of the EP4 receptor will reduce differentiation of hASMCs was proposed. To test this hypothesis, cells were treated with CAY10580, which is a potent EP4 receptor agonist. The experiments were performed under different cell culture conditions and the effect on the expression of differentiation markers was measured.

7.7.1 EP4 receptor activation by CAY10580 does not promote de-differentiation in hASMCs cultured under serum-deprivation conditions.

The effect of EP4 receptor activation on the induction of differentiation in serum-deprived cells was examined. Cultured cells were serum deprived over a 2 day or 3 day time period and then treated with 0.1 µM CAY10580 for 1 to 2 days. At each time point, cells were lysed, harvested and analyzed for α-actin,
SM22α and mPGES-1 protein levels via western blotting. Neither the 2 day nor 3 day treatment with CAY10580 in serum-free media resulted in a significant effect on protein expression of α-actin, SM22α or mPGES-1 (Figure 7.7.1).

**Figure 7.7.1 EP4 receptor activation by CAY10580 does not promote dedifferentiation in hASMCs cultured under serum-deprivation conditions.**

Representative western blots and densitometry quantitation showing protein levels of (A) α-actin (B) SM22α, (C) mPGES-1 in hASMCs serum deprived for either 2 or 3 days, and then treated with CAY10580 over a 2 day time period.

**2 day serum-deprivation**
Figure 7.7.1 (continued)

**B. SM22α**

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<tr>
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<th>Control</th>
<th>CAY10580 (µM)</th>
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**C. mPGES-1**

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Figure 7.7.1 (continued)

3 day serum deprivation

**α-actin**

A.

![Bar graph showing the percent of control (α-actin/α-tubulin) for different treatments with 3 days of serum deprivation.](image)

**SM22α**

B.

![Bar graph showing the percent of control (SM22α/α-tubulin) for different treatments with 3 days of serum deprivation.](image)
7.7.2 EP4 receptor activation by CAY10580 reduces α-actin levels in hASMCs cultured under conditions promoting differentiation

Cells were cultured under conditions promoting differentiation and treated with 0.3 μM CAY10580 for 1 day under the same conditions. Cells were fixed, permeabilized and incubated with α-actin antibody overnight and subsequently analyzed via immunocytochemistry. There was a significant decrease in the expression of α-actin in EP4 receptor agonist-treated cells, compared with vehicle-treated controls (Figure 7.7.2).
Figure 7.7.2 EP4 receptor activation by CAY10580 reduces α-actin levels in hASMCs cultured under conditions promoting differentiation

mRNA expression of α-actin in hASMCs treated with CAY10580 under conditions promoting differentiation for 1 day. Data represents the mean + SEM (n=4), **= p< 0.01 unpaired Student’s t-test.

Discussion

Among the COX-2-derived prostanoids, PGE₂ is a major component of the pro-inflammatory cascade which has been implicated in the pathology of numerous diseases including cancer, arthritis, atherosclerosis, and aneurysms [17, 123, 141, 149, 233, 248-250]. Furthermore, mPGES-1, which is the major PGE₂-synthesizing enzyme, has been shown to be co-expressed with COX-2 in
vitro and in vivo [17, 122, 231, 233]. Since an increase in COX-2 has been implicated in the reduced expression of differentiation markers during development of AAAs, and an increase in mPGES-1 and PGE₂ has been detected in aneurysmal lesions compared to normal aorta, the mechanism by which COX-2 may be inducing de-differentiation was proposed to be via the action of mPGES-1-derived PGE₂ [74, 126, 128, 147-150]. Using an in vitro model of cultured hASMCs, the role of PGE₂ in phenotypic modulation was investigated by measuring expression of markers of differentiation and de-differentiation after exogenous PGE₂ treatment and the inhibition of mPGES-1-derived PGE₂. It was proposed that COX-2 may be contributing to reduced differentiation of the vascular SMCs by increasing production of PGE₂.

Cells were cultured under conditions promoting differentiation using differentiation-promoting media and treated with PGE₂ under either de-differentiation-promoting or differentiation-promoting conditions. With differentiated hASMCs treated with PGE₂ under conditions promoting differentiation, both a concentration-dependent decrease in α-actin protein levels and a statistically significant reduction with a 1 µM PGE₂ treatment at the 1 day time point were observed (Figure 7.1.1). Although there was a trend toward reduction of α-actin protein levels with 1 µM PGE₂ at the 2-day time point, the effect was not statistically significant. This may be due to PGE₂ degradation in the media over time. The treatment of differentiated cells with PGE₂ under conditions promoting de-differentiation for 1 day also significantly decreased α-actin protein levels and the effect was more significant than that observed with
treatment under differentiation-promoting conditions (Figure 7.1.2). Treatment with PGE$_2$ for 2 days under conditions promoting de-differentiation however, did not yield significant reduction in $\alpha$-actin possibly due to degradation of the PGE$_2$ in the media over time. The reduction induced by PGE$_2$ under these conditions exceeded that observed with differentiation-promoting media possibly due to the fact that the increased serum and growth factors present in this de-differentiation-promoting media, may reduce differentiation, and thus potentiate the $\alpha$-actin–reducing effects of PGE$_2$ under these conditions (Figure 7.1.2). Media containing increased serum and growth factors have been shown to contribute to reduced expression of differentiation markers in vascular SMCs (45, 46, 54, 56-58). Therefore, treating the hASMCs with PGE$_2$ under these conditions may further potentiate this reduction in differentiation demonstrated by the greater decrease in $\alpha$-actin levels in these cells at the 1 day time point.

Since PGE$_2$ was observed to reduce differentiation in hASMCs, the effect of PGE$_2$ treatment on the induction of differentiation by serum-deprivation was examined. Vascular SMCs when deprived of serum over time have been shown to induce differentiation by upregulating the expression of markers of vascular SMC differentiation [23, 172-174]. This effect was also observed in the current studies with hASMCs as shown by the increase in $\alpha$-actin and SM22\(\alpha\) (Figure 4.2). hASMCs which were induced to differentiate under conditions of serum-deprivation with PGE$_2$ were treated for either 1 or 2 days in order to determine if PGE$_2$ would prevent induction of differentiation under these conditions. Treatment of serum-deprived cells with PGE$_2$ over time beginning either at the 2
or 3 day time point resulted in significant reduction in both mRNA and protein levels of differentiation markers. Even though serum-deprivation over time was expected to induce expression of differentiation markers, PGE₂ treatment inhibited this induction and also reversed differentiation in these cells (See section 7.2). Although the observed reduction in protein levels of α-actin and SM22α was significant, the reduction observed with the 0.2 µM treatment of PGE₂ was greater than that observed with the 1 µM treatment. This could be attributed to off-target effects occurring in cells treated with higher concentration of PGE₂ under serum-starved conditions.

The effect of PGE₂ treatment on the expression of markers of de-differentiation under conditions of serum-deprivation was also examined. The results showed that PGE₂ treatment increases expression of markers of de-differentiation, although the time required for producing the effect was different than for the differentiation markers. Although treatment with PGE₂ for 1 day did not increase expression of these markers, treatment for 2 days did result in the significant induction of mRNA expression of both HAS2 and MMP2 in these cells.

PGE₂ has been observed to alter the differentiation state of a variety of cell types in vitro and in vivo. For example, PGE₂ has been shown to induce the differentiation of myeloid-derived suppressor cells (MDSC) from bone marrow stem cells thereby promoting proliferation and malignancy of tumor cells in the pathogenesis of cancer [251]. In addition, PGE₂ has been shown to inhibit expression of differentiation markers in human lung fibroblast cells [252, 253]. In vascular SMCs, analogues of prostacyclin or PGE₂ increase expression of HAS2
mRNA and the synthesis of hyaluronic acid [34]. In SMCs induced to de-differentiate following treatment with interleukin-1beta, PGE$_2$ treatment was shown to enhance actin filament disorganization, although no changes in the expression of differentiation markers was reported[104]. These findings demonstrate that in hASMCs, exogenous PGE$_2$ treatment under a variety of culture conditions promotes a reduction in the expression of multiple markers of differentiation. Furthermore, PGE$_2$ treatment induces the expression of markers of the synthetic phenotype thus promoting the de-differentiation of these cells.

The isoprostane 15-deoxy-Δ(12,14)-prostaglandin J(2) (15d-PGJ$_2$), is a commercially available pharmacological agent which has been shown to be a potent inhibitor of mPGES-1 \textit{in vitro} [254]. Recent reports have demonstrated that 15-dPGJ$_2$ inhibits human mPGES-1 both covalently (by modification of residue C59) and non-covalently (by binding to the PGH$_2$ binding site) at concentrations of 1.0 µM or less [254, 255]. 15-dPGJ$_2$ has also previously been described as an endogenous ligand of PPAR$\gamma$, although this role is controversial because of the supraphysiological concentrations of 15d-PGJ$_2$ required to activate this transcription factor. In vascular SMCs, the concentration of 15-dPGJ$_2$ required to increase expression of differentiation markers has been shown to be 10 µM or greater [95, 97-99, 256]. Therefore, since 15d-PGJ$_2$ is more potent as an inhibitor of mPGES-1, as compared to an activator of PPAR$\gamma$, lower concentrations of 15d-PGJ$_2$ (0.1 µM, 1 µM, 2 µM) were primarily utilized to determine the effect of pharmacological inhibition of mPGES-1 in preventing hASMC de-differentiation.
15d-PGJ$_2$ treatment under conditions of serum-deprivation resulted in significant increase (approximately 6-fold) in α-actin protein levels compared with vehicle-treated cells (Figure 7.3.3A). Treatment with 15d-PGJ$_2$ under conditions of serum-stimulation, significantly reduced PGE$_2$ concentrations (Figure 7.3.2B) and produced a trend toward an increase in α-actin levels (Figure 7.3.3B). Under conditions of re-stimulation, the extent of reduction of PGE$_2$ synthesis was similar between the treatments using both concentrations of 15d-PGJ$_2$ (0.1 µM and 1 µM). The increase in α-actin levels under conditions of serum-deprivation far exceeded that observed under serum-stimulation. The presence of increased serum in media under conditions of serum-stimulation may have reduced the amounts of free 15d-PGJ$_2$ within the cell media available for mPGES-1 inhibition due to increased protein binding under these conditions. Therefore, to account for this, cells were treated with 10 µM of 15d-PGJ$_2$ under de-differentiation-promoting media containing increased serum (5% FBS) and after 1 day of treatment, a 9-fold increase in α-actin expression in treated cells compared with vehicle-treated controls was observed (Figure 7.3.3C).

To investigate if the effects of 15d-PGJ$_2$ on increasing differentiation is due to PPARγ activation and not mPGES-1 inhibition, cells were treated with Rosiglitazone, a well-established PPARγ activator under conditions promoting either differentiation or de-differentiation. Treatment of these cells with Rosiglitazone did not increase α-actin levels in treated cells like that observed with 15d-PGJ$_2$ treatment thus suggesting that 15d-PGJ$_2$ is not inducing differentiation in these cells by acting through PPARγ (Figure 7.3.3.1).
Although mPGES-1 is considered a valuable target for developing novel anti-inflammatory medications, few compounds have been identified with activity for inhibiting this enzyme. Although 15d-PGJ2 is a potent inhibitor of mPGES-1, the differentiation-promoting effects observed have the potential to occur through PPARγ-dependent mechanisms, rather than by inhibiting mPGES-1. Therefore, as an additional method to the pharmacological inhibition of mPGES-1, PGE2 levels were reduced by transient knockdown of mPGES-1 mRNA expression and the effect on cell differentiation was examined. Even though PGE2 may be synthesized by 3 synthases, mPGES-1 was selected for targeting because the majority of the PGE2 formed in response to COX-2 induction is dependent on this enzyme [121, 122]. Successful siRNA knock-down of mPGES-1 with consequent reduction in PGE2 levels has previously been reported in different cell culture models including vascular SMCs [109, 246, 257]. Furthermore, deletion of mPGES-1 in vitro and in vivo has been shown to reduce PGE2 levels [107, 127]. Upon siRNA-mediated mPGES-1 transfection, there was significant reduction in mPGES-1 mRNA expression and protein levels, with concurrent reduction in PGE2 concentrations. In addition, the reduction in endogenous PGE2 production was associated with significant increase in mRNA and protein markers of differentiation, as well as significant reduction in expression of de-differentiation markers (section 7.3.4). These results are in agreement with results from pharmacological inhibition of mPGES-1 as well as exogenous PGE2 treatment. Therefore, these findings have demonstrated that PGE2 is a primary product of
COX-2 in hASMCs and that activation of the mPGES-1/PGE₂ pathway is prominent mechanism contributing to hASMC de-differentiation.

The expression of COX-2 has long been known to be regulated by products dependent on COX-2 activity for their synthesis. Feedback mechanisms have been shown to either increase or decrease COX-2 expression in a variety of cell types and PGE₂ is the prostanoid most often implicated in affecting the expression of COX-2. In the regulation of cellular differentiation, a positive feedback mechanism for PGE₂ induction of COX-2 functions to suppress the differentiation of monocytes into dendritic cells [241]. In the kidney of mice, a negative feedback mechanism occurs in which activation of EP3 receptors by the PGE₂ analog, sulprostone, results in downregulation of COX-2 expression [242]. Similarly in arterial SMCs, the expression of COX-2 is strongly inhibited by treatment with exogenous PGE₂, thereby indicating the presence of a negative feedback mechanism [243, 244]. In the current studies, the role of PGE₂ in regulating the expression of COX-2 in these cells under different conditions was examined. Under conditions of serum-deprivation in which low levels of COX-2 are expressed, PGE₂ treatment resulted in a dose- and time-dependent increase in COX-2 mRNA expression (7.4.1). Following the reduction in PGE₂ resulting from siRNA-mediated mPGES-1 knockdown, the opposite effect was observed with significantly reduced COX-2 expression levels in siRNA-treated cells (7.4.2). These results indicate the presence of a positive feedback mechanism occurring with PGE₂ increasing COX-2 expression in hASMCs.
Numerous reports describing the expression characteristics of mPGES-1 have shown the synthase to be co-expressed and functionally coupled with COX-2 in response to a range of extracellular and intracellular stimuli [17, 106, 108, 111, 113, 117-120]. Furthermore, the majority of the PGE₂ formed in response to these various stimuli which induce COX-2, is dependent on the expression of mPGES-1 (10, 11, 52). In addition to these findings, other reports describe differences in expression between COX-2 and mPGES-1. In the murine mammary gland during lactogenic differentiation, mPGES-1 production of PGE₂ is dependent on the activity of COX-1 and not COX-2 [79]. In addition, in rat microglia cells, LPS-induced mPGES-1 synthesis was not coupled with corresponding increase in COX-2 levels [245]. Furthermore, in vascular SMCs, the time-course of IL-1β induced mPGES-1 expression and PGE₂ synthesis was delayed compared with expression of COX-2, and depending on the time of incubation with IL-1β, either COX-1 or COX-2 could be involved in the synthesis of PGE₂, thus demonstrating uncoupling of mPGES-1 expression with either COX-2 enzyme [246].

In these studies, profound reduction in PGE₂ synthesis in celecoxib-treated cells was observed (7.3.2), as well as a significant increase in PGE₂ concentrations when cells were cultured under conditions shown to induce COX-2 expression (serum-stimulation) (7.3.1). Therefore, it was expected of both COX-2 and mPGES-1 to demonstrate coupled expression in hASMCs. In contrast however, even though PGE₂ treatment significantly induced COX-2 mRNA expression, significant reduction in mPGES-1 mRNA expression following
PGE$_2$ treatment. Therefore, our findings suggest that PGE$_2$ may also promote de-differentiation by potentiating the actions of COX-2 through a positive feedback mechanism, and unlike other studies showing similar expression patterns between COX-2 and mPGES-1, in hASMCs the regulation of expression of these two enzymes may occur different mechanisms.

PGE$_2$ activity is modulated via activation of four different G-protein-coupled receptors known as EP1, EP2, EP3 and EP4. These four classes of receptors are structurally and functionally distinct, and their effects are mediated through different downstream signaling pathways (5, 14, 16, 45, 46). In addition to the four classes, the EP3 protein exists as 8 different forms that result from splice variants of the mRNA (86).

In order to investigate the receptors responsible for PGE$_2$'s effect on reducing differentiation, a PGE$_2$ analogue that is a selective agonist of the EP4 receptor was utilized. CAY10580 shows approximately a 100-fold selectivity for the EP4 receptor over the other EP receptors and has a disassociation constant for EP4 of approximately 50 nM. To examine the effect of EP4-selective activation, the initial studies were performed under serum-free conditions to eliminate the potential for interaction with serum proteins. In order to maintain selectivity for activating the EP4 receptor, these studies utilized a CAY10580 concentration of 0.1 µM, which is approximately twice the concentration of the disassociation constant. These studies also examined the effect of different durations of treatment and at different stages of the serum deprivation used to induce differentiation. However, neither treatment for 1 day or 2 days under these
varying conditions significantly reduced the protein expression of α-actin, SM22α or mPGES-1, as observed following treatment with PGE₂ (7.6.1). In addition to the treatments in serum-free media, the effects of EP4-selective activation under conditions promoting differentiation was also examined. Because of the presence of serum in the differentiation-promoting media, a higher concentration of 0.3 µM of the EP4-selective agonist was utilized. In contrast to our findings using serum-free media, the treatment of hASMCs with the EP4 receptor-selective agonist significantly reduced expression of α-actin, as determined by immunohistochemical quantitation (7.6.2). Therefore, although these findings suggest a role for the EP4 receptor in contributing to reduced differentiation under differentiation-promoting conditions, a role for this receptor being responsible for the potent de-differentiation effects of PGE₂ on hASMCs that was observed under multiple culture conditions is not conclusive.

In summary cultured human aortic smooth muscle cells (hASMCs) were utilized as an in vitro model to explore the role of components of the COX-2/mPGES1/PGE₂ axis on SMC phenotypic modulation to better understand the contribution of this to pathway to the alteration in differentiation observed with in vivo cardiovascular diseases such as AAAs. The reduced differentiation occurring in hASMCs may be mediated by the action of mPGES-1-derived PGE₂. PGE₂ added to the cell media exogenously will reduce differentiation and also prevent the induction of differentiation in quiescent cells. Furthermore, these findings demonstrate that inhibition of endogenous production of PGE₂ via
pharmacological or siRNA inactivation of mPGES-1 reduces PGE$_2$ production and promotes hASMC differentiation.

With the involvement of PGE$_2$ in the pathogenesis of numerous cardiovascular diseases including AAAs, the inhibition of mPGES-1 is expected to be an important future method of effectively reducing PGE$_2$ synthesis, thereby preserving the differentiated phenotype. Therefore, pharmacological inhibition of mPGES-1 may provide a mechanism of inhibiting PGE$_2$ synthesis that is more specific than celecoxib treatment, and may serve as a novel target for attenuating AAA progression by promoting and maintaining a differentiated SMC phenotype.
CHAPTER 8: EFFECTS OF OTHER PROSTANOIDS ON HUMAN AORTIC SMOOTH MUSCLE CELL PHENOTYPIC MODULATION

Introduction

Aside from the effects of PGE2 on the phenotype of vascular smooth muscle cells (SMCs), other prostanoids have been demonstrated in the literature to modulate the phenotype of vascular SMCs in a variety of physiological or pathological disease states. Prostacyclin (PGI2) and thromboxane (TXA2) are both prostanoids which have been demonstrated to produce opposing effects within the vasculature. Several studies have indicated that a balance between these prostanoids is important in the maintenance of the endothelial integrity of the vessels and overall vascular homeostasis [64, 88, 258].

Prostacyclin is the primary COX-2 product in endothelial cells and the synthase downstream of COX-2 responsible for PGI2 production is also expressed in vascular SMCs and platelets [33]. PGI2 is a potent vasodilator and an inhibitor of platelet aggregation and thrombus formation [87, 259]. These effects have been demonstrated to be produced by activation of the prostacyclin receptor (IP) by PGI2 resulting in cAMP production and stimulation of downstream signaling cascades [260]. In addition to its role as a potent anti-aggregatory [259, 261], anti-proliferative [262-265] and anti-migratory [266, 267] agent, prostacyclin and prostacyclin-mimetic agents have been also demonstrated to promote the contractile state by inducing the expression of markers of the differentiated phenotype in vascular SMCs [33, 92].
Thromboxane (TXA₂) on the other hand is primarily a derivative of COX-1-catalyzed arachidonic acid metabolism which is released by activated platelets [268]. Contrary to PGI₂, TXA₂ is involved in promotion of platelet aggregation and vasoconstriction [77]. In addition, TXA₂ and stable thromboxane-mimetics activate the thromboxane receptor (TP), stimulate DNA synthesis, and promote cell proliferation in vascular SMCs [265, 269, 270]. However, there is limited literature demonstrating the effects of TXA₂ or TP receptor stimulation on vascular SMC phenotypic modulation.

Therefore, the effects of IP and TP receptor stimulation on the differentiation state of human aortic smooth muscle cells (hASMCs) were examined. Using Iloprost, a specific agonist of the IP receptor, the effect of IP stimulation on hASMC differentiation was examined. For the TP receptor, a TP receptor agonist, IBOP, as well as 2 antagonists, SQ 29548 and seratrodast, to perturb the effects of these agents individually on the phenotype of these cells.

**Results**

8.1 Iloprost, a PGI₂ mimetic agent promotes differentiation in hASMCs after 1 day of treatment under de-differentiation-promoting conditions.

To examine the effect of PGI₂ on the phenotype of hASMCs, cells were cultured under conditions promoting differentiation for 2 days and then treated cells with iloprost under media conditions promoting either de-differentiation or differentiation for 1 or 2 days. Control cells were treated with vehicle (0.1% ethyl
acetate) in culture media. Cells were lysed, harvested and analyzed for α-actin protein expression via western blotting. Treatment of hASMCs with iloprost under conditions promoting de-differentiation resulted in a significant increase in α-actin protein levels at the 1 day time-point, as compared with vehicle-treated control, but not after 2 days of treatment (Figure 8.1A and B). In cells treated with Iloprost under conditions promoting differentiation, there was not a significant increase in α-actin protein expression, as compared to vehicle-treated cells (Figure 8.1C).

**Figure 8.1 Iloprost, a PGI₂ mimetic agent promotes differentiation in hASMCs after 1 day of treatment under de-differentiation-promoting conditions.**

Representative western blot and densitometry quantitation of α-actin in differentiated cells treated with 1µM of iloprost under de-differentiation-promoting conditions for (A) 1 day or (B) 2 days; (C) treatment with 1 µM of iloprost under differentiation-promoting conditions for 1 day. Data represents mean + SEM (n ≥ 3), p < 0.05, unpaired Student’s t-test.
Figure 8.1 (continued)

A. Percent of control (α-actin/α-tubulin)

B. Percent of control (α-actin/α-tubulin)

α-actin

α-tubulin
8.2 Effects of thromboxane A$_2$ receptor activation by IBOP on vascular SMC differentiation

IBOP, a stable thromboxane A$_2$ mimetic was used to examine the effects of TP receptor activation on the hASMC phenotype. Cells were cultured under conditions promoting differentiation and then treated with 1 µM IBOP in differentiation-promoting media for 1 or 2 days. For differentiated cells treated with IBOP under conditions promoting de-differentiation, a 5 µM IBOP concentration was used because of the increased potential for protein binding in the 5% serum-containing media. After incubation over time, cells were lysed, and harvested each day and subsequently analyzed for the expression of α-actin via western blotting. Control cells were treated with vehicle (0.1% ethanol) under both cell culture conditions over a 1 or 2 day time period.
After treatment of differentiated cells with IBOP under differentiation-promoting conditions at both time points, a trend toward reduction in \( \alpha \)-actin protein levels in treated cells compared with vehicle-treated cells was observed, but this decrease was not statistically significant (Figure 8.2 A & B). For differentiated cells treated with 5 \( \mu \)M IBOP under conditions promoting de-differentiation, there was a reduction in \( \alpha \)-actin protein levels at both time points compared with vehicle-treated controls at the 1 day time point (Figure 8.2 C) and the reduction was significant at the 2-day time point (Figure 8.2 D).
Figure 8.2 Effects of thromboxane receptor activation by IBOP on vascular SMC differentiation

Representative western blot and densitometry quantitation of α-actin in differentiated cells treated with 1 μM of IBOP under differentiation-promoting conditions for (A) 1 day or (B) 2 days; or with 5 μM of IBOP under de-differentiation-promoting conditions for (C) 1 day (n = 2), or (D) 2 days. Data represents mean + SEM (n ≥ 3), ** = p < 0.01, unpaired Student’s t-test.
Figure 8.2 (continued)

B. Percent of control (α-actin/α-tubulin)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IBOP (1μM)</th>
</tr>
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<tbody>
<tr>
<td>Percent of control (α-actin/α-tubulin)</td>
<td>100</td>
<td>150</td>
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</tbody>
</table>

α-actin

α-tubulin

C. Percent of control (α-actin/α-tubulin)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IBOP (5μM)</th>
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<tr>
<td>Percent of control (α-actin/α-tubulin)</td>
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<td>150</td>
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</table>

α-actin

α-tubulin
8.3 Effects of thromboxane receptor antagonism by SQ29548 on hASMC differentiation

Since the treatment of hASMCs with IBOP under certain conditions showed a reduction in α-actin protein levels, the effect of TXA₂ receptor antagonism by SQ 29548 on the expression of α-actin in these cells was examined. SQ 29548 is a potent TP receptor antagonist which binds to the human recombinant TP receptor with a Ki of 4.1 nM. Cells were cultured under conditions promoting differentiation for 3 days and then treated with either 50 or 300 nM SQ 29548 in de-differentiation-promoting media for 1 or 2 days. Control cells were treated with vehicle (0.1% ethanol) under the same conditions and
time points. Cells were lysed, harvested and examined for α-actin protein levels via western blotting.

Cells treated for 1 day with either 50 or 300 nM SQ29548 demonstrated only a trend towards a reduction in actin levels, as compared with vehicle-treated controls (Figure 8.3A and B). This trend was reversed by the 2-day time point where there was a trend towards an increase in α-actin levels observed in treated cells at both concentrations, as compared with vehicle-treated controls (Figure 8.3 C and D). However, neither of these effects was statistically significant.
Figure 8.3 Effects of thromboxane receptor antagonism by SQ29548 on hASMC differentiation

Densitometry and representative western blots showing protein levels of $\alpha$-actin in differentiated cells treated with (A) 50 nM SQ 29548 or (B) 300 nM SQ29548 under conditions promoting de-differentiation over time. Data represents mean $\pm$ SEM ($n = 3$)

1 day

A.

Percent of control ($\alpha$-actin/$\alpha$-tubulin)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SQ 29548 (50nM)</th>
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</thead>
<tbody>
<tr>
<td>$\alpha$-actin</td>
<td>$\uparrow$</td>
<td>$\downarrow$</td>
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<tr>
<td>$\alpha$-tubulin</td>
<td>$\downarrow$</td>
<td>$\uparrow$</td>
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</table>
Figure 8.3 (continued)

B.  

Percent of control  
(α-actin/α-tubulin)  

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SQ 29548 (300nM)</th>
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<td>150</td>
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<td>50</td>
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<tr>
<td>200</td>
<td>100</td>
<td>50</td>
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</table>

α-actin

α-tubulin

2 days

A.  

Percent of control  
(α-actin/α-tubulin)  

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SQ 29548 (50nM)</th>
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<tr>
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α-actin

α-tubulin

199
8.4 Thromboxane A₂ receptor antagonism by seratrodast does not increase α-actin protein levels in hASMCs

To further investigate the effect of TP receptor antagonism on the vascular SMC phenotype, cells cultured under different conditions were treated with varying concentrations of seratrodast and the effect on α-actin protein levels in the cells was examined. Seratrodast is another potent thromboxane A₂ receptor (TP) antagonist which has been demonstrated to block the TP receptor with an IC₅₀ of approximately 7 nM [271].

Cells were cultured under de-differentiation-promoting conditions and treated with 1, 10 or 100 µM of seratrodast in de-differentiation-promoting media. In addition, hASMCs were cultured under differentiation-promoting conditions
and then treated with 1, 10 or 100 µM of seratrodast in differentiation-promoting media. Control cells were treated with vehicle (0.1% of DMSO) in appropriate media. After 1 day of incubation, cells were lysed, harvested and then analyzed for α-actin protein levels by western blotting.

Treatment of cells under conditions promoting de-differentiation or differentiation did not significantly increase α-actin protein levels. A trend toward reduction in α-actin levels in cells treated with 100 µM seratrodast in de-differentiation-promoting media was observed and this reduction was significant compared with vehicle-treated controls in 100 µM seratrodast-treated cells under differentiation-promoting conditions.
Figure 8.4 Thromboxane receptor antagonism by seratrodast does not increase α-actin protein levels in hASMCs

Densitometry and representative western blots showing protein levels of α-actin in (A) growing cells treated with seratrodast under de-differentiation-promoting conditions for 1 day, and (B) cells cultured under differentiation-promoting conditions treated with seratrodast under conditions promoting differentiation for 1 day. Data represents mean ± SEM (n≥3), *** = p < 0.001; one-way ANOVA and post hoc analysis with Dunnett’s Multiple Comparison test.
Figure 8.4 (continued)

**Discussion**

The cyclooxygenase enzymes convert arachidonic acid via a two-step reaction to the unstable intermediate PGH₂, which is required for the synthesis of all prostanoids by the action of individual prostaglandin synthases. The prostaglandin synthases are specific isomerases which act on PGH₂ to convert this unstable intermediate to different prostanoids. Among these prostanoids, prostacyclin (PGI₂) and thromboxane A₂ (TXA₂) have emerged as key modulators of the vasculature with a balance between both prostanoids being necessary for the maintenance of cardiovascular homeostasis in vivo. In addition
to having opposing effects on the vasculature, the synthesis of both prostanoids is also coupled to different COX isoforms with thromboxane A\textsubscript{2} synthesis primarily coupled with COX-1 in platelets, and that of prostacyclin produced by endothelial cells resulting from the activity of COX-2 [219, 268, 272, 273]. Based on this coupling, it has been suggested that the increased risk of adverse cardiovascular events with the prolonged use of COX-2 inhibitors (such as celecoxib, rofecoxib and valdecoxib) may be due to the decrease in PGI\textsubscript{2} production without affecting TXA\textsubscript{2} levels, thereby disrupting the homeostatic balance between both prostanoids [33, 258]. Thus, the hypothesis was proposed that prostacyclin may contribute to maintenance of cardiovascular homeostasis by promoting the differentiated phenotype while TXA\textsubscript{2} would promote the de-differentiated phenotype.

In order to investigate the effects of prostacyclin (PGI\textsubscript{2}) on the SMC phenotype, the stable prostacyclin-mimetic iloprost was utilized to treat the cells under different conditions. Iloprost is a structural analog of prostacyclin (PGI\textsubscript{2}) which is more stable than prostacyclin and binds to the human recombinant IP receptors with a K\textsubscript{i} of 11 nM [274]. In addition, iloprost has been approved for clinical use in the management of pulmonary arterial hypertension. Treatment of differentiated hASMCs with iloprost for 1 day under conditions promoting de-differentiation showed a significant increase in \(\alpha\)-actin protein levels, as compared with vehicle-treated controls (Figure 8.1). These results are similar to those reported by other researchers who have demonstrated that PGI\textsubscript{2} mimetics promote the contractile state by inducing the expression of markers of the
differentiated phenotype in vascular SMCs [33, 92, 275]. Although the
differentiation-promoting effects of PGI2 on SMCs may be expected to be
beneficial in retarding the in vivo development of cardiovascular disease, the
limited synthesis of PGI2 by vascular SMCs reduces the potential significance of
PGI2 as an endogenous modulator of differentiation in our current culture model.

Thromboxane A2 has been shown to promote proliferation [265, 269, 270]
in vascular SMCs. However, though it was previously assumed that the
processes of proliferation and differentiation were coupled, with reduction in
proliferation being a prerequisite for differentiation, current findings have
demonstrated that these processes are not mutually exclusive [88]. Therefore,
since TXA2 promotes cell proliferation, it is not assumed that this product would
also inhibit vascular SMC differentiation. To investigate the effect of thromboxane
(TXA2) on the SMC phenotype, the effect of TXA2 receptor (TP) activation as well
as antagonism in these cells was examined. IBOP, a TXA2 receptor agonist,
produced an overall non-significant trend toward inducing de-differentiation in
these SMCs by reducing expression of α-actin in differentiated cells under both
conditions of differentiation and growth (Figure 8.2). Thromboxane has been
shown to promote hypertrophy of vascular SMCs by upregulating the synthesis of
basic fibroblast growth factor (FGF) endogenously, thereby inducing SMC
proliferation [276]. A variety of growth factors (including FGF) in cell culture
media have been shown to promote the de-differentiated state in cultured
vascular SMCs [6, 167, 174]. Therefore, TXA2 may promote reduction in hASMC
differentiation by increasing the expression of growth-promoting factors produced by the cells.

To further investigate the effects of thromboxane on hASMC phenotype, the effects of TP receptor antagonists on the expression of SMC differentiation markers in these hASMCs were examined. SQ29548 is potent TP receptor antagonist demonstrated to bind to the TP receptor with a ki of about 1.6 nM [277, 278]. hASMCs were treated with SQ29548 at concentrations exponentially higher than this (50 nM or 300 nM) in order to account for potential binding of this antagonist to proteins in the serum of the media. Contrasting effects of SQ 29548 were observed treatment in these cells, with treatment for 1 day showing a trend toward reduction in α-actin protein levels whereas, at the 2 day time point, treatment with SQ29548 at 50 and 300 nM produced a non-significant trend toward an increase in α-actin expression. The current studies also utilized the TP receptor antagonist, seratrodast. Despite treating cells with varying concentrations of seratrodast, there was no observed increase in α-actin protein expression, and the highest concentration of seratrodast (100 µM) decreased expression, likely the result of non-specific binding. Although TXA₂ release has been reported to be coupled with COX-1 in platelets, recent results have shown that serum-stimulation increases COX-2 expression and this is associated with TXA₂ release from the vascular SMCs. In vascular SMCs obtained from the type 2 diabetic mouse model (db/db mice), TP receptor antagonism by SQ29548 was demonstrated to reduce COX-2-dependent vascular smooth muscle hyper-reactivity mediated by endogenous TXA₂ [279]. Similarly, in another model, IL-1β
stimulation induced COX-2 expression thereby increasing the production of TXA₂ in vascular SMCs [81]. Therefore, although increased synthesis of TXA₂ that results from an induction of COX-2 expression has been shown to occur in other SMC culture models, these current studies suggest that there is limited importance for a role of TXA₂ as an endogenous regulator of hASMC differentiation.

Despite a thorough search of the literature, there are no reported studies indicating the effects of TXA₂ receptor activation or antagonism on the vascular SMC phenotype. This may be due to the fact that most of the focus has been on the detrimental pro-thrombotic, platelet aggregatory, and vasoconstriction effects of this prostanoid within the vasculature, since thrombus-formation and vasoconstriction induced by TXA₂ are implicated in the incidence of myocardial infarction, hypertension, and stroke. Even though there is a trend towards reduction in differentiation with IBOP treatment in these hASMCs, the concentrations of IBOP utilized are much greater than the EC₅₀ of IBOP (about 2 nM) reported in the literature and thus may not be physiologically relevant [280]. Therefore, it is difficult to ascertain how much, and to what extent the reduction in α-actin protein levels may be attributed to the action of IBOP activating the TP receptor or other off-target effects. Furthermore, studies have reported that stimulation of COX-2 in vascular SMCs promotes endogenous release of TXA₂ [81, 279]. Since the level of this prostanoid was not measured in these studies, the effect of the presence/absence of endogenous levels of TXA₂ in the cells with respect to modulation of the hASMC phenotype is unknown. Even though there
was an observed increase in α-actin protein levels in cells treated with the TP receptor antagonist, SQ29548, this effect was not consistent and treatment with Seratrodast did not induce differentiation in these cells. Therefore, to better understand the role of TXA₂ in hASMC phenotypic modulation, future studies would need to examine the endogenous production of this prostanoid as well as TXA₂ synthase expression and their correlation with the differentiation state of the cell.
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2013  Third place award, 3MT competition, Graduate school congress, University of Kentucky

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PEER-REVIEWED PUBLICATIONS AND PUBLISHED ABSTRACTS


**CONFERENCE POSTER PRESENTATIONS**

1. **Oreoluwa Adedoyin & Charles D. Loftin.** *Inhibition of Prostaglandin E2 promotes human aortic smooth muscle cell differentiation*. The 15th International Winter Eicosanoid Conference, Baltimore, Maryland (March 9-12, 2014)

2. **Oreoluwa Adedoyin & Charles D. Loftin.** *Inhibition of Prostaglandin E2 promotes human aortic smooth muscle cell differentiation*. Gill Heart Institute cardiovascular research day, Lexington, Kentucky (October 11, 2013)


6. **Oreoluwa Adedoyin & Charles D. Loftin.** *Prostanoid-induced smooth muscle cell phenotypic modulation in human aortic smooth muscle cells (hASMCs).* The 14th International Winter Eicosanoid Conference, Baltimore, Maryland (March 11-14, 2012)


10. **Oreoluwa Adedoyin & Heidi Mansour.** *Physicochemical characterization and Water Vapor Sorption of advanced spray dried D-Mannitol particles*
for pulmonary delivery. Pharmaceutics Graduate Student Research Meeting, Ohio State University (June 17-19, 2010)


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