

Myriam Iglewski, Urotensin II Signaling Mechanisms Result in Vascular Smooth Muscle Cell Proliferation and New Gene Induction. Doctor of Philosophy (Biomedical Sciences), April 2009, 156 pp, 3 tables, 7 diagrams, 20 figures.

Urotensin II (UII) plays an important role as an activator of vascular smooth muscle (VSM) cell proliferation and hypertrophy when sustained and unabated. UII receptor (UIIR) signaling results in the activation of phospholipase C (PLC) and second messengers IP<sub>3</sub>/ DAG, which trigger the release of Ca<sup>2+</sup> from the sarcoplasmic reticulum (SR). We hypothesize that UIIR- coupled Gq signaling in pulmonary arterial vascular smooth muscle cells (Pac1) and primary aortic vascular smooth muscle (PAVSM) cells activates downstream calmodulin (CaM) kinase kinase (CaMKK) as a primary downstream target. We show here that UIIR signaling in Pac1 cultures results in the immediate phosphorylation and activation of CaM Kinase I (CaMKI). Activated CaMKK phosphorylates and activates CaMKI. CaMKI activation was measured in response to UII stimulation in the presence/ absence of CaMKK inhibitor. We report here that activated CaMKK/ CaMKI has at least 4 downstream targets; ERK 1/ 2, PKD, ATF2, and P38 MAPK. Activation of these kinases were identified by measurement of UII induced phosphorylation of PKD, ERK1/ 2 kinase, P38, and ATF2 via western blot analysis. Reporter assays demonstrated that UII-induces the expression of gene markers for phenotypic modulation including SM-MHC, SM-22  $\alpha$ , and SM- $\alpha$  actin. CaMKK through UIIR stimulation results in the activation of SMC specific genes associated with phenotypic switching. Gene expression is transcriptionally driven by activation of the above kinases, which recruits and induces the expression three-transcription factors MEF2, SRF, and GATA. These genes are up-regulated for differentiation and recruitment of various factors which recovers matrix structure, and expression of adhesion molecules either during vascular injury or vascular pathological states. Taken together, these results

identify components of an important intracellular signaling pathway through which Angiotensin II induces UIIR up-regulation and leads to increased levels of intracellular  $\text{Ca}^{2+}$ , activation of  $\text{Ca}^{2+}$ -dependent kinases, phosphorylates stress-activated kinases, and stimulates cellular proliferation, as well as new gene expression.



UROTENSIN II SIGNALING MECHANISMS RESULT IN VASCULAR SMOOTH  
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## LIST OF ABBREVIATIONS USED

<b>Abbreviation</b>	<b>Defining Term</b>
$^3\text{H}$	Tritiated
ACSF	Artificial Cerebro-Spinal Fluid
A2 or AngII	Angiotensin II
ATR1	Angiotensin II Receptor
Alpha MHC or $\alpha$ -MHC	Alpha- Myosin Heavy Chain
ATF2	Activating Transcription Factor 2
CaM	Calmodulin
$\text{Ca}^{2+}$	Calcium
$[\text{Ca}^{2+}]_i$	Intracellular Calcium Concentration
CaMK	$\text{Ca}^{2+}$ / Calmodulin dependent kinase
CaMKI	$\text{Ca}^{2+}$ / Calmodulin dependent kinase I
CaMK II	$\text{Ca}^{2+}$ / Calmodulin dependent kinase II
CaMKK	$\text{Ca}^{2+}$ / Calmodulin dependent Kinase kinase
CBD	Calcium Binding Domain
DAG	Diacyl glycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
dn	Dominant Negative
ERK	Extracellular signal related kinase
FBS	Fetal Bovine Serum
GPCR	G-protein coupled receptor
HAVD	Hypertrophy Associated Vascular Disease
HBSS	Hank's Balanced Salt Solution
$\text{IP}_3$	Inositol Tri-Phosphate
JNK	Jun N-terminal kinase
MAPK	Mitogen Activated Protein Kinase
MEF2	Myocyte Enhancer Factor 2
MEK	MAP/ ERK Kinase
PBS	Phosphate Buffer Solution
PD90859	2' – amino 3'-methoxyflavone
PKC	Protein Kinase C
PKD	Protein Kinase D
SB203580	4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1H-imidazole
SERCA	Sarco/ endoplasmic reticulum calcium ATPase
SER	Serine

SR	Sarcoplasmic Reticulum
SRF	Serum Response Factor
STO609	7H-Benz[de]benzimidazo[2,1-a]isoquinoline-7-one-3-carboxylic Acid
TBS	Tris-buffered saline solution
TBST	Tris-buffered saline 0.1% Tween-20
TF	Transcription Factor
tg	Transgenic
Thr	Threonine
Tyr	Tyrosine
U0126	1,4-diamino-2,3-dicyano-1,4-bis {2-aminophenylthio} butadiene
UII	Urotensin II
UIIR	Urotensin II Receptor
199M	199 Formulation of Cell Medium

### EC<sub>50</sub> & IC<sub>50</sub> TABLE

<i>Compound</i>	<i>EC<sub>50</sub> or IC<sub>50</sub></i>	<i>Concentration</i>
<i>Urotensin II</i>	EC50	64 nM
<i>Angiotensin II</i>	EC50	75 nM
<i>Losartan</i>	IC50	5 µM
<i>STO609</i>	IC50	120 nM
<i>PD90859</i>	IC50	5 µM
<i>U0126</i>	IC50	4.2 µM
<i>Chelenthrene Chloride</i>	1C50	0.66 µM
<i>SB-203580</i>	IC50	340 nM



## CHAPTER ONE:

### INTRODUCTION:

Vascular diseases constitute the most common causes of death and disability today in Western Society (1). Thrombosis, atherosclerosis and hypertension are responsible for over \$100 billion dollars for medical expenses annually in the United States (2). Recently, especially within the last ten years, there has been an interest and an enhanced understanding of vascular systems and the treatment of vascular related diseases (1). Scientists using innovative imaging techniques (such as angiography, tomography scans & confocal microscopy), molecular biology and genetic tools combined with new animal disease models (both transgenic over-expressors & knockouts) we have learned the inner-workings of the vascular system and have delineated functional mechanisms involved in the etiology of the disease state (2). The latest advancements in vascular biology have led us to understand the complex, intricate pathological (intrinsic as well as extrinsic) and physiological processes that underlie these diseases (3). The importance of understanding intracellular signaling mechanisms that are stimulated during pathological conditions may be most useful as potential clinical targets in developing new and novel treatments and therapies.

### **Preface:**

Newly diagnosed patients with a vascular diseases caused in part by VSM cell hypertrophy affect a quarter of the nation's hospitalized patients annually. Most of these patients suffer from some form of hypertrophy-associated vascular disease (HAVD). Diseases related or classified under

the umbrella of HAVD include ischemia, atherosclerosis, arteriosclerosis, embolism, restenosis & remodeling post-angioplasty, thrombosis, diabetes, vascular neuropathy, atherosclerosis, arteriosclerosis, hypertrophy, and hypertension. Vasoactive factors including Angiotensin II and Urotensin II (UII) play an important role as activators of vascular smooth muscle cell proliferation and hypertrophy, often contributing to HAVD. Urotensin II receptor (UIIR) signaling results in the activation of phospholipase C (PLC) and second messengers inositol triphosphate (IP3), diacylglycerol (DAG) which triggers the release of calcium ( $\text{Ca}^{2+}$ ). These studies are designed to demonstrate that UII induces the release of intracellular  $\text{Ca}^{2+}$  through UIIR signaling mechanisms which results in the activation of  $\text{Ca}^{2+}$  dependent protein kinases, transcription factors, and new gene induction.

### **Structure of the Vascular Wall:**

VSM cells play a critical role in the arterial vascular system. Some of the more important physiological roles of the arterial system include, transport and delivery of oxygen, nutrients, and energy (4). The arterial vascular system also has the ability to monitor systemically critical components of blood supply under changing metabolic conditions, which helps the body adapt (5). Vessels through their structural components respond to metabolic or demand changes using contraction or dilation of various blood vessels for systemic control (5). There are three main types of vessels in the artery family, two of which include; 1) vascular beds of medium size arterioles which are pressure sensitive, and 2) there are large arteries which are non-sensitive to chemical mediators or pressure. Pressure sensitive arteries are one of the most important systems of vessels in the body. There are three layers that make-up the artery. Layers of the artery provide the artery with the ability to sense pressure changes, chemical mediators, and

environmental signals to adapt or compensate to a whole array of conditions and cardiovascular situations (6). Arteries are made of three cellular components, these cellular layers include, the *tunica intima*, *tunica media*, and the *tunica adventitia*. The layer that is predominantly made up of endothelial cells is the *tunica intima*; these types of cells commonly line many other parts and organs of the body and make-up the smooth surface (7). The endothelial layer of arteries has the important function of secreting various small molecules such as growth factors in response to various stimuli (7). As the name implies, the middle layer is the *tunica media* which has the primary role of maintaining pliability and provides the arteries with the ability to distend. The loss of pliability as well as distensability in the *tunica media*, is often the effect of aging (7). The outermost layer of the vessel, the *tunica adventitia*, acts as a structural and physiological support system for the small blood vessels that feed into the big arteries (7). This layer is innervated with many nerves and these nerves modulate the contraction and dilation of arteries. The three layers of the arteries give the arteries their elasticity and flexibility. Special sensory systems, called baro-recptors and endogenous mediators such as Angiotensin II and Urotensin II monitor blood flow in the arteries and mediate their flexibility and elasticity through an intricate feedback system (8, 9). The information processed by baroreceptors and chemical signaling through intracellular signaling pathways lead to new gene induction (7). This provides arteries with the capacity to constrict arteries to help propel blood through the system within a fraction of a second. These extraordinarily complex mechanisms by which the body manages pressure, involves hundreds of vessels, billions of nerves, and several chemical signaling systems all synchronized and working together, and thus signifying the importance of understanding this complex system.

## **Vascular Diseases:**

Recent cardiovascular studies have changed our understanding of the physiological, molecular, and biochemical characteristics of the VSMC during the etiology of the disease. One past study conducted by a group of scientists in Framingham Massachusetts began a critical study to determine the causes and characteristics of cardiovascular diseases (10). Some of the well studied cardiovascular diseases included atherosclerosis, arteriosclerosis, arterial remodeling, vascular hypertrophy and hypertension (10). The study involved 5000 people all between the ages of 30 to 60 years in age (10). This study has changed the worlds understanding of vascular diseases (10). The Framingham Study has provided the medical and scientific field with invaluable information regarding the structural & functional defining aspects of these disease conditions (10). This study also presented both genetic insights to predisposition, as well as symptomatic & diagnostic indications which promotes these vascular conditions (10). The studies also expanded our general understanding of both environmental & behavioral risks that can promote these vascular conditions (10). Despite the fact that this study did not evaluate cardiovascular disease conditions at a molecular level, these studies did contribute the basic knowledge necessary for our overall understanding of vascular pathological conditions.

## **Atherosclerosis & Arteriosclerosis:**

Atherosclerosis is one such vascular pathological condition that can be influenced by genetic, environmental, and behavioral lifestyles (11). Studies including the Framingham Study, have shown a direct correlation between high levels of cholesterol in the blood stream and atherosclerosis (10). Accumulation of cholesterol containing plaques generally results in increased resistance and reduced blood flow. The overall effect of accumulated atherosclerotic

plaques lead to “clogged” arteries. The decrease in artery compliance and distensability results in reduced flexibility and ultimately the arteries begin to “harden” either through proliferation of VSM cells, enhanced migration of inflammatory cells, or increased synthesis of extracellular matrix proteins (collagen & integrins) (12). Treatment of atherosclerosis & arteriosclerosis is limited to a few procedures such as angioplasty, and only a few cholesterol reducing pharmacological agents (13). There is an urgent need to identify better treatment strategies and therapeutic agents to regulate extracellular matrix synthesis, or VSM cellular proliferation, which would benefit patients suffering from atherosclerosis and arteriosclerosis.

### **Balloon Angioplasty/ Restenosis and Arterial Remodeling:**

Past studies, including the Framingham Study, identified the physiological changes that occur during the repair processes, post-vascular injury (10). Although recent studies regarding a new procedure, such as the application of balloon angioplasty resulting in restenosis has also been shown to lead in post-vascular injury. Angioplasty is a medical procedure that has expanded our general understanding of post-traumatic injury. Increased vascular injury has been recognized with balloon angioplasty, and this procedure leads to the reoccurrence of vessel narrowing (restenosis) (12, 14). Angioplasty involves the insertion of a balloon into the narrowing vessel, which was previously created by atherosclerotic plaques (12). The balloon is blown-up to “smash” the plaques and “break-up” the accumulation of plaques along the vessel wall (12, 14). Although, balloon angioplasty is characterized as a more successful and efficient procedure at breaking-up atherosclerotic plaques, it does however result in extensive damage to the artery walls (13, 15). Several stages observed post-procedure, involves a brief recovery period, development of thrombosis to the damaged area (small blood clots), arterial remodeling

(involving proliferation, migration of immune cells), and a mounted immune response which ultimately reduces blood flow (13-15). Individuals undergoing balloon angioplasty and restenosis typically require another angioplasty within 6 months (13-15). Although this has been the standard treatment for atherosclerotic plaques, the arterial remodeling process can be far more detrimental and may contribute to the progression of vascular pathology (12). Since this medical procedure leads to a more severe form of vascular pathological outcomes, this identifies the importance of elucidating the signal transduction pathways at the final stage to halt proliferation of cells and hyperplasia.

### **Vascular Hypertrophy and Hypertension:**

It is estimated that roughly 65 million Americans suffer from hypertrophy and hypertension. The indicator of hypertension is high blood pressure (1). Genetic disposition, age, gender, and behavioral/ environmental factors can predispose an individual to hypertrophy and hypertension (1). Vascular hypertrophy is a serious phenomenon that results in the reduction in the ratio of media thickness to lumen diameter due to vascular remodeling (16). Diseases categorized as hypertrophy and hypertension all involve vascular remodeling due to altered hemodynamic properties (2). The remodeling of these VSM cells leads to a decreased arterial compliance, as well as, an increase in the intima media thickness (2). Endogenous factors can also stimulate the transmigration of leukocytes within the inner lumen of the vessel wall, activate vascular proliferation, and trigger inflammatory/ immune mediators to migrate (16). These factors also stimulate collagen deposition, induce vasoconstriction, trigger the release of pro-inflammatory cytokines, and increases adhesion molecules (1). These various factors may also promote the progression of hypertrophy and activate an altered mechanistic change in force and

flow, as well as, change the arterial compliance (1). The reduction in arterial compliance stimulates VSM cell morphological changes, which activates structural remodeling of various vessels including small arteries. Small and large arterial wall thickness and stiffness results from the hypertrophic restructuring and remodeling of vascular wall (1). Phenotypic changes of vessels, such as those within small and large arteries, subsequently alters the pressure /volume relationship leading to an altered systemic vascular compliance (1). During the altered vascular compliance triggered by remodeling of vascular cells, the vessel wall is unable to adapt or compensate (16). This inability to adapt leads to continuous circulatory hemodynamic force and an overall load of shear stress (1). Understanding the molecular signaling pathways that lead to hypertrophy and hypertension of VSM cells will provide important insight into the treatment of aberrant VSM cell proliferation associated with the progression of pathological conditions.

#### **Incidence of VSM cell Phenotype Modulation:**

The VSM cell in adult mammals are highly specialized, functioning to primarily regulate blood flow, pressure, and distribution of blood and vessel tone through vessel contraction. The level of contractility, and its ability to regulate blood flow, ultimately depends on the phenotypic state of the VSM cell. Cells such as cardiac muscle cells, are typically terminally differentiated. Although this is not true for non-vascular SMC or VSM cells, these cells are remarkably plastic (more prone to plasticity). VSM cells exhibit greater cellular plasticity, because these cells undergo frequent and rapid phenotype changes in-response to its environment. There are three phenotypic states that VSM cells have the capacity to reversibly switch between. These three states of classification include: 1) proliferative (synthetic) or hypertrophied, 2) differentiated (contractile), and 3) quiescent state. Adult VSM cells proliferate at low rates and do not display

synthetic activity. Synthetic/ proliferative VSM cells generally express a unique repertoire of contractile proteins, signaling molecules, and ion channels that are necessary for this cellular phenotype. The cellular sensitivity to  $\text{Ca}^{2+}$  is one important change that results in response to a synthetic/ proliferative phenotype change seen in VSM cells. VSM cells under the proliferative phenotype may also exhibit higher rates of cellular proliferation, migration, or increased synthesis of extra-cellular matrix components. Recent studies have established under a proliferative phenotype, there appears to be a down-regulation of SMC specific marker genes (such as SM-MHC, SM-alpha actin, and SM-22 alpha). Also, other studies have noted that there is a reduction in contractility, and moreover, an overall decrease in the expression of contractile proteins. VSM cells undergoing a hypertrophic state are commonly observed as maintaining proliferative/ synthetic phenotypes. Although the phenotype that is most associated with mediating functions of vascular tone is the differentiated phenotype of VSM cells.

The differentiated phenotype is more likely to undergo morphological changes in-order to enhance VSM cells contractility. VSM cells display more of  $\alpha$  actin-stress fiber formation, as well as, more myofibrillar formation. Morphologically, differentiated VSM cells are observed to exhibit a “spindle-like-shaped”. Differences in expression of genes have also been noticed in VSM cells, including increasing the expression SMC maker genes. SMC marker genes have been closely linked to hypertrophic-pathological cellular states. There are a few cellular changes that have been commonly recognized in differentiated VSM cells, including an increase in the number of organelles, such as the golgi and ER. The increase in these cellular organelles gives the cell the capacity to increase protein synthesis, such as contractile proteins. Enhanced contractile proteins permit VSM cells to maintain the contractile state. Interestingly, inconclusive data has also trended towards the belief that differentiated cells, specifically differentiated VSM



cells that display increased protein synthesis and greater expression of SMC marker genes, which also makes-up hypertrophic VSM cells.

### **Gene Expression during Vascular Development and Phenotype Modulation:**

It is important to understand the processes that occur during both development and vascular pathology. Recognizing their similarities, as well as differences, will become a helpful tool in understanding VSM cells under pathological conditions. Formation and remodeling of vascular smooth muscle (VSM) cells is a very important process during development. Remodeling is also a prominent feature of VSM cell layers in a number of pathological disease states and during the etiology of these pathological conditions (17). Only during development does vasculogenesis occur, although gene induction and protein expression is a hallmark of development that may become a reoccurring event during vascular injury or disease (17). Studies have found that this hallmark event requires an environment rich in nutrients, induction gene expression (specific subset of genes), and importantly the addition of new cell mediators that provide the developing VSM cells to proliferate (18). Vasculogenesis is a very early developmental process in which a network of vessels begin to form; this process requires the assembly of a primary vascular plexus from endothelial progenitor cells (16). Vascular development also provides maintenance of organ systems and requires the coordination of several signaling pathways. Coordinated pathways during development result in the activation and the expression of a repertoire of certain vascular SMC genes (17). During early development stages of vasculogenesis, VSM cells are typically more migratory and they undergo rapid proliferation (17). These immature VSM cells during development also exhibit high rates of protein synthesis and an increased expression of extracellular matrix components such as

collagen, elastin, proteoglycans, and integrins (19). These extracellular matrix proteins, as well as unabated cellular proliferation, comprise a major part of the blood vessel mass and vessel wall thickness (20). Whereas, vessels that are under a normal and contractile/ differentiated phenotype undergo slow turnover of VSM cells, and little cellular proliferation. Studies have also revealed that differentiated cells exhibit decreased rates of protein synthesis, display decreased production of extracellular matrix components, and these cells are typically committed to performing contractions (as it is in the contractile state) (21). Under a quiescent or contractile state of VSM cell, the gene expression is limited to only a few subset of genes (21). While, the SMC genes that are required for vascular development also include SM-alpha actin, SM-MHC, SM-22 alpha (22). These genes are often referred to as SMC differentiation marker genes; because these genes typically are used to describe the differentiated phenotype of mature SMC during development. Under pathological conditions, the expression of these genes are also evident (23). Moreover, during pathological conditions this similar expression of genes are also recognized and this alters the overall phenotype of VSM cells (24). During pathological states, increased expression of these SMC genes and specific proteins contributes to arteriosclerosis, and ultimately results in the progression of vessel wall hardening (22). Phenotype switching and SMC plasticity is observed only during development. However, it is now shown under vascular pathological conditions and under states of vascular injury(22).

### **Smooth Muscle Cell Specific Genes Associated with Phenotypic Switching:**

The smooth muscle cell undergoes a variety of developmental processes throughout embryogenesis and these processes are often silent in the adult phenotype. Although these processes witnessed during development may appear during pathological conditions such as

differentiation, vasculogenesis, and lead to phenotypic switching of VSM cells (22). Phenotypic transition is viewed as an early even of vascular pathology, the VSM cell converts from a contractile (work) state to a synthetic (proliferative/ hypertrophic) state in which pre-mature embryonic isoform gene expression of contractile proteins are expressed. Gene markers for phenotypic modulation include smooth muscle cell myosin heavy chain (SM-MHC), smooth muscle alpha actin (SM  $\alpha$ -actin), and smooth muscle 22 alpha (SM22 $\alpha$ ) (22). These gene markers are transiently up-regulated for differentiation and during vascular injury or vascular pathological states (22). Under normal, controlled, and stabilized cellular conditions the expression of SM  $\alpha$ -actin, SM22 $\alpha$ , and SM-MHC are beneficial for pre-mature vascular repair due to minor injuries, although under pathological conditions the increased expression of these genes can result in compounding detrimental effects (17). Irregular expression of SM  $\alpha$ -actin, SM22 $\alpha$ , and SM-MHC contributes to vascular hypertrophy (24), which argues the importance of understanding the signaling mechanisms that leads to the increased expression of these genes.

### **SM-22 $\alpha$ :**

A prototypical smooth muscle cell phenotypic marker gene from the repertoire of adult differentiated genes is the SM-22 $\alpha$  gene (25). In a differentiated state SM-22 $\alpha$  gene expression is up-regulated, whereas in a undifferentiated state, VSM cell SM-22 $\alpha$  gene expression is down-regulated (25). These genes, such as SM-22 $\alpha$  offer a superior method for the study VSM cell specific gene expression associated with vascular hypertrophy (26). Recent hypertrophy studies identified that P38 and JNK pathways activate SM-22 $\alpha$  gene expression (26). Transcription and trans-acting factors that are critical for the SM-22 $\alpha$  gene expression includes CArG [CC(A/T)<sub>6</sub>GG] and CArG like binding elements such as SRF, DEFA, MEF-2, MADs box

transcription factors, and MCM1(26). SM-22 $\alpha$  gene promoter region contains two CArG box regions and also has a defined MEF-2 binding site (25). During normal artery formation involving SMC maturation, SM-22 $\alpha$  is expressed. This VSM cell marker gene, SM-22 $\alpha$ , is also expressed during the pathological conditions. SM-22 $\alpha$  is therefore, a key gene to monitor during changes in the etiology of the hypertrophy of VSM cells, and provides insight into the general status of VSM cells.

### **SM-MHC:**

Smooth muscle – myosin heavy chain (SM-MHC) is another differentiation specific marker gene that is associated with changes in phenotypic modulation(25). SM-MHC is primarily regulated via trans-activating factors in the CCTCCC region located between -89 and -61 and sequences surrounding CArG box in the -1,332 and -1225 bp fragment (25). Within the promoter region of SM-MHC, lies the CArG- box regions which provides an area of transcriptional modulation of gene expression (27). Regulation of SM-MHC gene expression is critical for contractile states of both vascular and non-vascular smooth muscle cells (27)(25). Phenotypic marker genes, SM-MHC is expressed under two conditions, during development (late stages of differentiation) and under vascular pathological conditions (25). Delineating molecular mechanisms that lead to the activation of gene transcription may provide some insight towards the development and design novel therapies for vascular pathological conditions.

### **SM-alpha Actin:**

Smooth muscle  $\alpha$  -actin is considered a fairly specialized SMC differentiation marker gene. SM- $\alpha$  actin gene expression is important for both vasculogenesis and vascular pathogenesis (25).

Recent studies have shown that SM- $\alpha$  actin exists in elevated concentrations of high resistance and pressure sensitive arterial vessels (24). SM- $\alpha$  actin is up-regulated during development. Moreover, SM- $\alpha$  actin expression is more abundant during the pathological states including under hypertrophic conditions (25). CArG – box promoter regions are important regions within the promoter sequence for identifying the transcription factors which regulate the expression of SM -  $\alpha$  –actin (27). Some regulators of SM  $\alpha$ -actin gene expression includes; SRF, c-Myc, and TEF-1 (25). The modulation of SMC marker genes play a pertinent role in the pathology of several vascular diseases. Taken together genes that alter SMC phenotype during development and in particular during vascular pathology are critical to understand, because currently the signaling mechanisms that lead to activation of SMC gene transcription are poorly understood.

#### **Transcription Factors that Mediate Phenotypic Dependent Gene Expression:**

SM  $\alpha$ -actin, SM22 $\alpha$ .and SM-MHC are pertinent marker genes that are expressed during development and phenotypic modulation (25). Analysis of the promoter region of these genes revealed an involvement of trans-acting factors, cis-regulatory elements, and a complex of cis/trans complex formations that regulate the expression of SMC marker genes (25). Transcription factors that are attractive candidates for regulation of SM  $\alpha$ -actin, SM22 $\alpha$ .and SM-MHC include (MEF2), serum response factor (SRF), and (GATA) (24).

#### **MEF2:**

Mycocyte enhancer factor -2 (MEF2) are a group of transcription factors in the MADS box family of transcription factors (28). Four types of MEF2 (MEF2- A, B, C & D) genes with three alternatively spliced isoforms of each MEF2 gene exist (28). MEF2 is ubiquitously expressed in

cardiomyocytes, smooth muscle, and vascular smooth muscle (28). Expression of MEF2 is also expressed in other cell types including neurons (29). MEF2 expression is required for the activation of gene expression and differentiation (29). MEF2 is considered a direct target for inducing transcription of SMC marker genes (28). Dimerization and DNA binding occurs at an A/T rich DNA sequence that targets the expression of specific sets of downstream genes (29). During cardiac, skeletal, smooth muscle and vascular smooth muscle cell development; MEF2 regulates the gene expression maintaining a normal developing phenotype (28). During mitogenic signaling, MEF2 is a pertinent downstream effector in the signaling processes (29). The complex regulation of diverse cellular processes such as differentiation and apoptosis are mediated by MEF2. MEF2 sends signals emanating various cell-surface receptors and modulates cell cycle progression via induction of c-jun promoter (29). P38 docking sites were identified within the MEF2 conserved region (30). Although MEF2 is critical for development of vascular smooth muscle and phenotypic switching, MEF2's transcriptional activation can determine the occurrence of an abnormal cellular phenotype (28). MEF2 may become a diagnostic determinant, identifying if VSM cells are likely to be abnormal and more predisposed to vascular pathological conditions on the basis of the cellular expression of MEF2.

#### **SRF:**

SRF is a key transcription factor in the MADS-box family, that regulates smooth muscle and VSMC gene expression and primarily modulates transcription of genes (31). In skeletal muscle, cardiac, and VSM cells an increase in the transcription factor SRF was observed under pathological conditions (25). The CArG box binding factor SRF, activates early response gene (25). Activation of SRF and co-activators form a functional multi-protein complex with the

dimerized forms of SRF within the CArG box sequence (31). Signaling pathways are believed to drive the activation of SRF, this includes RhoA and GPCR pathways (31). SRF can trigger morphological changes, such as spindle acquisition (31). SRF is currently recognized as being involved in gene expression related to VSM cell differentiation and phenotype switching (25). Currently the signaling mechanism that leads to SRF activation is unknown.

### **Function of GATA Transcription Factor Results in VSMC phenotypic Change:**

A transcription factor that consists of a highly conserved DNA binding domain and contains two zinc fingers which is apparently very important in the progression of SMC hypertrophy are the GATA family members 4 to 6 (32). The Cys-X<sub>2</sub>-Cys-X<sub>17</sub>-Cys-X<sub>2</sub>-Cys motif that directs GATA binding, also ultimately regulates gene expression (30). A variety of proteins such as SRF, ERK, SMAD (small mothers against decapentaplegic), MEF2, NFAT (nuclear factor of activated T-cells), CBP (CREB- binding protein), and FOG (megakaryopoiesis GATA- factor); have been discovered to interact with GATA (33)(30, 34). The GATA subfamily of proteins are split into two distinguishable groups, one group of GATA (GATA 1, 2, & 3) proteins regulate differentiation via regulation of gene expression in stem cells (34). Whereas the second group, (GATA 4, 5, & 6) which are expressed in vascular, smooth muscle and cardiomyocytes, which primarily functions as a mediator of tissue specific gene expression, development, and also play a critical role in hypertrophy (34). Recent studies have revealed that GATA4 is essential for survival and differentiation (34). GATA4 expression occurs in a normal pattern throughout development, although pathological states lead to unusual expression of SMC marker genes ultimately resulting in VSM cell hypertrophy (33).

Several endogenous small molecules can promote progression of vascular pathological conditions such as hypertrophy (16). One example of a small molecule that under elevated levels can lead to the progression of hypertrophy and hypertension is Angiotensin II (35). Understanding the role and the signaling mechanisms that angiotensin II induces in vascular disease will facilitate a better understanding of some molecular pathways that can be targeted to halt the progression of hypertrophy and hypertension.

### **Endogenous Angiotensin II and Characteristics of Angiotensin II Receptor:**

Angiotensin II is an example of an endogenous vaso-active factor that under elevated levels leads to the progression of vascular pathology by stimulating vascular remodeling, constricting blood vessels, and promoting proliferation of vascular cells (36). The mechanism by which angiotensin II triggers these vascular phenotypic changes is poorly understood, despite the abundant information that illustrates angiotensin II's role and its function under non-pathological states (37). Angiotensin II, under non-pathological conditions, is a natural hormone found in the kidney that plays an important role in overall physiological functioning of mammalian body systems (38). The rennin-angiotensin system consists of rennin and angiotensin (39). Renin, is a proteolytic enzyme that cleaves angiotensin I, an alpha globulin particle and forms angiotensin II, which is an octapeptide that functions in both intrarenal activities as well as regulates extrarenal functions (40). Extrarenal signaling of angiotensin II involves its affinity to bind two types of receptors; ATR1 and ATR2, both are Gq-GPCR receptors that triggers the activation of PLC and produces second messengers such as; IP3, DAG, and calcium (41). Extra-renal studies in VSM cells have also shown that ERK is activated through the stimulation of the ATR1 receptor-signaling pathway (42). Although the exact mechanisms in which ATR1 activates ERK



is unknown (43). Extrarenal regulation also includes the control of adrenal gland secretion and hemodynamic properties of peripheral blood vessels (41). Adrenal gland regulation includes modulating the secretion of aldosterone, whereby maintaining fluid balance and hemodynamic homeostasis (40). The vaso-active peptide angiotensin II regulates peripheral vascular systems via the regulation of blood flow, regulation of micro-circulation, aortic pressure, the total filling volume, vasoconstriction, and circulatory dynamics (44). Angiotensin II has been recently identified as a vaso-constrictor of major vascular systems (44). Angiotensin II induces contraction of smooth muscle cells including vascular smooth muscle cells (44). These angiotensin II characteristics are similar to the UII (45). Structure activity relationships identified a link between Angiotensin II and Urotensin II (45). Both are characterized as peptides that regulate the cardio-vascular system and are dual regulators of vascular tone, UII and Angiotensin II are endogenous agents that share these similarities (45). Studies comparing vasoactive agents such as UII and Angiotensin II found both endogenous peptides induce vasoconstriction (45). Angiotensin II as well as UII are hallmark factors that can trigger phenotypic conversion of VSMC resulting in pathological states (46). VSMC structural remodeling by UII and angiotensin II stimulate robust responses such as SMC proliferation, trigger hypertrophy, cellular migration and enhance the progression of hypertension (46). These similarities between the two peptides and their level of involvement in the progression of pathological states, provides a link and a possible mechanism of interaction, such as simple additivity or synergism (45). Thus, providing evidence that angiotensin with UII may act synergistically by the angiotensin II induced up-regulation of UIIR. The recent prediction that UII may interact with other endogenous vaso-active and vasopressor (resulting in constriction) agents evolved from the identification of shared similarities and a possibility of vaso-active synergism which was identified between angiotensin

II and specific growth factors (45). Previous findings did demonstrated that synergism already existed between angiotensin II and other growth-agents and vaso-active factors such as TGF-beta, VEGF, and the beta adrenergic receptor of norepinephrine (45). To understand and determine the synergism that exists between angiotensin II and UII, may lead us to a better understanding of the general role that similar endogenous agents play during vascular pathological conditions. Although, before angiotensin II signaling mechanisms can be determined, the comprehension and awareness of UII and the UIIR signaling mechanisms are equally important.

### **Autocrine and Paracrine Functions of UII:**

Recently several studies have observed the physiological and pathophysiological functions of UII as a neuro-humoral factor. Pre-pro-UII is a neuro-hormone that has been identified as being secreted from the urophysis of the goby fish (47). Mature UII is formed locally in specific tissues via cleavage by a converting enzyme. UII affects several tissues and has been identified in cardio-vascular diseases such as atherosclerosis and hypertension (51). UII mediates and induces several diseases including the induction of cardiac dysfunction through its autocrine and paracrine like activity (48).

UII affects hormone levels, induces an increase in cytokines, growth factors inflammatory factors and activates peptides through the adventitia of vascular tissue (49). The physiological role of UII results in secretion of vaso-active factors such as nitric-oxide (NO) and growth factors, which ultimately leads to the regulation of arterial structure and function (45). This UII induced modulation via NO is an example of UII's paracrine function. UII induced paracrine function often is most clearly demonstrated when UII transmits a molecular signal

from one cell type to another neighboring different cell type via diffusion in the intercellular gap junctions (47). Pre-pro-UII cleaved by urotensin II converting enzyme forms mature UII which leads to transport via diffusion of active UII through intracellular fluid channels (47). The diffusion of UII affects several cells including the adventitia of VSM cells. UII targets the adventitia of VSM cells to release inflammatory cytokines, growth factors, and hormones which ultimately alters other cells such as fibroblasts, and other cardio-vascular tissues (48).

Autocrine function of UII was recognized with UII induced inflammatory responses in specific cells (51). The studies that identified that UII exhibits autocrine function observed this characteristic of the peptide when it transmitted molecular signals to neighboring cells (of the exact same cell type) (47). Whereas other studies have shown that UII resulted in a feedback mechanism where cells that released UII were receiving signals of the higher levels of UII within the intercellular fluid (49). Therefore, a series of UII characterization studies have shown that UII exhibits both paracrine and autocrine action.

### **Endogenous Urotensin II:**

UII is an important vaso-active factor that under elevated UII levels appears to play an important role in the pathology of vasculature (8). Recently Urotensin II was suggested as a contributor of vascular diseases such as heart failure and hypertension (47). Characteristics of UII both structurally and functionally contribute to progression of disease (48). Urotensin II is a vaso-active cyclic endogenous peptide that was first isolated from the urophysis of a teleost fish (49). Recent studies have characterized UII structurally, identifying it as an 11 amino acid protein which resembles somatostatin (50). Structurally and functionally, UII has shown minimal inter-individual variation across species, which was demonstrated in studies analyzing various vascular smooth muscle preparations (51). There is a growing number of species including rat,

dog, rabbit, pig, monkey and human that have been identified as possessing UII (52). UII in a diverse group of species was isolated from blood vessels and was demonstrated as possessing a vasoconstrictive potency of 10 to 100 fold greater than endothelin-1 (53). The potent vasoconstrictive properties of UII also affects various organ systems within the mammalian body; such as cardiac tissue, central nervous system, and vascular smooth muscle systems (54). The UII found within a variety of these tissues, organ systems, and vessels had been first identified in a cat model. Renal artery, carotid, aortic, superior mesenteric femoral and arterial segments are among the many vessels UII is expressed in the vascular system of mammals (55). UII under non-diseased conditions respond to cellular changes, influences migration of cells, and modulates large vessels (56). Whereas UII under diseased conditions results in enhanced VSM cell remodeling, potent vasoconstriction, increased production of adhesion molecules, promotes collagen deposition, augments the productions of reactive oxygen species (ROS), and increases cellular proliferation (54). Recent studies indentified that UII stimulates cellular phenotypic changes such as inducing a hypertrophic phenotype, and triggering cellular proliferation through UII binding UIIR (55). These studies also demonstrated that UIIR signaling pathway influences as well contributes to vascular diseases (57). Few studies have identified the mechanisms by which, UII binding UIIR elicits its cellular effects and the signaling mechanisms that lead to a diseased vascular states (58). Thus more extensive research is necessary to elucidate the UII and UIIR signaling mechanism as well as the involved protein kinases as it contributes to vascular states.

### **Characteristics of Urotensin II Receptor:**

New findings of the de-orphanized the GPR14 receptor identified the receptor as the UIIR(59). Gene coding studies and selective binding assays demonstrated its affinity to activate the Gq coupled GPCR UIIR, formerly known as GPR14 orphan receptor (48). Studies determining the maximal effects and high potency with UII binding of UIIR was displayed in HEK293 cells cloned and transfected, to model an isolated system (60). Activating UIIR, a Gq coupled GPCR, results in a complex signaling cascade activating second messengers such as inositol triphosphate (IP3) and diacylglycerol (DAG) (61). Mitogen activated kinases (MAPK) regulating ERK signaling phosphorylation is believed to be activated by UIIR stimulation, leading to induction of cellular proliferation (8). Remodeling, hypertrophic and proliferative effects of human UII in an atherosclerotic/ myocardial ischemia model are mediated by the activation of MAPK, indicating the importance of understanding the UIIR signaling cascade (60). In the pulmonary vasculature and other vascular systems, the signaling mechanisms are largely unknown. UIIR stimulation has been suggested as being involved in the induction of proliferative pathways involving ERK phosphorylation (47). UIIR activation through the phosphorylation of various kinases and altered gene transcription can induce vascular phenotypic changes resulting in hypertrophy of the vascular systems.

#### **UIIR Stimulates the Release of $\text{Ca}^{2+}$ from the Sacroplasmic Reticulum:**

UII binding to the UIIR mediates cardiac and vascular cell function (62). Recently recognized as a pertinent agonist that mediates  $\text{Ca}^{2+}$  release from the sacroplasmic reticulum, UII binding UIIR results in intracellular chemical modulations (60).  $\text{Ca}^{2+}$  is a critical second messenger that controls VSM cell contraction, regulates metabolism, and alters gene expression (63). Cellular responses such as modification of vascular tone or stimulation of cellular

secretions are activated by proteins, such as UII via UIIR signaling which induces the release of intracellular  $\text{Ca}^{2+}$  (64). UIIR is a GPCR that releases  $\text{Ca}^{2+}$  through the production of  $\text{IP}_3$  (65). VSM cells respond to UII binding of UIIR by mobilizing the small cation  $\text{Ca}^{2+}$  (66). Normal endogenous UIIR stimulation results in both temporal and spatial release of intracellular  $\text{Ca}^{2+}$  (63). Under pathological situations which, involves elevated UIIR stimulation, UIIR signaling leads to the release of intracellular  $\text{Ca}^{2+}$  and promotes a phenotype switch (67). Under both pathological and non-pathological conditions,  $\text{Ca}^{2+}$  regulates several downstream events such as mediating the activation of  $\text{Ca}^{2+}$  / calmodulin (CaM) dependent kinases. The physiological role that the second messenger,  $\text{Ca}^{2+}$  plays in all Gq signaling mechanisms, highlights its significance in the activation of other downstream kinases, such as CaMKK and CaMKI.

#### **B6. CaMKK and CaMKI:**

Members from the CaM Kinase family, which characteristically phosphorylate at serine/threonine residues, are recognized as playing pivotal roles in various signaling pathways and participate in diverse cellular processes (18). The calcium /calmodulin system is stimulated by the release of calcium from the sarcoplasmic reticulum (SR) triggered by Gq coupled signaling events (63). This signal transduction pathway regulates many cellular processes including contraction of smooth muscle. Upon release of the calcium ion, calcium binds calmodulin and undergoes a conformational change, leading to modulation of function. The calcium/ calmodulin complex (CaM) formation exposes the methionine residues in the hydrophobic binding region and allows interaction with CaMKK (63). CaM interacts with CaMKK by binding to the catalytic binding domain (CBD) this leads to CaMKK auto-phosphorylation (68). CaMKK phosphorylation activates CaMKI, which is a kinase from the CaM Kinase family (68). CaMKI

has been identified as being pertinent in the transduction of calcium signals during cellular events (68). For example, CaMKI has been demonstrated as an in-vitro substrate for synapsin I and II, a critical interaction in neuronal vesicle release (68). CaMKI has a variety of down stream targets including cyclic- AMP – response element binding protein (CREB) (69). CaMKI recognizes sequences approximately 7 amino acids in length, and in-vivo studies have been reported to possess the potential to recognize multiple substrates (70). CaMKI has been identified as activating specific kinases involved in cellular proliferation, such as ERK (71)(72). CaMKI and its ability of activate multiple substrates identifies its potential involvement in cardio-vascular pathological conditions (72, 73). The wide spread tissue distribution of CaMKI, emphasizes its importance in signaling and in phosphorylation of physiological targets such as  $\text{Ca}^{2+}$  dependent kinases including ERK and PKD.

### **$\text{Ca}^{2+}$ Dependent Kinases:**

Pathological vascular conditions accompanied by vascular remodeling are accompanied by a diverse alteration in protein expression and phosphorylation states (74). Protein phosphorylation states are often influenced by the generation of the second messenger  $\text{Ca}^{2+}$ , the elevated levels of  $\text{Ca}^{2+}$  occurs through Gq coupled signaling (75). The release of a finite concentration of  $\text{Ca}^{2+}$  can initiate cell signaling. Increases in intracellular  $\text{Ca}^{2+}$  have been demonstrated to induce the activation of CaM dependent kinases such as CaMKK and CaMKI (29). Although, little is understood about the  $\text{Ca}^{2+}$  induced activation of  $\text{Ca}^{2+}$  dependent kinases, such as PKD and ERK. Recent studies have broadened our overall understanding of these pertinent protein kinases and the possible signaling mechanisms that leads to their activation (72)(74). Studies conducted in neuroblastoma cells have previously demonstrated that CaMKI

directly phosphorylates ERK kinases (72). Although, no studies have determined the protein kinases involved in the signaling mechanism that directly phosphorylates ERK kinase (72). Studies examining PKD activation have identified PKD also as a  $\text{Ca}^{2+}$  dependent kinase, even though the direct signaling mechanisms that leads to PKD phosphorylation has not yet been identified (74-76). Both ERK and PKD have been revealed as being activated by GPCR signaling and also have been identified as downstream targets of DAG and PKC (75). Other studies have demonstrated that both ERK and PKD in several cell types and cell lines have been shown to elicit pro-proliferative effects (75, 77-81). Therefore in connection with identifying the signaling mechanism that leads to UII-induced cellular proliferation, understanding the role that these kinases play in this signaling pathway may provide insight to the kinases involved in this cellular proliferation pathway.

#### **Urotensin II Induced Phosphorylation of ERK1/2 and Proliferation:**

Urotensin II has recently been recognized as an enhancer of activating cellular growth capacity by stimulating proliferation via the inhibition of growth-regulated factors (such as insulin and epidermal growth factor) and the stimulation of signaling cascades that stimulate proliferation (57). UII has been shown to stimulate ERK phosphorylation in both a time and dose dependent manner in primary thoracic aortic cells (42, 82-84). Recent studies report that ERK activation has been recognized as a target for several GPCR's (75). ERK commences the cellular proliferative state by increasing the biosynthesis of macro-molecules, stimulating the production of organelles, and synthesizing lipid bilayer for the production of cellular membranes (42). Initial studies recognized ERK as a pertinent protein involved in proliferation was first identified with the observation of specific kinases that were phosphorylated due to mitogenic stimulation (75,



85). Since the findings of these critical experiments, ERK has been widely accepted as a possible marker for proliferation, depending on level of intensity and duration of ERK activation. ERK1/2 is a ubiquitously expressed protein found in almost all mammalian cells, and is activated through up-stream MAP kinase effectors such as Ras, Raf, and MEK (83). Vaso-active factors, such as Angiotensin II and UII have been recognized as activating ERK phosphorylation through their respective Gq GPCR, (ATR1/ ATR2) and UIIR (82, 86). Thus the kinases that are involved in the UIIR signaling transduction that leads to ERK phosphorylation and ultimately cellular proliferation has not yet been elucidated. These facts suggest the importance of identifying the  $\text{Ca}^{2+}$  dependent kinases (such as ERK & PKD) that are involved in the UII-induced UIIR cellular proliferation pathway.

#### **Cellular Proliferation and PKD as a $\text{Ca}^{2+}$ Dependent Kinase:**

PKC-mu which is also known as PKD is a serine/ threonine kinase with a distinct catalytic domain similar to  $\text{Ca}^{2+}$  regulated kinase (74). PKD has been recognized as regulating a variety of cellular functions including gene expression. PKD in GPCR signaling has been identified as a downstream target of DAG and PKC (87). PKD in several cell types and cell lines has been demonstrated as triggering a pro-proliferative effect. Vascular endothelial cells also revealed that PKD increases ERK1/ 2 phosphorylation status and stimulates cellular proliferation (78). Other studies have indentified a direct correlation with ERK over-expression increase PKD activity. PKD has been revealed as a factor in several pathological states including proliferative disorders, cancer progression hypertrophy, aging and degenerative disorders (39). PKD in correlation to its pro-proliferative effects promotes anti-apoptotic effects, as shown in studies conducted in a tumor cell line (76). PKD metabolism promotes pathological states such as cardiac failure and

hypertrophy (74). Although PKD has not been identified as an intracellular target of the UIIR signaling pathway. Studies have shown that PKD leads to hypertrophy. Similar to PKD studies, stress activated kinases have also been reported to play a pertinent role in the progression of hypertrophy associated vascular diseases.

### **Stress Activated Pathways and Kinase Activity:**

Simple architecture of VSM cells has been built upon a myriad of pathways that basically fine tunes cellular responses to a diverse set of stress stimuli (88). Stress activated pathways respond to a multitude of cellular or extracellular stresses such physical shear stress and biological stress such as those induced by cytokines or DNA damage (89). There are several proteins that are ubiquitous and conserved evolutionarily that relay signals through post-translational modifications (89). Proteins such as, specific kinases, elicit an appropriate cellular response through the phosphorylation of numerous downstream proteins (90). P38 is one such MAP kinase that is strongly activated by environmental stresses and physical stresses, such as shear or hemodynamic stresses (88). ATF2 kinase is also involved in the signaling that stimulates stress activated cascades and induces mitogenesis (88, 91). Studies have demonstrated that P38 directly phosphorylates ATF2 (30, 91). Both P38 and ATF2, protein kinases sense shear stress and trigger changes in gene expression (30). Cells that sense ischemia and cellular stress (88), begin to induce transcriptional changes through P38 or transcription factors such as ATF2 (88, 89, 92). P38 and ATF2 signals function primarily to regulate cellular differentiation and ischemic cellular stress pathways through the induction of specific genes linked to cellular stress or phenotype change (30, 90, 93). Enhancing our knowledge of the signaling mechanisms that

occur during cellular stress may provide potential therapeutic agents to target specific kinases involved in these pathways.

### **Stress Activated Pathways and P38 Phosphorylation:**

P38 MAP kinase is a ubiquitously expressed serine/ threonine kinase that plays a critical role in the regulation of transcription factors and gene expression (36). P38 is the first protein identified with a similar sequence homology to ERK and with the unique 38kDa protein size bearing the qualities of a protein kinase. Distinctly different from other MAP kinases, p38 has different downstream targets and results in different functional cellular changes (36). Currently p38 is accepted as the stress-activated protein kinase with the ability to undergo dual phosphorylation at both tyrosine/ threonine residues, with its activation leading to the phosphorylation of certain transcription factors (Elk-1 and MEF2) (26) . Cellular growth, differentiation, and apoptosis are altered with the stimulation of p38 (36). Cellular migration, structural cellular changes, death, and differentiation during vascular remodeling have identified p38 as a major contributing factor in the intracellular signaling cascades during pathology (36). Human UII in studies have displayed the capacity to activate p38- MAP kinase and activation was shown via p38 phosphorylation in primary rat aortic cells (40). Therefore, general comprehension of the UII-induced P38 activation may facilitate our overall understanding of the signal transduction that occurs during vascular disease and cellular stress. ATF2 is one direct target of P38 and understanding the cellular stress signaling pathways that directs gene transcription may help elucidate the biological importance of these kinases. Establishing the role of P38 and ATF2 will permit the pharmacological targeting of pathway-specific actions.

### **Stress Activated Pathways and Phosphorylation of ATF2:**

Because studies have shown P38 mediates the phosphorylation of ATF2, this kinase is now characterized as cellular stress activated kinase. ATF2 is typically represented as transcription factors that form dimers activating oncogenic cellular transformations, but ATF2 has recently been identified as a kinase that is phosphorylated in response to cellular stress or activation of specific cytokines (30). ATF complexes form heptameric or octameric binding conformation complexes to the AP-1 binding sites (30). ATF transcription factors are characterized as a class of b-Zip dimers at specific promoter elements (30). Regulation of growth, development, anchorage independence, and growth factor independence in the oncogenic states are the primary aspects of ATF2 under pathological states (30). MAP kinases such as P38 have been demonstrated as an up-stream regulator of ATF2 activity (32). Moreover ATF2 has not been identified as a transcription factor involved in the UIIR signaling pathway, although since UII induces P38 and studies have shown that P38 directly phosphorylates ATF2(91). The hypothesis to be tested is if UII potentially activates ATF2 through P38 cellular stress signaling pathways.

During cellular stress or hemodynamic stress which induces shear stress on the wall of the inner lumen of VSM cells, often induce new gene induction (genes associated with phenotype switching)(22). Proteins, such as SM- $\alpha$  actin, SM-MHC, SM-22 $\alpha$ , are expressed in response to a subset proteins that are associated with SMC differentiation, VSM cellular development, and vascular pathology (24). Therefore it is critical to understand if cellular stress pathways and more specifically which molecular mechanisms are involved in driving the expression of these genes.

### **Projected Hypothesis and Specific Aims:**

We have reported that UIIR activation of calcium dependent kinases & stress-activated kinases, stimulation of transcription factors, and initiation of new gene induction, plays an important role in VSMC cellular proliferation and new gene expression. In our preliminary studies for this proposal we show UIIR signaling in Pac1 cultures and PAVSMC results in the immediate activation and phosphorylation of specific kinases that leads to proliferation and the stimulation of SMC specific genetic markers. Using a combination of pharmacological inhibitors and UII stimulation, we want to identify CaMKK/ CaMKI as primary downstream targets in the UIIR signaling pathway. We hypothesize that UIIR up-regulated by Angiotensin II in Pac1 and PAVSMC activates CaMKK/ CaMKI, P38, PKD, and ERK which results in initiating cellular proliferation, and inducing new gene expression. The hypothesis will be tested in the following specific aims.

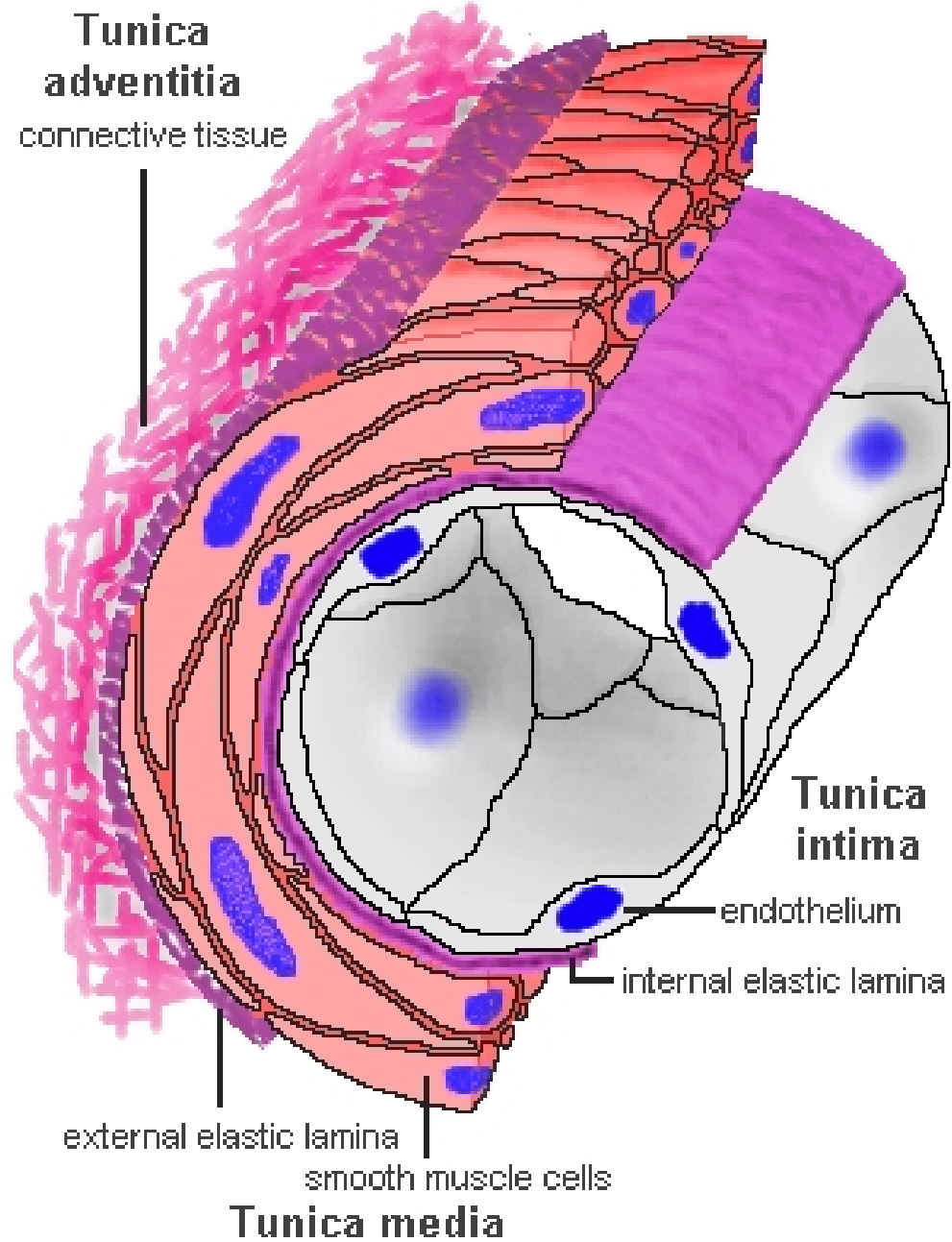
**Aim 1a:** To determine UII induced signaling mechanisms that leads to the activation of calcium dependent kinases in Pac1 cells and PAVSMC cells. Experimental Approach: Pac1 cells and primary aortic cells isolated from rat will be cultured and exposed to UII in a time and dose dependent manner. Phosphorylation of UIIR down-stream targets will be measured via western blot analysis. Using a CaMKK inhibitor, we will determine if CaMKK is involved UII induced phosphorylation of CaMKI, PKD, and ERK.

**Aim 1b:** To determine in Pac1 and PAVSMC cells if UII induced signaling mechanisms triggers cellular proliferation. Experimental Approach: Using both Pac1 and PAVSMC cultures exposed to UII, we will measure cellular proliferation via <sup>3</sup>H thymidine incorporation. Using various inhibitors, including the CaMKK inhibitor, we will identify the signaling proteins that are involved in the UII induced cellular proliferation.

**Aim 2:** To determine if activated of UIIR induces stress activated kinases and gene expression of transcription factors & SMC specific genes. Experimental Approach: UII induced phosphorylation studies of P38 MAP kinase and ATF2 via western blot analysis. Gene expression analysis of transcription factors (SRF, MEF-2, and GATA) and the expression of SMC specific gene markers (SM-alpha-actin, SM-MHC, and SM-22 alpha) will be measured via reporter assays. Using a CaMKK inhibitor & P38 inhibitor, we will determine if CaMKK and P38 kinases are involved in the UII induced transcription activation of new gene induction.

**Aim3:** To determine signaling mechanisms by which Angiotensin II induces UIIR gene expression in Pac1 cells. Experimental Approach: Pac1 cells will be treated in a dose and time dependent manner and the UIIR mRNA and protein levels will be measured using RT-PCR and western blot analysis. Phosphorylation of downstream targets identified in the ATR1 signaling cascade will be measured via western blot.

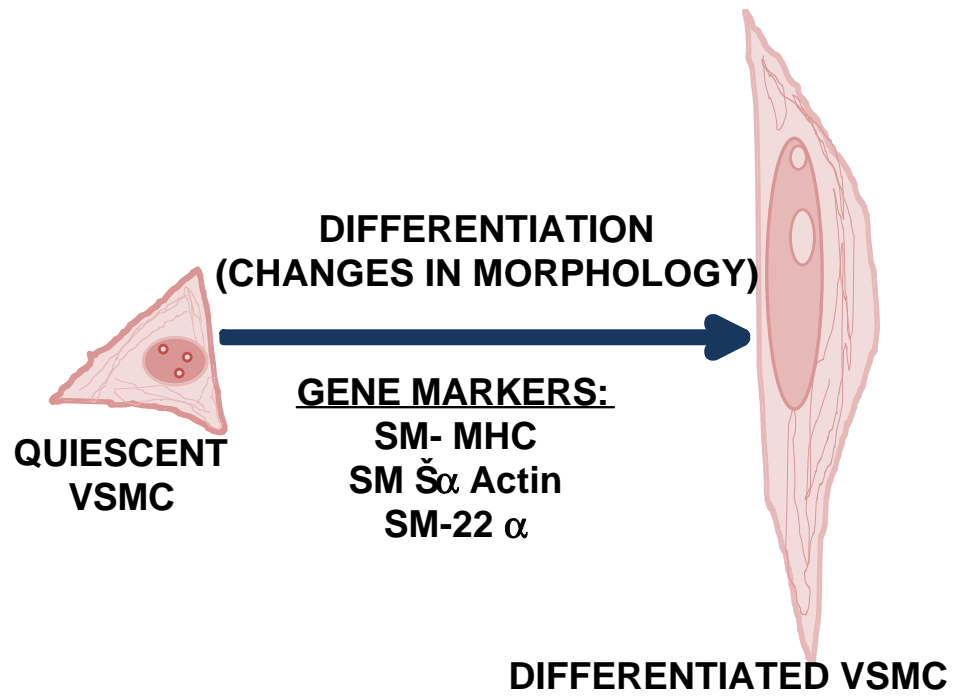
**Diagram 1. A diagram of a healthy artery.** There are three layers that provide the vessels with both the functional capacity as well as distensability, these layers include tunica intima, tunica media, and tunica adventitia. Taken from: Wilkie, Collins, Moonstone & Blue Histology – Vascular System ([www.lab.anhb.uwa.edu.au](http://www.lab.anhb.uwa.edu.au)).





**Diagram 2. Expression of SMC Marker Genes during VSM Cell Differentiation.**

Environmental cues and external stimuli trigger VSM cells to modulate their phenotype to various phenotypic states including the differentiated state. Upon vascular pathological conditions the incidence of phenotype modulation to a more differentiated phenotype is more likely. Alterations to a differentiated phenotype involve changes in morphology to a more “spindle-like” formation and altered SMC- specific gene expression. Figure modified from *Atherosclerosis*. 1990; 10:966-990.



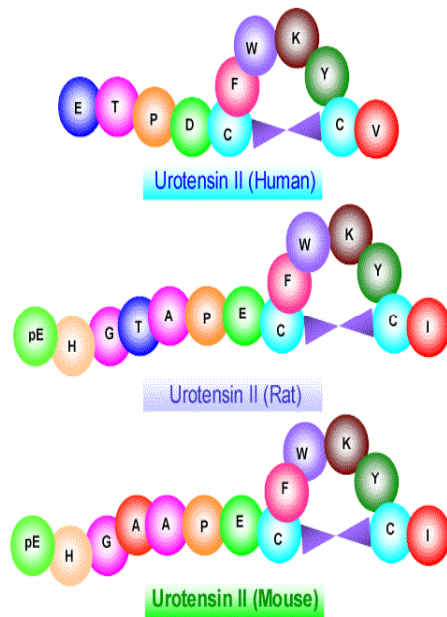
**Table 1. Gene Expression During VSM Cell Development, Differentiation, & Proliferative Phenotypes.** VSM cell development involves the expression of a specific sub-set of genes that may also be expressed during VSM cell differentiation. Cells that undergo VSM cell differentiation typically express genes that elevate the expression of contractile proteins. Whereas, the proliferative phenotype of VSM cells involves; the expression of genes that increase extracellular matrix proteins, organelles, and protein synthesis machinery. Figure modified from *Physiolo Rev.* 1995;487-517.

VSMC STATES	GENE EXPRESSION
Development of VSMC Embryonic	SM- $\alpha$ - Actin Calponin SM- $\alpha$ Tropomyosin SM-1-MHC SM-2-MHC
Differentiated VSMC Contractile	SM- MHC SM- 22 $\alpha$ SM- $\alpha$ - actin
Proliferative Synthetic	Collagen I $\alpha$ , III $\alpha$ , IV Fibronectin Laminin Tropoelastin Integrins $\alpha$ / $\beta$ Pro-MMP2 Focal Adhesion Kinase (FAK) Proteoglycans

**Table 2: Transcription Factors Regulate the Expression of SMC-Marker Genes.** The contractile or differentiated phenotype involves the expression of SMC-marker genes, such as SM- $\alpha$  Actin, SM-MHC, and SM-22  $\alpha$ . Gene expressions of SMC-marker genes are primarily regulated by a sub-set of transcription factors. Figure modified from *Arterioscler Thromb Biol.* 2003; 23:737-747.

Transcription Factors	Target Genes	Differentiation or Proliferation
<b>SRF</b> (MADS Box Protein)	<b>SM-<math>\alpha</math> Actin</b> <b>SM-22 <math>\alpha</math></b> <b>SM-MHC</b>	<b>Differentiation</b>
<b>MEF2B</b> (MADS Box Proteins)	<b>SM-MHC</b>	<b>Differentiation</b>
<b>Phox1/ Mhox</b> (Homeodomain Proteins)	<b>SM- <math>\alpha</math> Actin</b>	<b>Proliferation/ Differentiation</b>
<b>Barx1b</b> (Homeodomain Proteins)	<b><math>\beta</math> -Topomyosin</b>	<b>Proliferation</b>
<b>GATA 4/5/6</b> (Homeodomain Proteins)	<b>SM- <math>\alpha</math> Actin</b> <b>SM-22 <math>\alpha</math></b> <b>SM-MHC</b>	<b>Differentiation</b>
<b>Myocardin</b> (Homeodomain Proteins)	<b>SM- <math>\alpha</math> Actin</b> <b>SM-22 <math>\alpha</math></b> <b>SM-MHC</b>	<b>Differentiation</b>
<b>NFATc1</b>	<b>SM-MHC</b>	<b>Differentiation</b>
<b>AP-1</b>	<b>Osteopontin</b>	<b>Proliferation</b>

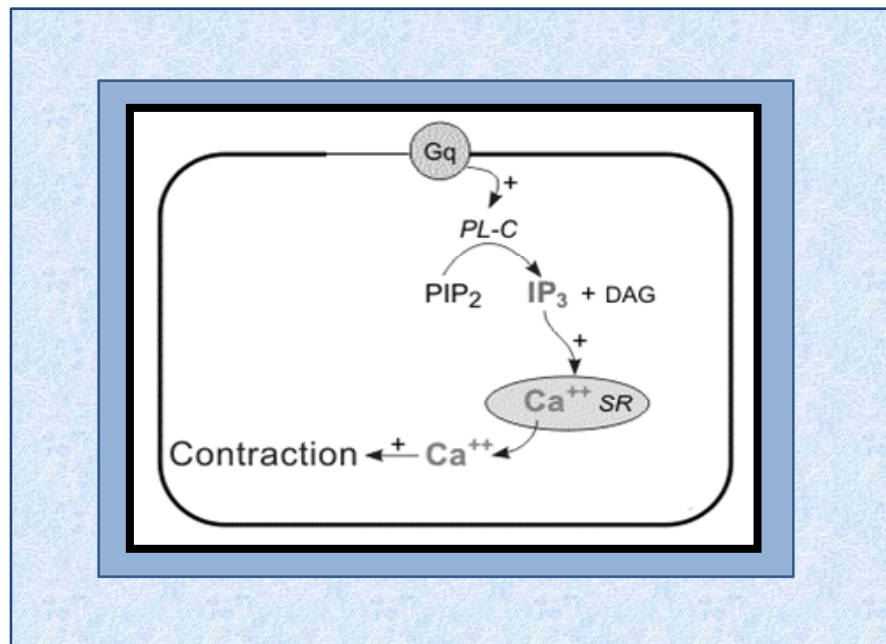
**Diagram 3. UII Structure and Function:** UII is an 11 amino-acid peptide that resembles somatostatin. UII has been shown to show minimal inter-individual variation across species. Urotensin II is a vaso-active agent, that structurally has been identified as a cyclic endogenous peptide. UII under elevated levels of UII appears to play an important role in vascular pathology. It has been suggested that UII is a major contributor of vascular diseases, including hypertension. Figure modified from *Curr Hypertens Rep*; 2006;6:479-483.



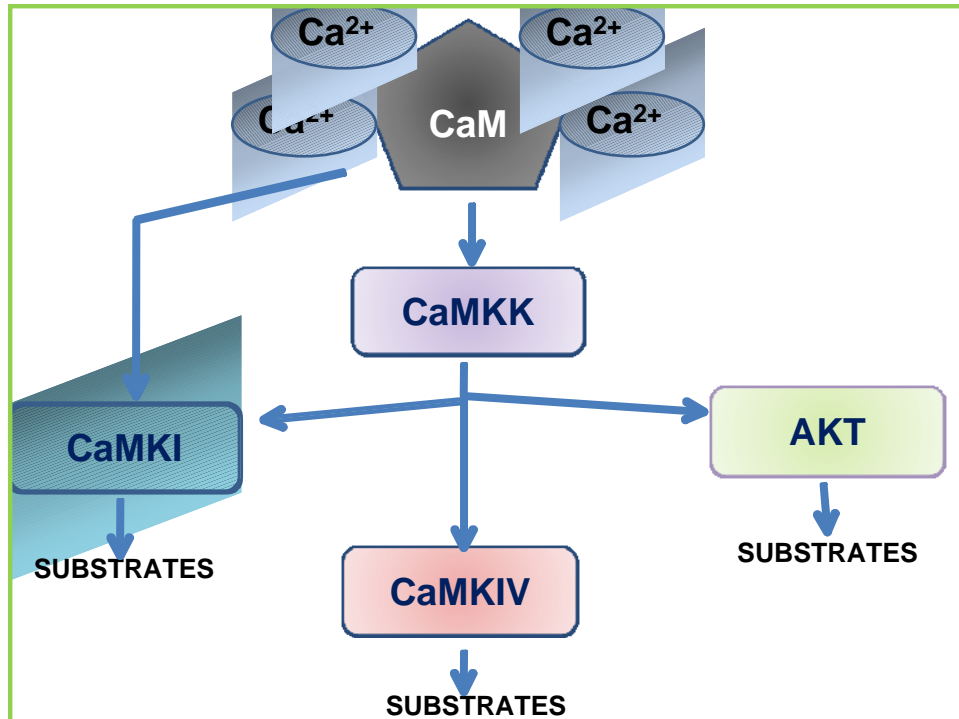
Peptide Properties	Ull - Characteristics
<input type="checkbox"/> 11 amino acid peptide	<input type="checkbox"/> Vaso -active
<input type="checkbox"/> Resembles the Structure of Somatostatin	<input type="checkbox"/> Induces Vasoconstriction in Specific Vessels
<input type="checkbox"/> Peptide Inter-Individual Variation Across Species	<input type="checkbox"/> Contributor to Vascular Diseases Such as Hypertension
<u>Peptide Expressed in:</u> -Dog -Rat -Rabbit -Pig -Monkey	<u>Ull Š Vessel Expression:</u> -Renal Artery -Carotid -Aortic -Superior Mesenteric -Arterial Segments



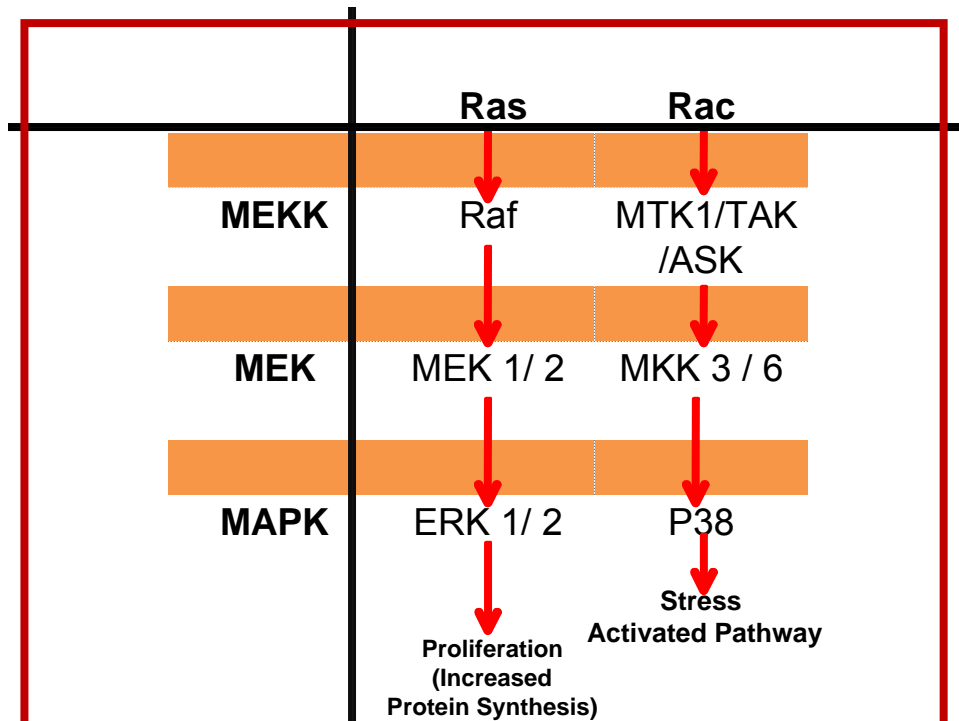
**Diagram 4. Activation of ATR1 & UIIR, which are Gq-coupled GPCR.** Both ATR1 and UIIR are considered Gq-GPCR receptors. Upon binding of UII to UIIR receptor or A2 to ATR1 the Gq-protein is activated. Upon Gq-GPCR activation, PLC is stimulated to cleave PIP2 to IP3 and DAG. The second messenger IP3 binds to the IP3-receptor on the SR and releases calcium from the SR. DAG on the other hand activates other downstream targets such as PKC, where as elevated intracellular calcium levels triggers the phosphorylation of other calcium-dependent kinases. Figure modified from *J Cell Physiol*; 2007:213:589-602.



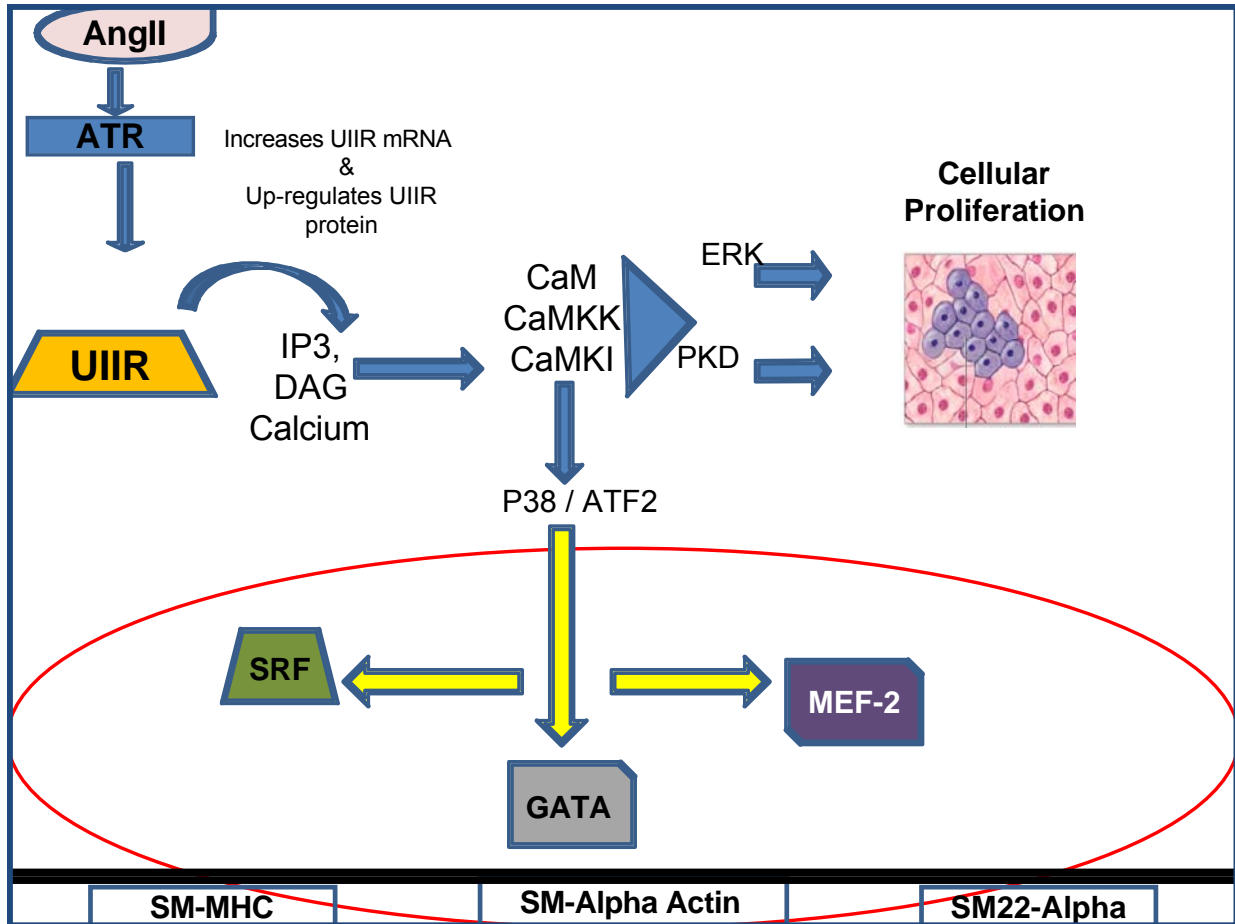
**Diagram 5. CaMKK Activation of Kinases from the CaM Kinase Family.** The calcium/ CaM system is stimulated by the release of calcium from the SR triggered by Gq signaling events. Upon release of the calcium ion, calcium binds CaM and undergoes conformational change, leading to the modulation of function. The calcium/ CaM complex formation exposes the methionine residues in the hydrophobic binding region and allow interaction with CaMKK. CaM interacts with CaMKK by binding to the CBD, this leads to CaMKK autophosphorylation. There are only three known substrates for CaMKK. CaMKK has the capacity to activate CaMKI, CaMKIV, or AKT. This signal transduction pathway regulates many cellular processes including contraction of smooth muscle.



**Diagram 6. The MAP Kinase Signaling Pathway.** There are 3 tiers of kinases in the MAP kinase pathway. MAPK's are recognized as serine/ threonine kinases that result in the dual phosphorylation of conserved threonine and tyrosine residues. P38 MAP kinase and ERK are the most well studied MAP kinases that have been associated with vascular proliferation, increased protein synthesis, and hypertrophy.



**Diagram 7. Hypothesized Model.** UII through UIIR may regulate cellular proliferation through the activation of CaMKK/ CaMKI, ERK, and PKD. The down-stream activation of PKD, CaMKI, and ERK via the UIIR signaling pathway are CaMKK dependent. The UII- induced activation of stress-activated kinases P38 and ATF2, and hypertrophy-associated new gene induction maybe dependent on CaMKK/ CaMKI as well as P38 activity. A2 possibly plays a pertinent role in the up-regulation of UIIR, and this A2 induced UIIR up-regulation maybe ERK dependent.





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## CHAPTER II

Urotensin II-Induced Signaling Involved in Proliferation of Vascular Smooth Muscle Cells

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## ABSTRACT:

Recent studies demonstrate that Urotensin II receptor (UIIR), not present on the surface of a healthy cardiomyocyte, is abundantly up regulated on the myocyte of a failing heart. In contrast, UIIR is present on normal arterial vascular smooth muscle cells (VSMC) and can be up regulated by Gq-coupled receptor signaling. In most cell types, UIIR signaling activates PLC, generates second messengers, IP3 and DAG, releases calcium ( $\text{Ca}^{2+}$ ), mobilizes calmodulin, and activates calcium-dependent kinases. Preliminary studies by us suggest that UIIR signaling via a CaM kinase kinase (CaMKK)-dependent mechanism may drive the proliferation of VSMC and be operative during pathological processes of restenosis. We hypothesize UIIR signaling both in a pulmonary arterial vascular smooth muscle cell line (Pac1) and in primary aortic vascular smooth muscle cells (PAVSMC) activates CaMKK, which then phosphorylates and activates  $\text{Ca}^{2+}$ /CaM-dependent Kinase I (CaMKI). This signaling then drives smooth muscle cell proliferation. Our results demonstrate that Pac1 cultures exposed to UII increase intracellular  $\text{Ca}^{2+}$  and activate CaMKK and CaMKI. Activated CaMKI then phosphorylates and activates two critical downstream kinases involved in proliferation, ERK 1/ 2, and PKD (PKC- $\mu$ ). Urotensin II (UII) exposure to either Pac1 or PAVSMC cultures results in proliferation as measured by  $^3\text{H}$  thymidine uptake and is inhibited by prior exposure to the CaMKK specific inhibitor, STO-609.

Moreover, Pac1 or PAVSMC cell exposure to STO-609 blocked CaMKK induced CaMKI phosphorylation and the subsequent downstream activation of ERK1/2 and PKD. Taken together, results presented here demonstrate that UII exposure to smooth muscle cells leads to a proliferative phenotype driven by UIIR signaling and regulated solely by CaMKK-dependent activation.

## INTRODUCTION:

Studies have recognized that vascular cell hypertrophy is associated with abnormal vascular function (5, 9, 24). Proliferation of vascular cells are related to hypertensive circulation manifested by increased arterial stiffness and narrowing of the vessel wall. This results in increased vascular resistance with increased mean arterial pressure (5, 24).

Proliferation or hypertrophy of vascular cells alters the overall vascular function and underlines the importance of experimental research in this area of vascular biology (24). Vascular hypertrophy is a difficult condition to treat due to the lack of pharmacological agents that can specifically target the proliferating vascular cells (9, 24). Experimental and clinical studies during the past decades have provided strong evidence for a correlation between ERK activation and cellular proliferation (26).

Activated ERK is an important target that has been linked to various vascular disease conditions (20). Studies conducted in cell lines such as mouse fibroblast cell line (NIH 3T3) and hamster fibroblast cell line (CCL39), have shown that ERK phosphorylation is required for cellular proliferation (8, 26). Under vascular pathological conditions, these effects often involve the activation of MAP kinases (22). Studies in thoracic aortic cells demonstrated that UIIR signaling stimulates the phosphorylation of ERK (26, 30). Since UII has been associated with the

progression of various vascular diseases (11), and UII studies have shown that UII induces ERK phosphorylation in various cells, this signifies the importance of understanding the mechanisms by which UII induces ERK phosphorylation.

Recent studies have suggested that UII and their receptors play an important role in the cellular proliferation associated with hypertrophy (11). Understanding the role of UII and UIIR signaling in VSMC cells is critical for delineating the progress of certain vascular diseases. UII, is an endogenous peptide, that under non-pathological conditions, influences vascular constriction/dilation and responds to hemodynamic changes during vascular smooth muscle (VSM) contraction (38). UII mediated vasoconstriction occurs in various vascular beds, including coronary, radial, pulmonary, saphenous, and aortic arteries (36). UII under pathological conditions responds to cellular changes, influences migration of cells, and modulates large blood vessels as shown in studies conducted in human aortic smooth muscle cells (SMC) (16). UII, originally isolated from fish urophysis, is an undecapeptide cleaved from a precursor molecule (17, 37) that stimulates vasoconstriction and VSMC proliferation (11). Elevated levels of UII induce pathological conditions by activating cellular pathways. This occurs when UII recognizes the orphan G- protein coupled GPR-14, now identified as UIIR(18).

UIIR bound and activated by UII results in the generation of the second messengers IP3 and DAG(23). These second messengers trigger the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR) (15). Studies have identified UIIR in many tissues including vascular smooth muscle (37). The manner in which the UIIR transduces signals in VSMC is largely unknown. Activation of UIIR via Gq coupling influx, and increased intracellular  $\text{Ca}^{2+}$  may activate CaM (23). Allosterically activated CaM subsequently leads to the stimulation of CaM-dependent

kinases (CaM-K) such as CaMKK (33). Phosphorylated members of CaM-K result in the activation of  $\text{Ca}^{2+}$ -dependent protein kinases such as ERK and PKD (19, 29). Previous studies in neuroblastoma cells have shown that  $\text{Ca}^{2+}$  activates CaMKI through CaMKK and subsequently leads to the phosphorylation of ERK 1/ 2 (27). Understanding the mechanisms by which UII through UIIR induces  $\text{Ca}^{2+}$  mobilization and activation of these kinases, such as PKD kinase, could provide additional knowledge of the direct signaling effectors that leads to vascular pathology.

Previously described studies have linked PKD to pathological conditions associated with vascular hypertrophy (4, 19). Recent findings have shown that ERK activation is directly correlated with PKD phosphorylation in several cell types (4, 6). In addition to ERK activation studies, intracellular  $\text{Ca}^{2+}$  influx has also been shown to modulate the activity of PKD (4). Vaso-active agents, such as endothelin-1, that bind Gq coupled receptors have been demonstrated as mediating PKD phosphorylation in various cell types (13). Therefore understanding UII activation of various protein kinases, such as PKD may provide a better understanding of the signaling pathways and effectors involved in UII-induced signaling under vascular pathological conditions.

In the present work, we demonstrate that UII, a rat 14 amino acid peptide, induces intracellular  $\text{Ca}^{2+}$  release, which stimulates CaMKI phosphorylation in Pac1 cells independent of extracellular  $\text{Ca}^{2+}$ . Moreover, we demonstrate that UIIR stimulation leads to CaMK-dependent phosphorylation of ERK and PKD. Also, we have shown that the acute application of UII results in cellular proliferation independent of  $\text{Ca}^{2+}$  in the growth media, and that cellular proliferation can be blocked by CaMKK inhibitor. We have shown that UII-induced CaMKI, ERK, and PKD

phosphorylation was blocked by CaMKK inhibitor in VSMC. These observations potentially indicate that UII-induced signaling triggers proliferation and may contribute to hypertrophic pathological conditions.

## MATERIALS AND METHODS:

### *Cell Culture:*

*Pac1:* Rat Pac1 cells (obtained from Rothman A.) were cultured according to Rothman A. et al (25). In brief, rat pulmonary arterial smooth muscle cells (Pac1) were cultured in medium 199 (Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (FBS) based on a previously described protocol (Atlanta Biologicals) with gentamicin (Fisher Scientific, Pittsburgh, PA). The cells were carried only through passages 3 to 15.

*Primary Aortic Smooth Muscle Cells:* Rat aortas were isolated from 3 month old Sprague-Dawley rats based on an established protocol (34). The aortas were incubated for 5 minutes in 10% FBS + Medium 199 then incubated at 37°C for 30 minutes in Hanks Balanced Salt Solution (HBSS) (GIBCO- Invitrogen, Grand Island, NY) with 70 units/ml of collagenase (Worthington Biochemical Company, Lakewood, NJ). Adventitias were stripped from the aortic tissue with watchmaker forceps and discarded. Aortas were digested in HBSS (GIBCO) with 70 units/ml collagenase and 40 units/ml elastase (Sigma Chemicals Co., St. Louis, MO). Aortas were gently agitated at 37°C for 90 minutes. The aortic solution was passed through a strainer with a 70 µm pore size, and centrifuged at 900 rpm for 5 minutes. Cellular suspensions were dispersed by pipetting the suspensions up and down. Cells were then plated in 6 well plates containing 10% FBS in 199 media.

*Calcium Imaging:* Pac1 cells ( $1 \times 10^5$  cells / ml) were grown on coverslips at 70%-80% confluency, and then bathed in HBSS solution with  $\text{Ca}^{2+}$  (GIBCO) and without  $\text{Ca}^{2+}$  {HBSS containing: 0.137M NaCl, 5.4mM KCl, 0.25mM  $\text{Na}_2\text{PO}_4$ , 0.44mM  $\text{KH}_2\text{PO}_4$ , 1.0mM  $\text{MgSO}_4$ , 4.2mM  $\text{NaHCO}_3$ ) for measuring intracellular  $\text{Ca}^{2+}$ . Detection of intracellular  $\text{Ca}^{2+}$  was quantified

using a ratiometric technique recognized by Fura-2-AM (Invitrogen) (excitation at 340 nm and 380 nm with emission at 510 nm) according to Prasanne et. al. (10). A Nikon Diaphot microscope using Metafluor software (Universal Imaging, West Chester, PA) was used to measure intracellular  $\text{Ca}^{2+}$ . Concentration (nM) of intracellular  $\text{Ca}^{2+}$  was calculated using the Grynkiewicz equation. EDTA (ethylene diamine tetra-acetic acid) (0.5 $\mu\text{M}$ ) (Fisher Scientific), a membrane impermeable chemical chelating agent that binds  $\text{Ca}^{2+}$ , was used to reverse the elevated levels of  $\text{Ca}^{2+}$ . BAPTA-AM (1, 2-bis(2-aminophenoxy)-ethane-N,N,N', N'-tetra-acetic acid) (10 $\mu\text{M}$ ) (Invitrogen) was also used as a  $\text{Ca}^{2+}$  chemical chelating agent. BAPTA-AM, a membrane permeable compound, also reverses the elevated levels of intracellular  $\text{Ca}^{2+}$ .

*Immunostaining:* Pac1 and PAVSMC cells were cultured ( $1 \times 10^5$  cells / ml) on glass coverslips and were washed two times with phosphate buffer solution (PBS) and fixed for 15 minutes with paraformaldehyde at room temperature. Cells were blocked for 1 hour in blocking solution (3.0% BSA in PBS), and then incubated for 1 hour in primary antibody SMC specific anti-Myosin IgG (Biomedical Technologies Incorporated, Stoughton, MA) diluted 1:200 or anti GPR14R (Antibody for UIIR – Alpha Diagnostic Intl. Inc., Woodlake Center, San Antonio) antibody 1:200 with blocking solution. The secondary fluorescent labeling was incubated with cells using Alexa 488 goat/anti-rabbit (Molecular Probes- Invitrogen, Eugene, OR) in blocking solution. The cells were washed three times with PBS. Coverslips were mounted on slides with Pro-Long Gold Anti-Fade with DAPI (Molecular Probe- Invitrogen). A DP70 Olympus digital camera and AX70 fluorescent microscope was used to visualize stained cells (40X objective).

*$^3\text{H}$  Thymidine Incorporation Assay:* Pac1 and PAVSMC cells were seeded ( $8 \times 10^4$  cells / ml) in RPMI (GIBCO- Invitrogen Corporation) medium containing 10% FBS. After 70% confluency, cells were washed with PBS and serum starved in serum free media (SF) to induce G1 arrest.

Cells were serum starved for 24 and 48 hours and were incubated in serum free RPMI media (GIBCO) or ACSF (142mM NaCl, 5mM KCl, 10mM Glucose, 1.3mM  $Mg^{2+}$  10mM HEPES, and in the presence or absence of 3.1mM  $Ca^{2+}$ ) containing 1  $\mu$ Ci/ml of  $^3H$  thymidine (Specific Activity = 48.0Ci/mmol) (Amersham Pharmacia, Buckinghamshire, UK) for 4 hours in the  $CO_2$  incubator at 37 ° C in the presence and absence of UII (100nM) (Sigma) and STO609 (250nM) (Sigma). After the 4 hour incubation period, cells were washed with PBS. Cells were precipitated with 15% trichloroacetic acid and incubated overnight at 4°C. Cells were then lysed with 1 Normal NaOH and were incubated for 30 minutes at room temperature. Cell lysates were transferred to tubes containing scintillation fluid for analysis using a (Beckman 1539) scintillation counter. Proliferation was measured by incorporation of  $^3H$  thymidine.

*Western Blot Analysis:* Pac1 cells were seeded at a density  $4 \times 10^4$  cells/mL and cultured until cells were 80% confluent in 100 mm<sup>3</sup> dishes. The day prior to treatment, the cells were serum reduced to 0.1% FBS in medium 199 with gentamicin (GIBCO) or 0.1% FBS in ACSF. 24 hours after serum reduction, cells were treated with 100nM UII (Sigma Aldrich), and 250nM STO609 (Sigma). All treatment conditions were carefully selected based on preliminary dose and time dependent studies. Experiments with the use of inhibitors were conducted under optimal conditions, and the concentrations were based on previous studies as referenced above. Inhibitors were incubated 30 minutes prior to UII treatments. Dose response and time course investigations were conducted with UII treatment. Cells were lysed using RIPA buffer (50mMTrisHCl, 150mM NaCl, 2mM EDTA, 1% NP-40, & 0.1% SDS pH 7.4) and protease inhibitor cocktail (Sigma Aldrich). Protein concentration was measured by BCA method (bicinchoninic acid – Pierce Corp). Equal amounts of protein (20 $\mu$ g - 30 $\mu$ g) were loaded and electrophoresed in 10% SDS- acrylamide gel. Proteins were transferred to PVDF membrane



(Millipore, Billerica, MA). Membrane was incubated with respective phospho-specific primary antibodies at 4°C overnight and with corresponding HRP-conjugated secondary antibody at room temperature for 1 hour. After washing with 1XPBS three times at room temperature, phosphorylation state of the proteins were detected by chemiluminescence (GE Healthcare Amersham, Piscataway, NJ). Phospho-specific antibodies used for the western blots are: p-ERK1/2 (Cell Signaling-9106S), p-PKD (Cell Signaling, Boston, MA) and pCaMKI (Threonine 178 - T. Soderling Vollum Institute, Oregon Health Science University, Portland, Oregon). Loading control was determined by stripping the blot and re-probing with anti Beta Actin antibody (Santa Cruz Biotech, Santa Cruz, CA). Expression of UIIR was measured using anti GPR14R (Antibody for UIIR – Alpha Diagnostic Intl. Inc.) antibody.

*Statistical analysis:* Western blot densitometry values were normalized and evaluated relative to control.  $\text{Ca}^{2+}$  imaging data was presented relative to control in terms of percent change. Densitometry,  $\text{Ca}^{2+}$ , and proliferation data were subjected to ANOVA (GraphPad – Prism, San Diego, CA) and the multiple range test Newman-Keuls, determined the pair-wise comparisons of the means. Statistical significance was indicated with at least a  $p \leq 0.05$ .

## RESULTS:

***UIIR is expressed in Pac1 & PAVSMC cells:*** To investigate UII-induced signaling we used both Pac1 and PAVSMC cells to verify the expression of UIIR. Pac1 cells are rapidly dividing cells (25), and therefore may not accurately represent the characteristics observed in normal VSMC. Thus, we incorporated the two cell types to identify the UIIR signaling mechanisms. Western blot analysis confirms that Pac1 cells compared to PAVSMC expresses greater UIIR (Fig 1A). Previous studies have identified that UIIR is expressed in PAVSMC, but UIIR expression in

Pac1 cell lines have not been examined (35). Therefore to identify if Pac1 cultured cells express UIIR, we immunocytostained Pac1 cells using an anti-UIIR antibody. Immunocytostaining results established that UIIR is expressed in Pac1 cells, as demonstrated with green staining of the plasma membrane (Fig 1B). Consequently, these results establish that UIIR is expressed in both PAVSMC and Pac1 cells (Fig 1).

***UII-induces mobilization of  $Ca^{2+}$  in Pac1 and PAVSMC cells:*** It has been well investigated that UII bound UIIR leads to intracellular mobilization of  $Ca^{2+}$  (3). We measured intracellular  $Ca^{2+}$  transients in UII treated Pac1 and PAVSMC cells using Fura-2-AM dye and digital imaging micro-fluorometry. Figure 2A & 2B shows that 100 nM UII treated Pac1 and PAVSMC cells mobilize  $Ca^{2+}$ . Figure 2A & 2B demonstrates a trace of  $Ca^{2+}$  concentration vs. time in Pac1 and PAVSMC cells. This trace demonstrates that UII induces an 2 to 3 fold increase in  $Ca^{2+}$  concentration within 30 seconds in Pac1 cells (figure 2A) and in PAVSMC cells 100 nM UII induces a 600 nM increase in  $Ca^{2+}$  concentration within 30 seconds (figure 2B). The Fura-2-AM scale, as shown in figure 2C, illustrates the level of intracellular  $Ca^{2+}$ . Our results (Fig 2D, Pac1 cells) and (Fig 2E, PAVSMC cells) depict cells with and without UII treatment. Figure 2D and 2E results demonstrate that UII induces an increase in  $Ca^{2+}$ , which is represented with the bright colors in figure 2D-bottom and figure 2E-bottom. In figure 2F we show that UII-induced  $Ca^{2+}$  release does not depend on extracellular  $Ca^{2+}$ . Here we identified that UII treatment in both  $Ca^{2+}$  free HBSS, as compared to HBSS with  $Ca^{2+}$ , results in similar levels of  $Ca^{2+}$  mobilization as measured by % increase. Because UII treatments with  $Ca^{2+}$  free HBSS and UII treated HBSS with  $Ca^{2+}$  are not statistically different, this provides evidence that the  $Ca^{2+}$  source is predominantly intracellular. EDTA (membrane impermeable) & BAPTA-AM (membrane permeable) served as our controls in Figure 2F, and our UII treatments in both  $Ca^{2+}$  free HBSS

and HBSS with  $\text{Ca}^{2+}$  were statistically significant as compared to EDTA and BAPTA-AM treated Pac1 cells. Therefore these results in figure 2 validate that with 100 nM UII treatments; there is an increased release of  $\text{Ca}^{2+}$  in Pac1 and PAVSMC cells.

***UII induces phosphorylation of CaMKI, ERK and PKD in Pac1 cells:*** We determined UII induced the phosphorylation of CaMKI, PKD, and ERK through western blot analysis (Fig 3A – 3F) in Pac1 cells. Our results in Pac1 cells demonstrated that UII in dose (Fig 3A) and time dependent studies (Fig 3D) induced CaMKI phosphorylation. Phosphorylation of CaMKI was maximally increased with the treatment of 100 nM UII (Fig 3A) for 10 minutes (Fig 3D). In western blot studies measuring the phosphorylation status of PKD, we show that UII induced the phosphorylation of PKD in Pac1 cells in a dose (Fig 3B) and time dependent manner (Fig 3E). These results demonstrate that with 100nM UII (Fig 3B) for 15min (Fig 3E) PKD is maximally phosphorylated. To determine if UII induces the phosphorylation of ERK, western blot analysis was conducted in a time (Fig 3C) and dose dependent (Fig 3F) manner using ERK phospho-specific antibody. With exposure to 100 nM UII (Fig 3C) in Pac1 cells for 15 minutes (Fig 3F), we revealed that this was the optimal dose and treatment time for UII-induced phosphorylation of ERK. Therefore UII treatment in Pac1 cells induces phosphorylation of CaMKI, PKD, and ERK in a time and dose dependent manner (Fig 3).

***UII -induced phosphorylation of CaMKI, PKD, and ERK is reduced in the presence of CaMKK inhibitor in Pac1 cells:*** To test if CaMKK is an integral target that affects the activation of several downstream effectors in UIIR signaling cascade, we used the CaMKK inhibitor STO609 in the presence of UII. Our previous UII dose and time dependent studies established that UII induces the phosphorylation of CaMKI, PKD, and ERK in our cells, although we wanted to also identify if CaMKK is involved in the UIIR signaling pathways that induces the

phosphorylation of these targets. Using 250 nM STO609 for a 30 minute pre-treatment in the presence of 100 nM UII for 10 minutes, we measured CaMKI phosphorylation via western blot analysis (Fig 4A & 4B). Our results in Fig 4A & 4B revealed that STO609 blocked the UII-induced phosphorylation of CaMKI. Pac1 cell treatment of STO609 in the presence of UII reduced CaMKI phosphorylation as compared to UII induced phosphorylation of CaMKI. We also pre-treated Pac1 cells with 250 nM STO609 for 30 minutes, followed by a treatment of 100 nM UII for 15 minutes to measure PKD phosphorylation (Fig 4C & 4D). Our findings demonstrated a visible reduction of PKD phosphorylation with the treatment of STO609 in the presence of UII (Fig 4C & 4D). We used 250 nM STO609 for a 30 minute pre-treatment, followed by a treatment of 100 nM UII for 30 minutes. Under these treatment conditions, we then measured ERK phosphorylation (Fig 4E- 4F in Pac1 cells). Our results demonstrate that STO609 elicits a reduction in UII-induced ERK phosphorylation in both Pac1 and PAVSMC cells. By and large, the inhibitor of CaMKK presents an overall reduction in UII-induced phosphorylation of CaMKI, PKD, and ERK (Fig 4). Therefore, our results with STO609 establish CaMKK's involvement in UIIR signaling (Fig 4).

***Characterization of Primary Cell Cultures:*** UII induced signaling mechanisms are poorly understood in VSMC, therefore we looked at UIIR signaling in both a cell line and a primary culture. To determine if PAVSMC cells were isolated from rat aortas the culture composition was verified by immunostaining the cells with a SMC specific antibody – Anti Myosin primary antibody for smooth muscle cells. Figure 5A shows the merged image image of PAVSMC cells. As shown in figure 5A, greater than 70% of PAVSMC are SMC positive. To determine if UII treatment of PAVSMC cells induced ERK phosphorylation, western blot analysis was conducted to measure the ERK phosphorylation status. Our results demonstrate that PAVSMC cells

exposed to 100 nM UII in a time dependent manner induces the phosphorylation of ERK maximally within 15 minutes (Fig 5B). To determine if UII-induced phosphorylation of ERK is reduced in the presence of CaMKK inhibitor in PAVSMC cells (Fig 5C & 5D); western blot analysis was conducted using a phospho-specific ERK antibody. Our results in figure 5C & 5D, show that with 30 minutes pretreatment of 250 nM STO609 in the presence of 100 nM UII, STO609 blocked UII-induced ERK phosphorylation in PAVSMC cells. Therefore our results demonstrate that UII induces the phosphorylation of ERK in PAVSMC cells, and CaMKK exposure to PAVSMC cells blocks UII-induced phosphorylation of ERK (Fig 5).

***UII Stimulates Proliferation in a Dose Dependent Manner in Pac1 & PAVSMC cells:*** Studies have linked UII induced ERK phosphorylation with cellular proliferation in thoracic aortic cells (30). Therefore, we investigated UII induced cellular proliferation in Pac1 and PAVSMC cells. We measured <sup>3</sup>H thymidine incorporation uptake in a dose dependent manner as a proliferative assay in Pac1 (Fig 6A) and PAVSMC cells (Fig 6B). Results demonstrate that with 100 nM UII, the highest level of proliferation occurred (Fig 6).

***CaMKK Inhibitor Blocks UII-Induced Cellular Proliferation in Pac1 & PAVSMC cells:*** Previous studies have also identified a correlation between the activation of ERK and the stimulation of cellular proliferation (26). Thus, we examined the role of CaMKK in UII-induced cellular proliferation. Our results showed that in both 24 hour and 48 hour serum starved cells with a 4 hour 100 nM UII treatment, cellular proliferation occurred (Fig 7A & 7B). To further evaluate if UII induces proliferation through CaMKK, we used 250 nM CaMKK inhibitor and/or 100 nM UII, and we measured proliferation by <sup>3</sup>H thymidine uptake. We show STO609 treatment in the presence of UII in PAVSMC (Fig 7C) and Pac1 (Fig 7A & 7B) blocks UII-

induced proliferation. Our results demonstrate that STO609 reduced UII induced stimulation of cellular proliferation (Fig 7).

***Extracellular  $Ca^{2+}$  does not alter UII induced phosphorylation of CaMKI and cellular proliferation:*** To determine if extracellular  $Ca^{2+}$  alters the 100 nM UII induced phosphorylation of CaMKI, the UII induced phosphorylation status of CaMKI was measured in 0.1% FBS in ACSF media in the presence and absence of  $Ca^{2+}$  via western blot analysis (Fig 8A). Results indicated no statistical difference between treatment groups [ACSF + UII] and [ACSF + UII +  $Ca^{2+}$ ] ( $n = 3$ ) (Fig 8B). To address the role of extracellular  $Ca^{2+}$  and 100 nM UII induced cellular proliferation, we treated cells for 24 hours with reduced serum ACSF in the presence and absence of  $Ca^{2+}$  and measured cellular proliferation via  $^3H$  thymidine incorporation assay (Fig 8C). Results in figure 8C demonstrate that UII treatment in ACSF media, in the presence or absence of  $Ca^{2+}$ , did not alter the levels of cellular proliferation.

## DISCUSSION:

In the present study we showed that UII-induced signaling in VSM cells results in the phosphorylation of  $Ca^{2+}$  / CaM dependent kinases leading to cellular proliferation. Also we demonstrated that inhibition of CaMKK attenuates UII-induced phosphorylation of  $Ca^{2+}$  / CaM dependent kinases and cellular proliferation in VSM cells.

UIIR expression in various cells and cell lines had not been completely determined (36), therefore we evaluated UIIR expression in both Pac1 and PAVSMC cells. Previous studies have shown that UIIR is expressed in thoracic aortic cells, although little is known about the UIIR expression in Pac1 cells (36). In figure 1 we show UIIR expression in both Pac1 (Fig 1A & 1B)

and PAVSMC cells (Fig 1A). Therefore, these findings support our use of both Pac1 and PAVSMC cells to study UIIR signaling.

In addition to identifying the expression of UIIR in Pac1 and PAVSMC cells, we have also found that activation of UIIR leads to the mobilization of intracellular  $\text{Ca}^{2+}$  in these cells. Intracellular  $\text{Ca}^{2+}$  mobilization has been linked to a diverse group of functions in arterial SMC (12). Cellular responses to  $\text{Ca}^{2+}$  increase can be sizable or sudden, and is a pertinent factor that contributes to SMC proliferation (7). Our results demonstrate that UII induces an increase in intracellular  $\text{Ca}^{2+}$  (Fig 2A, 2B, 2D, and 2E). This increased release of  $\text{Ca}^{2+}$  from intracellular stores as shown with studies using  $\text{Ca}^{2+}$  free HBSS and HBSS with  $\text{Ca}^{2+}$  (Fig 2F), signifies the independence of extracellular  $\text{Ca}^{2+}$ . According to other  $\text{Ca}^{2+}$  signaling studies, elevated levels of  $\text{Ca}^{2+}$  have been shown to activate CaM and other CaM-dependent kinases. Thus, our data indicates that UII induces the mobilization of intracellular  $\text{Ca}^{2+}$  in Pac1 and PAVSMC cells and this finding supports our rationale for studying UII-induced activation of CaM dependent kinases.

In this current study we demonstrated that UII-induces the phosphorylation of CaMKI, and that UII-induced CaMKI phosphorylation is CaMKK dependent in Pac1 cells. By identifying that UII induces an elevation in intracellular  $\text{Ca}^{2+}$ , these experiments further supports the notion that CaM-dependent kinases are involved in the UIIR signaling pathway. Previous studies have shown that elevated levels of  $\text{Ca}^{2+}$  stimulate the activation of  $\text{Ca}^{2+}$ -dependent protein kinases (1, 2). The Gq- GPCR signaling mechanism activates the release of  $\text{Ca}^{2+}$  from the SR and stimulates  $\text{Ca}^{2+}$ / CaM kinases (21). Upon the release of these  $\text{Ca}^{2+}$  ions,  $\text{Ca}^{2+}$  binds CaM and undergoes a conformational change, leading to modulation of function such as phosphorylation of downstream targets (15). CaMKI has been identified as pertinent in transduction of  $\text{Ca}^{2+}$  signals

during cellular events (21). Here we show that UII treatment of Pac1 cells demonstrates an increase in phosphorylation of CaMKI (Fig 3D & 3A). The consequence of pre-treatment with STO609 and UIIR stimulation results in a sizeable reduction of CaMKI phosphorylation (Fig 4A & 4B). Under pharmacological doses used in these studies, STO609 specifically inhibits CaMKK(31-33). This study supports our hypothesis that CaMKK is involved in the UII-induced phosphorylation of CaMKI in Pac1 cells. Thus putting forward the question of whether CaMKK inhibition blocks UII-induced phosphorylation of other downstream targets.

Our preliminary studies have linked ERK kinases as a  $\text{Ca}^{2+}$  dependent kinase, which has been demonstrated by other studies as well (27). Therefore UII-induced mobilization of intracellular  $\text{Ca}^{2+}$  provided a link to UII-induced phosphorylation of ERK. Our present findings in this study demonstrate that UII induces the phosphorylation of ERK, and CaMKK inhibitor pre-treatment blocks UII-induced phosphorylation of ERK in Pac1 and PAVSMC cells. Previous studies demonstrated that ERK activation is dependent on several intracellular signals including an increase in intracellular  $\text{Ca}^{2+}$  (14). ERK has been recognized as a target for several GPCR's, and its activation is correlated with proliferation (26). UIIR is one such Gq-coupled GPCR, which triggers ERK phosphorylation and induces cellular proliferation (26). One proliferation study identified UIIR signaling as stimulating the phosphorylation of ERK via the classical pathways involving Ras and Raf in primary thoracic aortic cells (30). Although we did not measure UII induced Ras/ Raf activation, we did examine UIIR signaling as a Gq-coupled mechanism. Our results demonstrate that UII induces ERK phosphorylation in a time and dose dependent manner in Pac1 cells as shown in figure 3 (Fig 3C - 3F-in Pac1 & Fig 5B in PAVSMC). Therefore, our results demonstrating that UII induces ERK phosphorylation supports our conjecture and is consistent with previous findings. Although no studies have investigated



$\text{Ca}^{2+}$ / CaM-dependent kinase's role in the UII-induced phosphorylation of ERK, we postulate that  $\text{Ca}^{2+}$ / CaM-dependent kinases are involved in the regulation of UII-induced ERK phosphorylation. Other studies have demonstrated that binding of  $\text{Ca}^{2+}$ / CaM complex results in the modulation of numerous intracellular kinases, many of which are serine/ threonine protein kinases such as ERK kinase (29). Detailed investigations identifying ERK as a target of CaMKI have been reported in non-SMC (29), therefore we examined CaMKK/ CaMKI activation of ERK via UIIR signaling in Pac1 and PAVSMC cultures. We determined that CaMKK plays an important role in UII-induced ERK phosphorylation, which we extrapolated by using CaMKK inhibitor. Inhibitor studies in both Pac1 (Fig 4E & 4F) and PAVSMC cells (Fig 5C & 5D) exposed to CaMKK inhibitor, resulted in a substantial reduction in ERK phosphorylation. These findings support the projected hypothesis and indicate that CaMKK plays an essential role in the phosphorylation of ERK in the UIIR signaling pathway.

Our data in this study demonstrates that UII-induces the phosphorylation of PKD and that CaMKK inhibitor pre-treatment blocks the UII-induced phosphorylation of PKD in Pac1 cells. Studies have demonstrated a link between  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -dependent kinases with the activation of PKD (19). Since UII-induced UIIR signaling results in the mobilization of intracellular  $\text{Ca}^{2+}$ (22), to our knowledge no studies have linked PKD phosphorylation to UIIR signaling and the mobilization of  $\text{Ca}^{2+}$ . In figure 3B and 3E we have shown that UII via UIIR induces PKD phosphorylation in a time and dose dependent manner in Pac1 cells. In figure 4C & 4D our results show that the CaMKK inhibitor reduced PKD phosphorylation in the presence of UII. Thus, our results show that CaMKK is involved in UII-induced phosphorylation of PKD.

According to our data we present here, UII induces cellular proliferation and cells exposed to CaMKK inhibitor blocks UII-induced cellular proliferation in both Pac1 and

PAVSMC cells. Previous studies have shown that UII induces cellular proliferation in human umbilical vein cells, and other studies have linked the activation of specific signaling kinases such as PKD and ERK to cellular proliferation (28). Our findings in figure 5 revealed that UII induces proliferation in a dose dependent manner in both Pac1 (Fig 6A) and PAVSMC cells (Fig 6B). In figure 6 the levels of thymidine incorporation were greater in Pac1 cells compared to PAVSMC cells, and may be due to the rapid doubling time of Pac1 cells. In figure 7C - PAVSMC and figure 7A – 7B Pac1, we show a reduction in proliferation with STO609, a specific CaMKK inhibitor in the presence of UII. Furthermore, these findings support the notion that a signaling correlation exists between CaMKK and UII-induced cellular proliferation in both Pac1 and PAVSMC cells.

Our results also show that extracellular  $\text{Ca}^{2+}$  does not alter UII-induced phosphorylation of CaMKI (Fig 8A & Fig 8B) and cellular proliferation (Fig 8C) in Pac1 cells. Because our studies have demonstrated that  $\text{Ca}^{2+}$  plays a critical role in the UII-induced UIIR signaling pathways leading to the activation of CaM dependent kinases. Our rationale was to define if extracellular  $\text{Ca}^{2+}$  plays a significant role in UII-induced phosphorylation of CaMKI and cellular proliferation. Our results demonstrate that the presence or absence of extracellular  $\text{Ca}^{2+}$  did not alter the UII-induced phosphorylation of CaMKI and UII induced cellular proliferation. Thus, confirming our hypothesis that UII-induced release of intracellular  $\text{Ca}^{2+}$  and stimulates CaMKI phosphorylation and subsequently leads to cellular proliferation.

In summary these results identify the importance of UII-induced  $\text{Ca}^{2+}$  mobilization and the activation of  $\text{Ca}^{2+}$  / CaM-dependent kinases in Pac1 and PAVSMC cells. Interestingly, we demonstrate the UII-induced increase of  $\text{Ca}^{2+}$  suggests that additional kinases, such as  $\text{Ca}^{2+}$ /

CaM-dependent kinases, are involved in the UIIR signaling pathway. Important findings in our present study identify that UII-induced phosphorylation of UIIR downstream targets can be blocked using a CaMKK inhibitor in VSM cells. We also establish a therapeutically relevant finding, which reveals that CaMKK inhibitor can block UII induced cellular proliferation in Pac1 and PAVSMC cells. Understanding CaMKK's role in UII induced proliferation may provide insight into potential therapeutic targets for hypertrophy of VSM cells.

*Future Studies:* Although our studies indirectly identify  $\text{Ca}^{2+}$  / CaM kinases as the target signaling kinases in the UII-UIIR pathway, future studies are needed to elucidate UIIR signaling mechanisms using a UIIR- specific antagonist. These studies will determine the direct involvement of  $\text{Ca}^{2+}$  / CaM kinases in the UII-induced proliferation pathways of VSM cells.

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## FIGURE LEGENDS:

**Figure 1 – UIIR is expressed in Pac1 and PAVSMC cells:** (A) A representative immunoblot using anti GPR14 (antibody to UIIR) demonstrates that UIIR is expressed in both Pac1 and PAVSMC cells. (B) Merged image UIIR immunocytochemistry in Pac1 cells is highlighted by the green stain using anti GPR14 antibody (antibody to UIIR) and the nuclei staining is identified with DAPI, which is shown in blue.

**Figure 2 – UII induces elevated levels of intracellular  $[Ca^{2+}]_i$  in Pac1 and PAVSMC cells:** Modulation of intracellular  $Ca^{2+}$  in response to UII treatment in (A) Pac1 and (B) PAVSMC cell. Cells preloaded with Fura-2 and treated with UII. Cells were captured at the 340/380 fluorescence ratio of Fura-2 and cells were monitored to detect changes in  $Ca^{2+}$ . UII concentrations were chosen according to our previous dose response studies. Data is from an average of  $Ca^{2+}$  response from a population of Pac1 cells (A) and PAVSMC cells (B) stimulated with UII (n=14) and a representation of 3 independent experiments of UII treatment in Pac1 and PAVSMC cells were graphically plotted. UII induced mobilization of  $Ca^{2+}$  response was plotted as  $Ca^{2+}$  concentration versus time. (C) A representative scale demonstrating the correlation between color and  $Ca^{2+}$  levels. High levels of  $Ca^{2+}$  are shown with bright colors (yellow/red/orange) and low levels correspond to the dark colors (violet/blue/green). Fluorescent images of Pac1 (D) and PAVSMC (E) cells were captured with preloaded Fura-2AM and UII treatment. Images depict (D-top & E-top) control {Fura-2AM & no treatment} and (D-bottom & E-bottom) {Fura-2 + UII treatment}. (F) Pac1 cells were treated with (UII + HBSS) and (UII + HBSS +  $Ca^{2+}$ ), these treatment groups were compared to determine if intracellular  $Ca^{2+}$  is the



predominant source. Pac 1 cells exposed to (EDTA), a membrane impermeable compound, and (BAPTA- AM) membrane permeable and were used to reverse the elevated levels of  $\text{Ca}^{2+}$ . Experiments were conducted in 3 separate experiments (n = 14, \*  $p \leq 0.05$ ).

**Figure 3- UII induces phosphorylation of CaMKI, PKD, and ERK in Pac1 cells:** Western blot analysis of the time course of CaMKI, PKD, and ERK phosphorylation [A, B, & C] and dose response of UII stimulated phosphorylation CaMKI, PKD, and ERK [D, E, and F] in Pac1 cells.

**Figure 4 – UII induced phosphorylation of CaMKI PKD and ERK is inhibited by CaMKK inhibitor in Pac1 cells:** UII induced phosphorylation of CaMKI (A) PKD (B) and ERK (C) is inhibited by CaMKK inhibition (C - Control, U- Urotensin II, U/S – Urotensin II + STO609, S – STO609) (n=4, \*  $p \leq 0.05$ , #  $p \leq 0.05$ ).

**Figure 5 – Characterization of PAVSMC:** (A) PAVSMC cells were immunostained with anti-myosin SMC specific antibody to identify if cultured cells are composed of VSMC. Merged image of cultured PAVSMC cells depicts positive anti-myosin SMC staining (A –left image). Anti-myosin SMC antibody is highlighted by the green stain (A - top), and the nuclei staining is identified with DAPI, which is shown in blue (A- bottom). (B) A representative immunoblot demonstrates that UII induces ERK phosphorylation in a time dependent manner. (C & D) A representative western blot demonstrates that CaMKK blocks UII-induced phosphorylation of ERK (10% Serum – 10% FBS in Media, U – Urotensin II, U/S – Urotensin II + STO609, S – STO609, SF – Reduced Serum Media) (n=2, \*  $p \leq 0.05$ ).

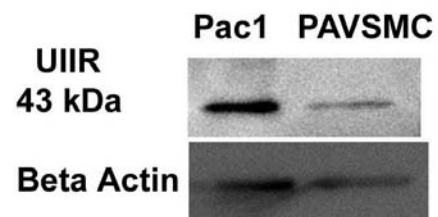
**Figure 6 – UII induces Cellular Proliferation in a Dose Dependent Manner:**  $^3\text{H}$  thymidine uptake, (A) Pac1 and (B) PAVSMC cells, treated with UII in a dose dependent manner stimulates proliferation (n=4, \*  $p \leq 0.01$ ).

**Figure 7 – CaMKK Inhibitor Reduces UII-Induced Cellular Proliferation in Pac1 and PAVSMC cells:** Proliferation studies involved serum starvation for 24 hours (A) and 48 hours (B) and Pac1 cells were treated with UII and were measured via  $^3\text{H}$  –thymidine incorporation assay. Inhibitor studies using CaMKK inhibitor in the presence of UII were conducted in Pac1 (A & B) and PAVSMC cells (C) and were analyzed through  $^3\text{H}$  thymidine incorporation as a measurement of cellular proliferation (10% Serum – 10% FBS in Media, U – Urotensin II, U/S – Urotensin II + STO609, S – STO609, SF – Reduced Serum Media) (n=4, \*  $p \leq 0.05$ , ++  $p \leq 0.01$ , +++  $p \leq 0.001$ , & \*\*\*  $p \leq 0.001$ ).

**Figure 8 – Intracellular  $\text{Ca}^{2+}$  is the predominant source of  $\text{Ca}^{2+}$  involved in UII-induced phosphorylation of CaMKI and cellular proliferation in Pac1 cells:** (A) A representative immunoblot demonstrates that UII-induced phosphorylation of CaMKI is independent of extracellular  $\text{Ca}^{2+}$ . Pac1 cells were treated with (ACSF + UII +  $\text{Ca}^{2+}$ ) and (ACSF + UII), (B) densitometry analyses of treatment groups are compared in pair-wise t-test. The y-Axis data is plotted as a fraction of control (n=2). (C) Proliferation studies conducted in Pac1 cells were measured via  $^3\text{H}$  thymidine incorporation in Pac1 cells. Treatment groups (ACSF + UII +  $\text{Ca}^{2+}$ ) and (ACSF + UII) were compared in a pair-wise t-test (n=4).

FIGURE 1.

**A.**



**B.**

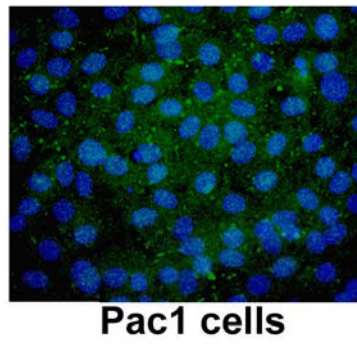


FIGURE 2.

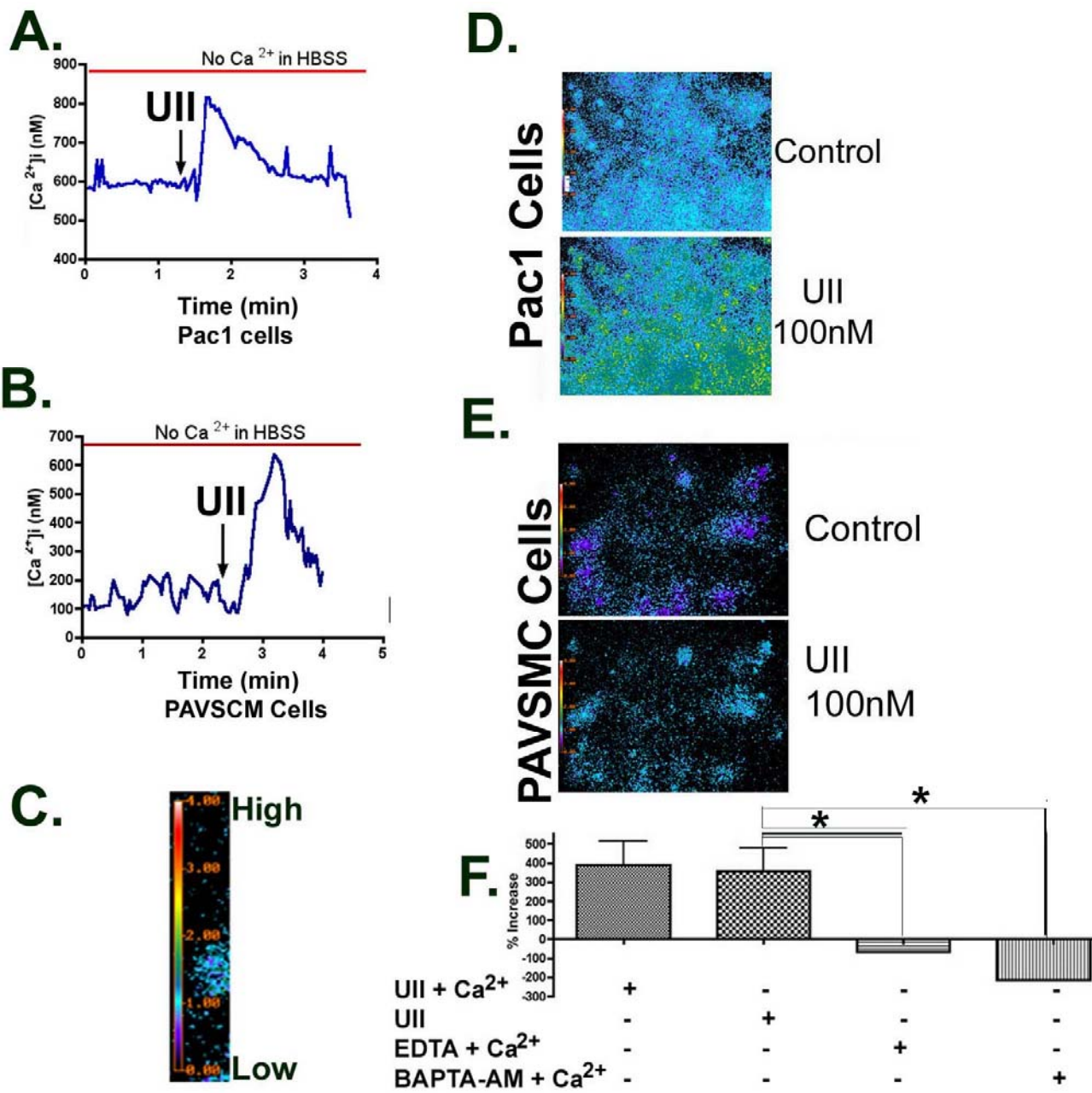


FIGURE 3.

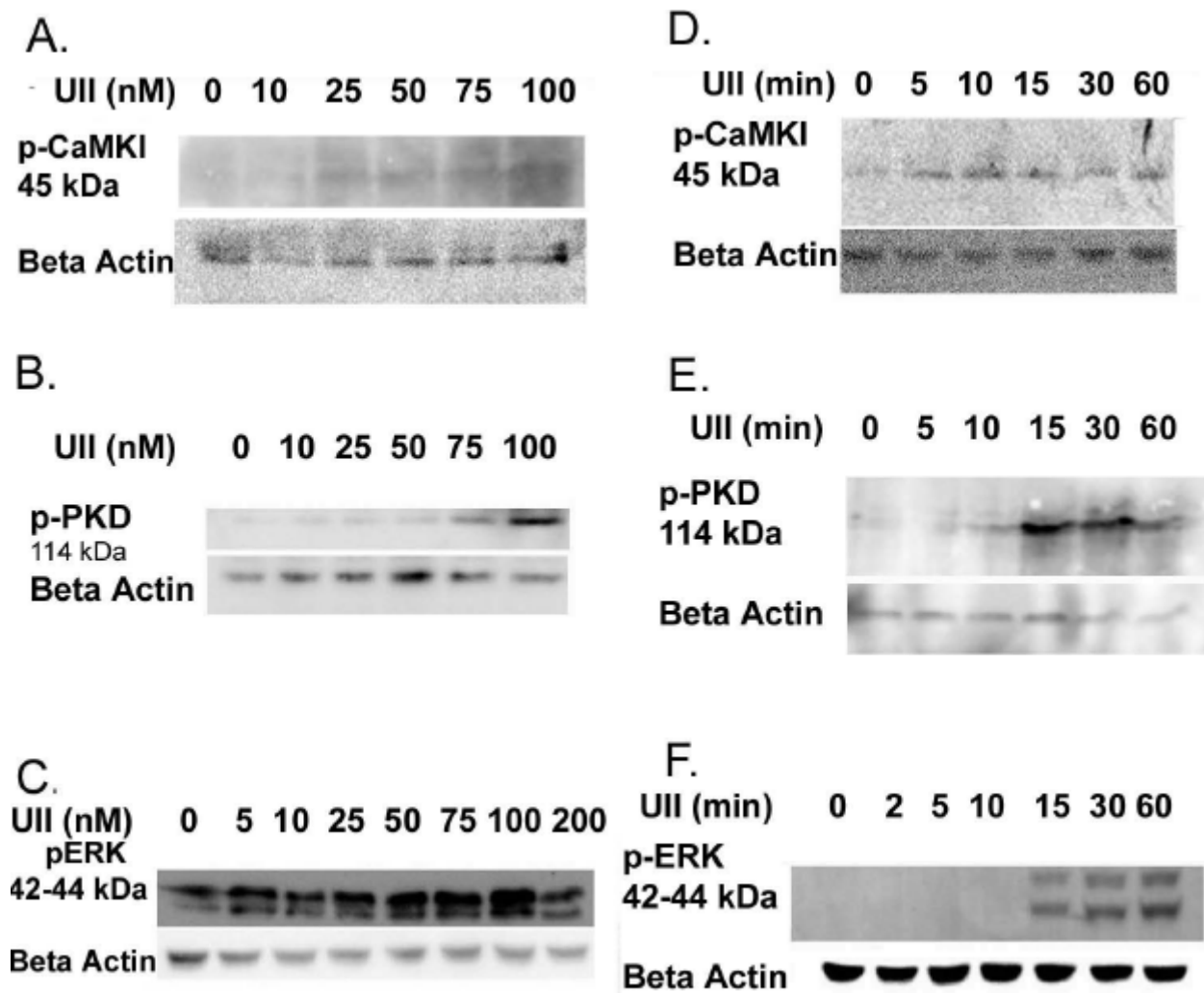


FIGURE 4.

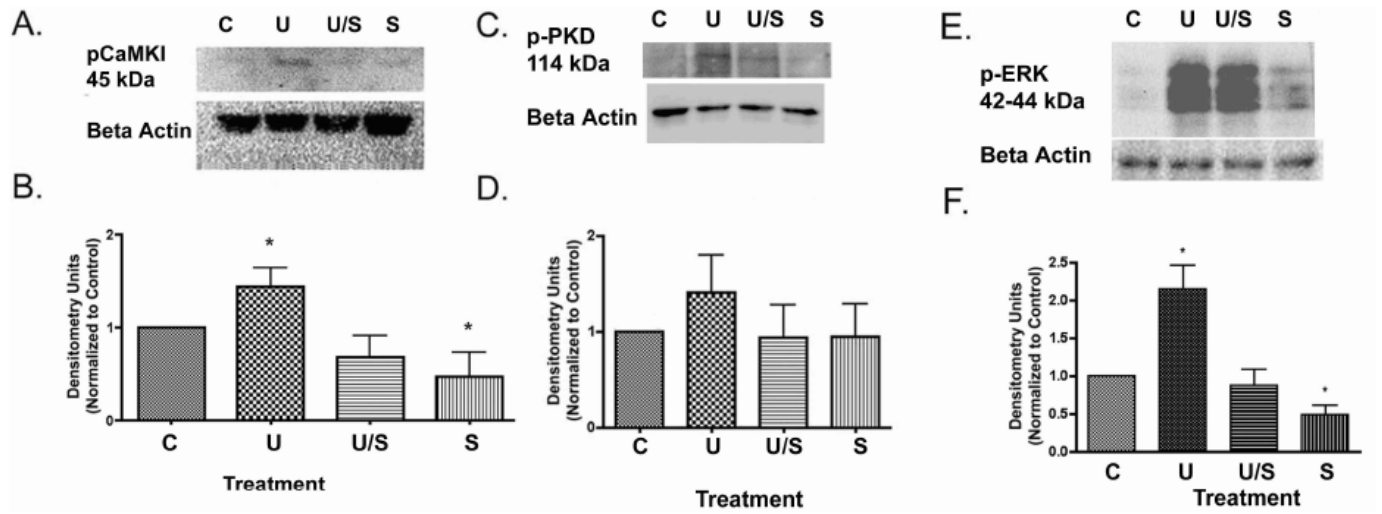


FIGURE 5.

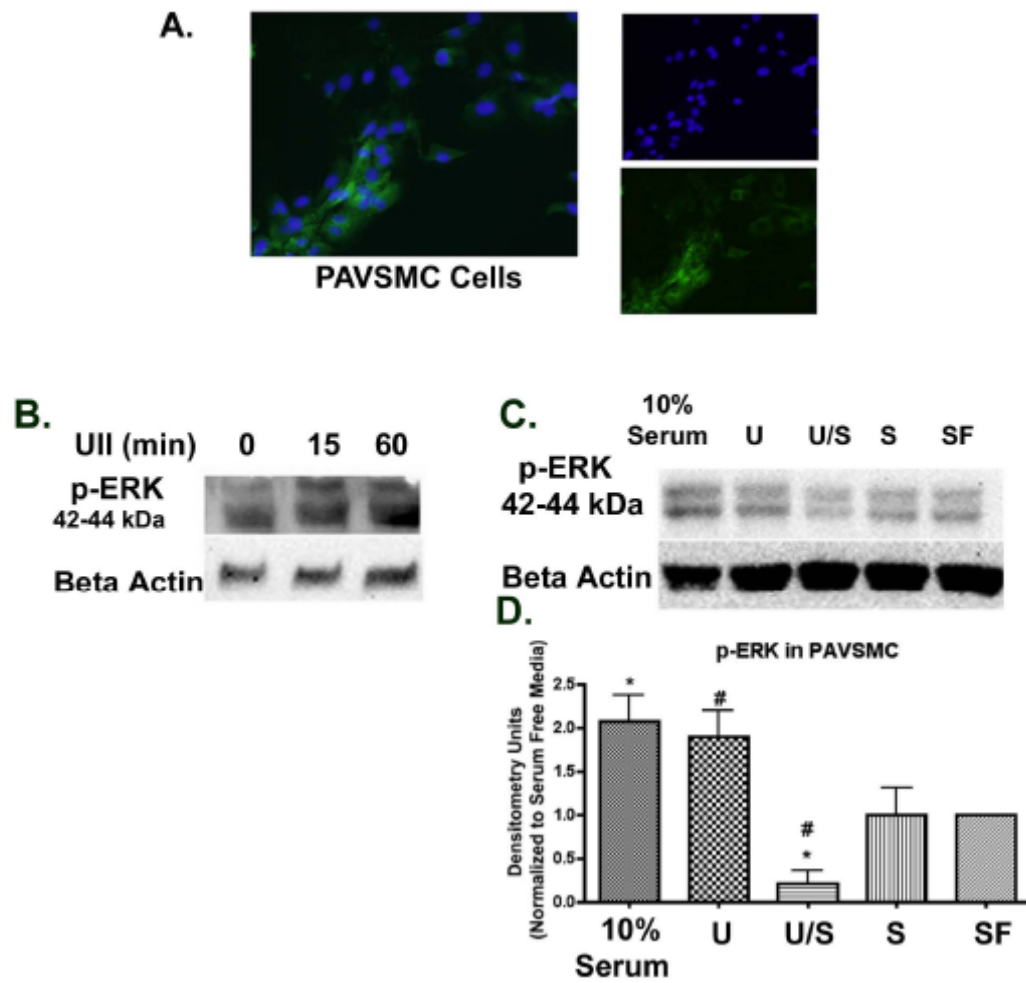


FIGURE 6.

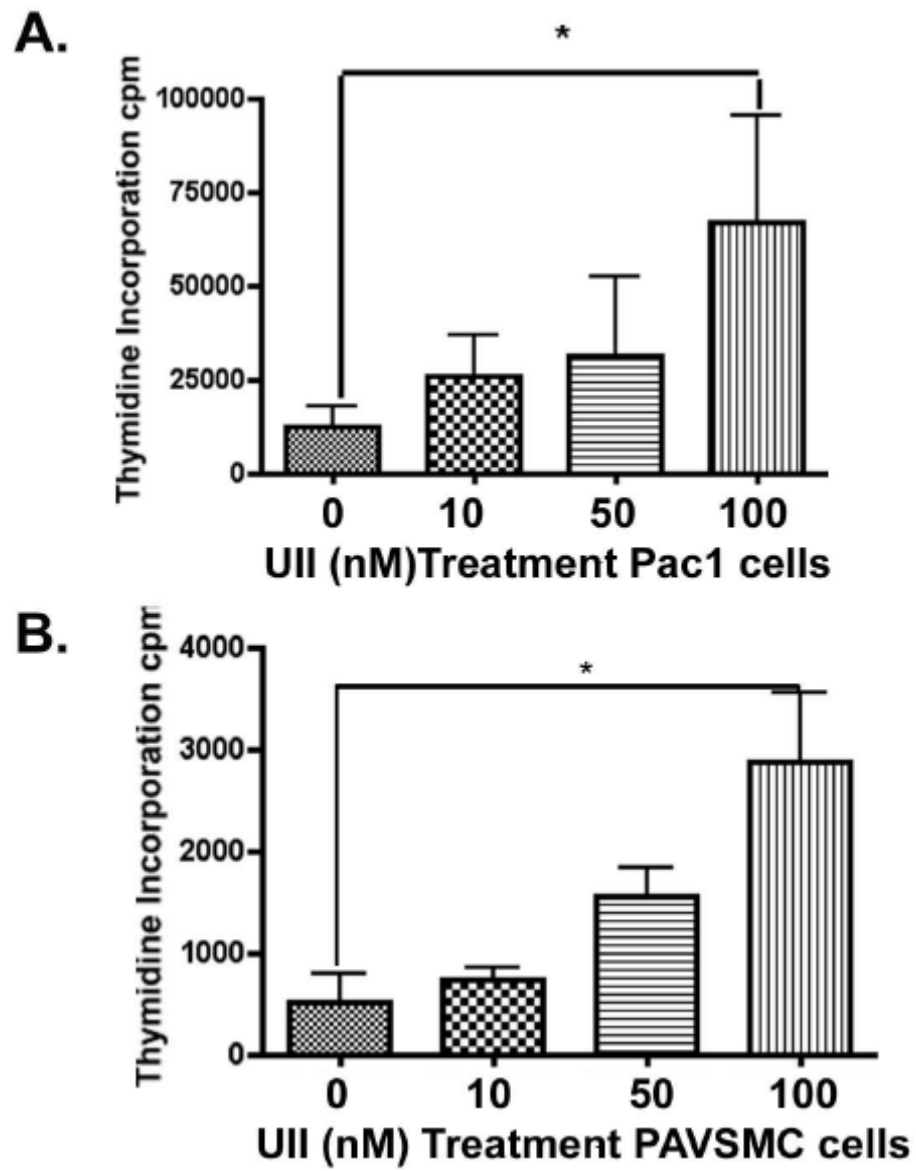




FIGURE 7.

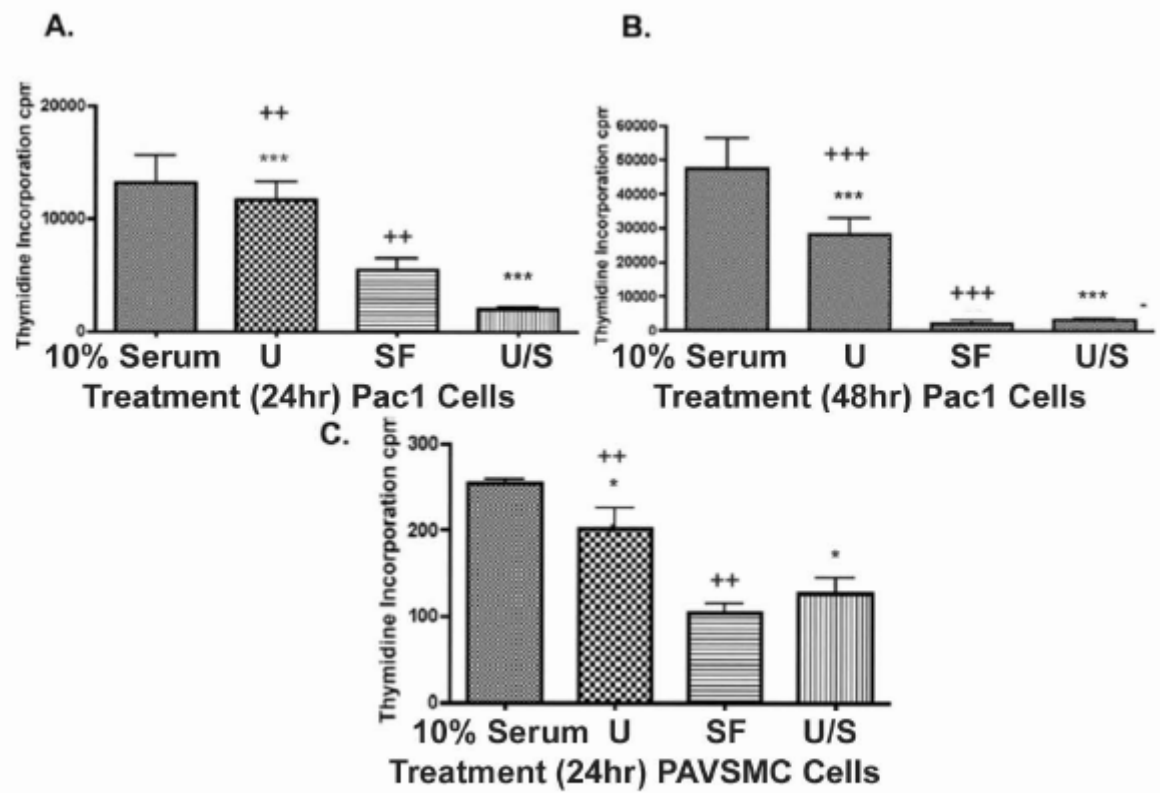
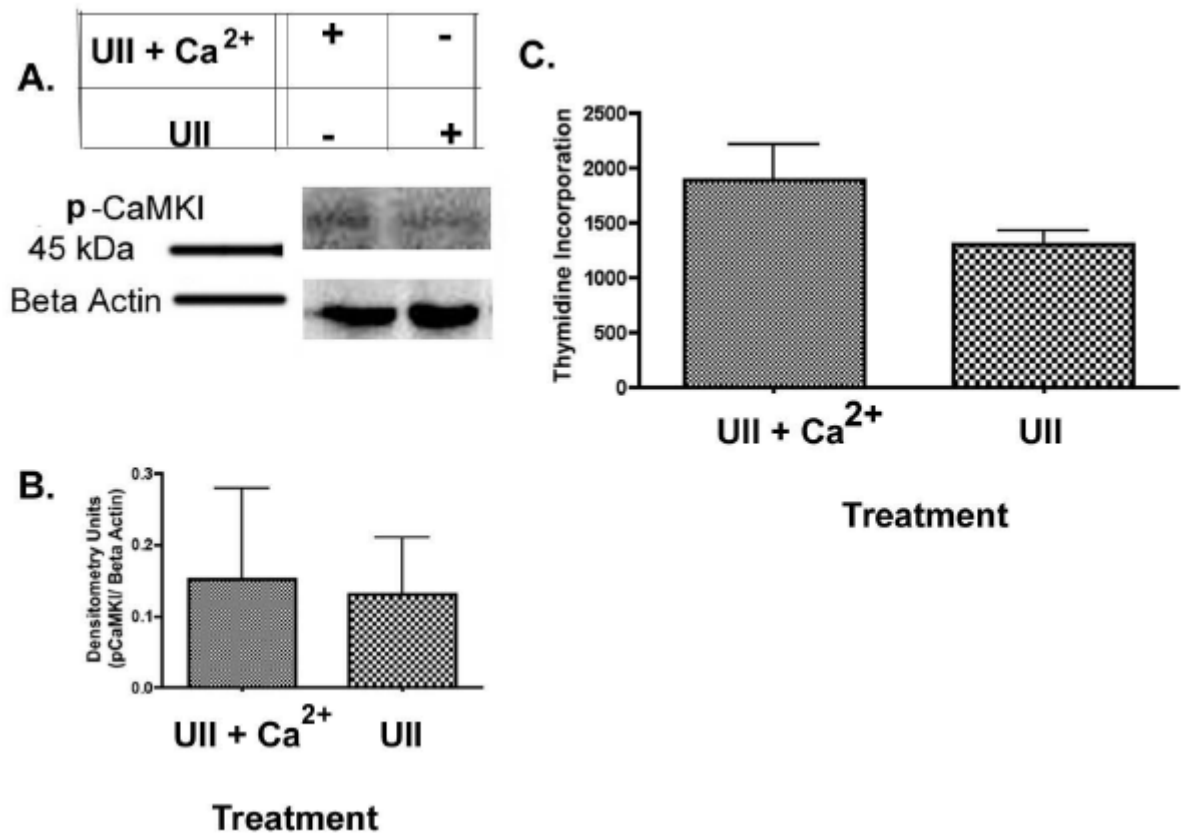


FIGURE 8.



## CHAPTER III

Urotensin II Induces Hypertrophy Signaling and New Gene Induction in Pac1 cells

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## ABSTRACT:

Elevated levels of Urotensin II (UII) triggers the stimulation of stress activated pathways and hypertrophy of vascular smooth muscle (VSM) cells. Several signaling pathways have been implicated in vascular hypertrophy; including  $\text{Ca}^{2+}$ /CaM kinase's (CaMK) and P38 stress activated mitogen activated protein (MAP) kinases. Both of CaMK's and MAP kinases are activated as a result of Gq coupled signaling. We hypothesize that UII exposure to Pac1 cell cultures activates P38 MAP kinase via  $\text{Ca}^{2+}$ /CaM-dependent kinases (CaMKK), and increases the expression of transcription factors which responsible for regulating SMC marker genes. Western blot analysis and luciferase reporter assay data demonstrates that UII induces P38 /ATF2 phosphorylation, as well as expression of transcription factors (SRF, MEF, and GATA) and SMC marker genes (SM-22 $\alpha$ , SM-MHC, and SM  $\alpha$ - actin). Previous studies in our laboratory demonstrated that TGF- $\beta$  induces the expression of SMC marker genes. Therefore TGF- $\beta$  comparisons were drawn in relations to UII- induced expression of transcription factors and SMC marker reporter activation. Inhibitors studies using CaMKK inhibitor (STO609) and P38 inhibitor (SB203580) demonstrated the involvement of CaMKK and P38 in the expression of transcription factors and SMC marker genes. This data indicates that perturbation of vascular homeostasis leads to elevated levels of UII which promotes activation of cellular stress activated pathways involving CaMKK, P38, ATF2, and increased expression of SRF, SM-MHC, SM- $\alpha$  Actin.

## INTRODUCTION:

Stress kinase phosphorylation plays an important role in signal transduction pathways. Stress activated kinases are involved in various cellular responses with respect to extracellular signals (1 – 3). Stress activated pathways play a crucial role in various disease conditions such as hematopoiesis, myocardial contractility/ cardiomyopathies, and atherosclerosis. For instance recent reports have observed pressure overload induces the activity of p38 MAP kinase (1). A variety of patho-physiological stimuli such as myocardial infarction, hypertension, viral myocarditis, and cardiomyopathies can lead to an increase in cardiac work leading to an increase in mechanical stress on cardio-vascular tissue (3). In response to increase cardiac workload, studies have reported that P38 MAP kinase increases its activity during these pathological conditions including following pressure overload exhibited in disease states such as hypertrophy (1 – 3). Recent studies linking P38 to vascular hypertrophy was observed with the inhibition of P38 in vascular cells which resulted in reduced vascular hypertrophic conditions (3). Stretching, increased pressure overload, and vascular injury of VSM cells increases P38 phosphorylation (3).

Current studies have identified that UII simulation of thoracic aortic cells results in the activation of P38 MAPK.(4) Activation of Urotensin II receptor (UIIR) by its ligand UII, generates second messengers IP3, DAG, and calcium ( $\text{Ca}^{2+}$ ) in VSM cells(5-7).  $\text{Ca}^{2+}$  influx leads to the activation of  $\text{Ca}^{2+}$  dependent kinases through calmodulin binding, resulting in phosphorylation of downstream targets such as CaMK (8, 9). Data has demonstrated that

CaMK's regulate hypertrophic gene expression (8, 9). Other studies conducted in neurons have shown that CaMK cross talk with MAPK(10, 11).

The current study investigates UII exposure to Pac1 cell cultures, the activation of P38 MAP kinase, increased expression of hypertrophy associated transcription factors, and SMC gene markers via  $\text{Ca}^{2+}$ /CaM dependent kinases (CaMKK). Through western blot analysis we conducted phosphorylation and inhibitor experiments to identify if UII induces P38 and ATF2 phosphorylation, and inhibitors to CaMKK as well as P38 blocked the phosphorylation of these kinases. The UII-induced phosphorylation (ATF2) and increase in expression of transcription factors (SRF, MEF2 and GATA) were measured or quantified in the presence and absence of CaMKK and P38 inhibitors in Pac1 cells.

TGF- $\beta$  in previous studies has been shown to induce the expression of SMC marker genes (12, 13). Recent reports have also identified that elevated levels of TGF- $\beta$  contribute to VSM hypertrophy and restenosis (12-16). In the present study TGF- $\beta$  induced expression of transcription factors and SMC gene markers via reporter activation were compared to UII induced expression in Pac1 cells.

SMC marker genes are also typically synchronously up-regulated under conditions of vascular mechanical stress (15, 16). Thus, UII-induced expression of SMC marker genes were measured in the presence and absence of CaMKK and P38 inhibitors via the luciferase reporter assay.

Taken together, our results demonstrate that UII stimulation of smooth muscle cells leads to the phosphorylation of stress activated kinases and induces the expression of

transcription factors and SMC marker genes. Delineating the UIIR signaling pathways will provide insight into the mechanisms by which UIIR activates MAPK resulting in cellular stress activated signaling and hypertrophy of VSM cells.

#### MATERIALS AND METHODS:

*Cell Culture- PAC1:* Established cell line (17), rat pulmonary arterial smooth muscle cells were cultured in 199 media (Invitrogen, Calsbad, CA) supplemented with FBS based on a previously described protocol (Atlanta Biologicals, Atlanta, GA) with gentamycin (Fisher, Pittsburgh, PA). The cells were carried only through passages 3 to 15. At passage 15, Pac1 cells lose their normal phenotypic properties.

*Western Blot Analysis:* Pac1 cells were seeded at a density  $4 \times 10^4$  and cultured till cells were 80% confluent in 100mm<sup>3</sup> dishes. The day prior to treatment of the cells, the cells were serum reduced to 0.1% FBS in 199 media with gentamycin. 24 hours after serum reduction cells were treated with UII (Sigma Aldrich, St Louis, MO) AngII (Sigma Aldrich), TGF-B (Sigma Aldrich), STO609 (Sigma Aldrich), and SB203580 (Sigma). Inhibitors were incubated 30min prior to Gq-agonist treatments. Dose response with UII was performed using doses ranging from 0 nM to 200 nM. Time course experiments involved the use of 100nM UII for time points ranging from 0 minutes to 60 minutes. Detection of the following phosphorylated proteins were analyzed using antibodies; p-P38 (Cell Signaling, Boston, MA), and p-ATF2 (Cell Signaling). Total protein levels were determined using GAPDH (Alpha Diagnostics, Cell Signaling) and Beta Actin (Santa Cruz Antibodies, Santa Cruz, CA). Cell lysates were collected using RIPA buffer and protease inhibitor cocktail (Sigma). Lysates were stored in -80 degrees Celsius.

*Source of Vector Constructs:*

Reporter	Source of Vector Construct
MEF & SRF	The 3xMEF2-luciferase enhancer reporter and the 4xSRF – luciferase enhancer reporter (4xSM22 $\alpha$ CArG-luc) were obtained from Eric Olson (UT Southwestern, Dallas, TX).
6X GATA	The 6XGATA-luciferase enhancer (p(G1)6-tk-luc) was provided by T. Yamagata (Joslin Diabetes Center, Harvard Medical School, Boston, MA).
SM-22 $\alpha$	The SM-22 $\alpha$ actin promoter reporter (p441 SM22 $\alpha$ -luc) containing 482 bp of DNA from -441 to +41 bp was provided from M. Parmacek (University of Pennsylvania, Philadelphia, PA).
SM-MHC	The smooth muscle myosin heavy chain promoter reporter (SM-MHC) containing 1303 bp of DNA from -1259 to +45 bp was provided by S. White (University of Vermont, Burlington, VT).
SM- $\alpha$ Actin	The smooth muscle – $\alpha$ promoter reporter (SMP-2 luc) containing 767 bp of DNA from -724 to +43 was provided by J.Cook (Oshner Clinic Foundation, New Orleans, LA).

*Transient Transfection and Luciferase Assay:* PAC1 cells, seeded at a  $2.4 \times 10^4$  density and were transfected 48 hours after seeding in 1 well of a 6 well tissue culture plate. Once cells are 80-



90% confluent, cells were transfected with Lipofectamine<sup>TM</sup> with 10% Serum and 199 media without gentamycin and either of these vectors were used (depending on the experiment); MEF-2 (10ng/ well/ transfection), SRF (1ng/well/transfection), 6xGATA4 (1ng/well/transfection), SM a-actin (30ng/well/transfection), SM22- $\alpha$  (30ng/well), and SM-MHC (10ng/well/transfection). After 3 to 6 hour transfection, the cells were fed 10% Serum in 199 without gentamycin. 24 to 48 hours post-transfection cells were treated for 24 hours with UII (Sigma Aldrich), AngII (Sigma Aldrich), TGF-B (Sigma Aldrich) and STO609 (Sigma Aldrich). Treatment after 24hour incubation at 37 degree is removed and cells are washed two times with 1x Phosphate Buffer Solution (PBS). Cells are lysed with 1x Reporter Lysis Buffer (Promega Corp). Luciferase substrate (Promega, Madioson, WI) is added to the cell lysates and luciferase activity is measured by luminometry (Model TD 20/20 Luminometer, Turner Designs, Sunnyvale, CA) using a commercially available kit (Promega, Madison, WI).

*Statistical Analysis:* All results are expressed as the mean  $\pm$  S.E. Data was analyzed with GraphPad Prism software (Version 4.0, GraphPad Software Inc.). One-way ANOVA analysis of variance and Neuman Kheuls post –hoc test for inter-group comparisons were analyzed.  $P < 0.05$  were considered statistically significant.

## RESULTS:

***UII-induces time dependent activation of stress activated kinases:*** To determine if UII-induces time dependent activation of stress activated kinases P38 and ATF2, western blot analysis with UII treatment of Pac1 cells in a time dependent manner was performed. Currently, P38 is widely accepted as a stress activated kinase, and plays a major role in intracellular signaling cascades during vascular pathology (2, 18). Previous studies measuring

P38 phosphorylation in response to UII treatment were performed in primary VSM cells (1, 19, 20), although UII studies have not been conducted in Pac1 cell lines. Therefore, these results in **Figure 1A** demonstrate that UII-induces the phosphorylation of P38 within 5 minutes of exposure to Pac1 cells. VSM cell studies have previously demonstrated that P38 is a downstream target in the UIIR signaling cascade (1, 20). Although, to our knowledge, no study has identified ATF2 as a kinase involved in the UIIR signaling pathway in VSM cells and non-VSM cells. To determine if UII induces ATF2 phosphorylation, Pac1 cells were stimulated with UII in a time dependent manner, and western blot analysis was performed using a phospho-specific ATF2 antibody. Our results in **Figure 1B** demonstrate that UII treatment induces maximal ATF2 phosphorylation within 10 minutes. Despite ATF2 studies having not been previously shown in UIIR signaling pathways studies, the correlation between P38 and ATF2 in non-UIIR signaling studies has been shown in non-SMC types (21-23). Therefore, these results have revealed that UII induces the phosphorylation of P38 and ATF2.

***UII induces the time dependent gene expression of hypertrophy associated transcription factors and SMC gene markers:***

A hypertrophic vascular state has been linked to a specific subset of transcription factor gene expression and activity (3, 24, 25). Cis- and trans-acting transcription factors mediate the expression of a variety of genes, including genes linked to cellular stress and vascular hypertrophic conditions. SRF, MEF2, and GATA are a subset of transcription factors that have been identified as inducing the expression of SMC marker genes (24-26). Therefore, vectors for SRF, MEF2, and GATA containing the luciferase gene were transfected into Pac1 cells. 24 hours later Pac1 cells were stimulated with UII, and gene expression was measured via relative luciferase activity in turner units (TU). These results in

**Figure 2A, 2B, and 2C** demonstrate that within 24 hours, exposure to UII, induces the increase in GATA, SRF, and MEF2 ( $7.875 \pm 0.323$ ) gene expression. Signaling through hypertrophic pathways induced by cellular stress and mechanical stress ultimately leads to the increased expression of SMC marker genes (24, 25). Hypertrophic-sensitive SMC marker genes have been shown in many studies to be associated with vascular phenotype change (26). To determine if UII-induces the expression of hypertrophic-sensitive SMC marker genes, Pac1 cells were transfected with vectors containing both the luciferase gene and the promoter sequences of SM- $\alpha$  actin, SM-MHC ( $2.25 \pm 0.354$ ), and SM-22 $\alpha$  ( $7.813 \pm 0.427$ ). 24 hours post-transfection Pac1 cells were treated with UII in a time dependent manner and luciferase activity was measured. In **Figure 2D, 2E, and 2F** UII-induces the expression of SMC marker genes within 24 hours of UII exposure. This data demonstrates that UII induces the expression of both hypertrophy associated transcription factors and also the expression of SMC marker genes in a time dependent manner in Pac1 cells.

***UII-induced the activation of stress-activated kinases is CaMKK dependent:*** STO609 is the only pharmacological inhibitor to date that has been described as possessing the specificity and the efficiency to inhibit the activation of CaMKK (27-29). CaMKK has been demonstrated in various cardio-vascular studies to be involved in hypertrophic conditions (30-32). However, to our knowledge, there has been a lack of studies examining the phosphorylation of P38 and the correlation of CaMKK activation in the UIIR signaling pathway or other P38 activated signaling pathways (33, 34). Therefore, to determine if CaMKK inhibitor (STO609) blocks UII induced phosphorylation of P38, Pac1 cells were treated with 100 nM UII in the presence and the absence of 250 nM STO609. Western blot

analysis studies were conducted using the P38 phospho-specific antibody and levels of P38 phosphorylation were analyzed. Results in **Figure 3A**, demonstrate that STO609 blocks the UII-induced phosphorylation of P38 MAP kinase. Typically represented as a transcription factor that regulates cellular transformations such as phenotype switch, ATF2 has not been examined in response to  $\text{Ca}^{2+}$  /CaM dependent signaling in response to UII stimulation in VSM cells (21, 35, 36). Thus, to determine if STO609 blocks UII- induced phosphorylation of the transcription factor ATF2, Pac1 cells were treated with 100 nM UII in the presence and absence of 250 nM STO609. Western blot analysis data determined the phosphorylation status of P38 in the presence of the CaMKK inhibitor. **Figure 3B** shows that CaMKK inhibitor blocks the UII-induced phosphorylation of ATF2. Therefore, these results demonstrate that CaMKK is involved in the UIIR signaling pathway and the subsequent phosphorylation of P38 and ATF2.

***UII-induced expression of stress associated transcription factors and SMC marker genes***

***are CaMKK dependent:*** Few studies have reported that SMC differentiation transcription factors are expressed under a variety of circumstances, including conditions of elevated  $\text{Ca}^{2+}$  levels in response to cellular/mechanical stress or hypertrophy, examples to this are observed with SRF and MEF2 (24, 37, 38). Increased  $\text{Ca}^{2+}$  levels are associated with the activation of other  $\text{Ca}^{2+}$  dependent signaling pathways under situation of cellular stress(2, 3, 39, 40). The objective of these studies was to determine whether  $\text{Ca}^{2+}$ / CaM dependent kinases are modulating the expression of transcription factors that regulate SMC marker genes involved in differentiation(31, 33, 41, 42). A series of reporter assays were conducted using vectors containing sequences of the luciferase genes and the enhancers. Previous studies have shown that TGF- $\beta$  induces the expression of these hypertrophic-associated transcription factors, thus

TGF-beta comparisons were drawn and represented as a positive control in the present UII studies (13)(12). Luciferase measurements using the luminometer in TU determined the level of gene expression induced by UII stimulation of transfected Pac1 cells. Results in **Figure 4A, 4B, and 4C** illustrate that UII-induces expression of stress associated transcription factors; MEF2, SRF, and GATA and that this expression is blocked in the presence of CaMKK inhibitor. TGF- $\beta$  stimulation in Pac1 transfected cells (**Figure 4G**), and the expression of transcription factors, appeared to be more prominent for GATA and SRF (**Figure 4G**) transfected cells when compared to UII stimulation. In many studies of cardio-myocytes,  $\text{Ca}^{2+}$  sensitivity appeared to influence the expression of hypertrophy- associated SMC marker genes(25, 26, 43, 44). Preceding studies in our laboratory have focused on the role of CaM-dependent kinases in the expression of these SMC marker genes. Therefore, to determine if CaMKK inhibitor blocks the UII-induced expression of SMC marker genes SM- $\alpha$  actin, SM-MHC, and SM-22  $\alpha$ , vectors containing luciferase gene and the promoter sequences were transfected in Pac1 cells. Gene expression was measured in direct correlation to relative luciferase activity. These findings in **Figure 4D** ( $4.325 \pm 0.384$ ), **4E** ( $0.726 \pm 0.265$ ), and **4F1** reveal that CaMKK inhibitor (STO609) blocks the UII-induced expression of SMC marker genes. TGF- $\beta$  comparisons were drawn between UII induction of SMC marker gene expression **Figure 4G** ( $8.663 \pm 0.278$ ), and results display that TGF- $\beta$  elicits a greater expression of SM- $\alpha$  actin and SM-22  $\alpha$  (**Figure 4G**). Therefore, the results in **Figure 4** exhibit the importance of CaMKK in the UII induction and UIIR signaling pathway leading to expression of hypertrophic-associated transcription factors and SMC marker genes.

***UII-induced phosphorylation of ATF2 is P38 dependent:*** The SB-compound is a pharmacological inhibitor to P38 that has been revealed to possess a great degree of potency in blocking P38 activity (2, 45). The SB-compound has been commonly used in several mechanical stress studies in VSM cells (3, 46). To determine if the P38 inhibitor (SB-203580 compound) blocks UII-induced phosphorylation of ATF2 western blot analysis using 100 nM UII in the presence and absence of 10 uM SB203580, and a phospho-specific ATF2 antibody was used to measure the phosphorylation status. Results in **Figure 5** establish that P38 inhibitors (SB203580) block the UII-induced phosphorylation of ATF2. Taken together this study reveals that UII-induced phosphorylation of ATF2 is P38 dependent.

***UII-induced expression of stress associated transcription factor and SMC specific genes are P38 dependent:*** Progress has been made in the past decade in identifying the signaling mechanisms that regulate the expression of specific transcription factors (25). Distinguishing between hypertrophic and stress activated pathways using chemical inhibitors that block the expression of transcription factors which modulate gene expression has recently become an area of focus (25). By abolishing the expression of transcription factors that regulate hypertrophic or mechanical stress – associated gene expression could potentially provide a method to manipulate the progression of abnormal vascular conditions (25). Therefore, a series of experiments were conducted to delineate a signaling pathway that induces hypertrophy gene expression via chemical inhibition. Pac1 cells transfected with enhancer reporters to SRF, MEF2, and GATA were detected in the presence of UII treatment and P38 inhibitor (SB203580) exposure. Data not included revealed that UII-induced activation of MEF2 and GATA were not P38 dependent. Although, SRF transfected Pac1 cells illustrated that UII-induced increase in SRF expression is P38 dependent. Reporter assay studies in

**Figure 6A** demonstrated that P38 inhibition blocks the UII-induced reporter activation of SRF. Signaling through the Gq coupled receptor results in the induction of hypertrophic gene expression in cardio-myocytes, and this expression is dependent on MAP kinase activation (1, 47, 48). Thus, to determine if UII induced SMC marker gene expression is P38 MAP kinase dependent, a sequence of reporter assays were carried out. Promoter reporter assays conducted in the presence and absence of a P38 inhibitor, measuring luminescence of UII-induced expression of SM-22  $\alpha$ , were inconclusive. Although, the studies reported here revealed that UII-induced expression of SM- $\alpha$  actin and SM-MHC is P38 dependent. **Figure 6B & 6C** demonstrates that P38 inhibits UII-induced SMC marker gene expression of SM- $\alpha$  actin and SM-MHC via increase in relative luminescence, as shown in promoter reporter activity. These data demonstrate that UII-induced activation of SRF, SM-MHC, and SM- $\alpha$  actin requires activated P38.

## DISCUSSION:

In the present study we have shown that UII exposure to Pac1 cells induces signaling pathways associated with cellular/ mechanical stress and hypertrophy -associated SMC gene expression. Studies have shown that VSM cells perform a specialized subset of functions dictated through a repertoire of certain genes under contractile and differentiated state (25, 26, 43, 49). Whereas, under a hypertrophied state the result of cellular or mechanical stress involves a different subset of genes expressed (3, 46). This subset of genes expressed includes SM- $\alpha$  actin, SM-MHC, and SM-22  $\alpha$  (25, 43, 44, 50). These genes are often referred to as hypertrophy-associated genes which mediate phenotype modulation, as seen under circumstances of vascular

injury (arterial restenosis due to angioplasty) or cellular/ mechanical stress due to pressure overload of VSM cells(26, 43, 51).

Recent studies have shown that P38 is the primary kinase that is activated under situations of cellular or mechanical stress, although other kinases have also been linked to pathological conditions, such as JNK and SAPK (2, 3, 34, 39, 52, 53). Studies have shown that activation of SAPK and JNK kinases are involved in the signaling that induces hypertrophic conditions in myocardial tissue. Although, for the purposes of this study our focus was directed towards p38 MAP kinase and the signaling pathway that results in cardiac hypertrophy. Cardiac hypertrophy findings have established that P38 signaling is involved in the progression hypertrophy (5, 54, 55). Moreover recent UII studies in thoracic aortic VSM cells have demonstrated that P38 phosphorylation is stimulated by UII treatment (56, 57). Since UII studies in Pac1 cells do not exist, the UII-induced phosphorylation status of P38 was consistent in other cell types, including Pac1 cells (56). These results reveal that UII induces the phosphorylation of stress activated kinase, P38 MAP kinase (**Figure 1A**). Other studies have reported that P38 directly phosphorylates the transcription factor ATF2, thus categorizing ATF2 as a stress activated kinase, as well. Therefore, in addition to measuring the UII- activation of P38, we also measured UII-induced phosphorylation of ATF2. The results in **Figure 1B** establish that UII exposure to Pac1 cells results in ATF2 phosphorylation. Therefore, UII-induces the phosphorylation of stress activated P38 and ATF2.

Previous studies in our laboratory have shown that UII-induces the activation of SMC marker genes and a subset of transcription factors that regulate this gene expression in cardiomyocytes (58, 59). SMC gene expression has previously been demonstrated in Pac1 cell in



response to TGF- $\alpha$  stimulation in our laboratory (12, 13). In this current study, these results revealed that UII in VSM cells induces the expression of transcription factors (SRF, MEF2, GATA) and SMC marker genes (SM- $\alpha$  actin, SM-MHC, and SM-22 $\alpha$ ) within 24 hours (**Figure 2**). To date, the expression of the majority of SMC hypertrophy associated marker genes have been dependent on CArG elements within the promoter region (24, 25, 43). SM-MHC, SM- $\alpha$  actin, and SM-22  $\alpha$  all have CArG elements within their promoter sequences (24, 50). Moreover, SRF, MEF2, and GATA are all transcription factors that have the general sequence motif that permits binding to the CArG elements in the SMC gene promoter region (25, 50). Therefore, the rationale for selecting this subset of transcription factors is due to the preliminary studies which identified UII-induction of SMC marker genes and provided a logical link to UII stimulated new gene induction in Pac1 cells.

In nearly all VSM cell types, Ca<sup>2+</sup> is a major second messenger that is important for the regulation of vascular contraction and also for the activation of Ca<sup>2+</sup> dependent kinases(8, 60-62). Signaling mechanisms that induce elevated levels of Ca<sup>2+</sup> have been associated with both cellular/mechanical stress and vascular hypertrophy (61, 62). UIIR is a Gq coupled receptor which is activated under similar conditions of mechanical/ cellular stress(18, 63, 64). Moreover, studies have shown that UIIR signaling induces an increase in Ca<sup>2+</sup> mobilization and other studies have demonstrated that P38 phosphorylation is stimulated through Ca<sup>2+</sup> dependent signaling mechanisms(2, 4, 64). Few studies have shown that UIIR signaling is Ca<sup>2+</sup> dependent and that stimulation of P38 phosphorylation requires CaMKK (33, 65). In this present study CaMKK has been considered as a possible signaling mechanism that leads to UII-induced P38 phosphorylation. These results reveal that UII-induced phosphorylation of P38 can be interrupted or blocked by Pac1 cells exposure to the CaMKK inhibitor (**Figure 3A**). Since ATF2

phosphorylation has been reported to be directly mediated by P38(22, 36, 66), CaMKK inhibitor studies were performed in the presence of and the absence of UII, and the phosphorylation status of ATF2 was evaluated. These results demonstrate that UII- induced ATF2 phosphorylation is CaMKK dependent (**Figure 3B**). The results also reveal that CaMKK is involved in the UIIR signaling pathways that lead to the activation of P38 and ATF2.

Signaling mechanisms that cause the release of  $\text{Ca}^{2+}$  are maintained in cellular/mechanical stress or hypertrophic states (1, 3, 31). Transcription factors SRF, MEF2, and GATA are recognized as transcription factors involved in hypertrophy associated gene transcription (25, 43). SM- $\alpha$  Actin, SM-MHC, SM-22  $\alpha$  are SMC marker genes that have also been reported to be directly correlated with hypertrophic gene expression (43, 50). Thus, the link between  $\text{Ca}^{2+}$  and CaMKK's with UII induced expression of SMC marker genes and associated transcription factors are suggested in previous studies (43), although not directly measured in response to UII or in VSM cells. To our knowledge our laboratory is the first to identify that UII-induced expression of these transcription factors and SMC marker genes can be blocked with CaMKK inhibitor treatment (**Figure 4**).

P38 has been reported to control the activity of several genes through ATF2(21, 22, 36, 65). Therefore, since several studies in non-VSM cell types have established that ATF2 activation is P38 dependent (65, 66), we set out to confirm if ATF2 and P38 reports were consistent in Pac1 cells. A series of experiments were conducted, measuring UII-induced ATF2 phosphorylation using the P38 inhibitor. The ATF2 phosphorylation status was evaluated with P38 activated and P38 blocked.

These results in Pac1 cells established that UII-induced ATF phosphorylation is P38 dependent (**Figure 5**). Therefore, our results were consistent with previous reports; that the signaling pathways that lead to ATF2 phosphorylation require P38 activation.

The local environment plays an important role in phenotype switch, which often involves changes in either mechanical or cellular stress, and depends on activated P38 MAPK (3, 46). In response to vascular injury or stress, these environmental cues are disrupted and VSM cell undergo profound phenotypic changes either through the suppression or expression of SMC marker genes(43, 46). Since cellular and mechanical stresses are distinctly linked to P38(3, 21, 40), then a subset of SMC specific gene expression could depend on activated P38. These observations provided the rational for measuring UII-induced SMC gene expression in the presence or absence of a P38 inhibitor. The gene expression via reporter activity of transcription factors and SMC marker genes, were tested in response to UII induction and P38 inhibition in this study.

We tested all of the transcription factors and SMC marker genes in this study, although these results identified SRF (**Figure 6A**) and SM- $\alpha$ -actin (**Figure 6B**) and SM-MHC (**Figure 6C**) as the only UII-activated reporters that were blocked in the presence of the P38 inhibitor (**Figure 6**). UII- induced MEF2 expression was not found to be P38 dependent, which is consistent with the literature (40). Previous studies in SMC have revealed that activated P38 directly induces MEF2 activity (40). These findings were not observed in our studies possibly because of our use of a serum dependent cell line that does not adequately represent the findings seen in primary cells. Taken together, these results demonstrate that UII-induced activation of SRF, SM- $\alpha$  actin, and SM-MHC are P38 dependent.

In summary we demonstrate that UII induces P38 and ATF2 phosphorylation, and this phosphorylation is both CaMKK and P38 dependent in Pac1 cells. Furthermore, we show that UII induces the increased expression of transcription factors (SRF, MEF2, & GATA) and SMC marker genes (SM- $\alpha$  actin, SM-MHC, & SM-22  $\alpha$ ). The UII-induced expression of SMC marker genes and transcription factors are CaMKK dependent. We also report that the UII-induced expression of the transcription factor SRF and SMC marker gene SM-MHC and SM- $\alpha$  actin are P38 dependent.

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#### FIGURE LEGENDS:

**FIGURE 1: UII induces the time dependent activation of stress activated kinases.** Western blot data demonstrates that UII induces the time dependent activation of P38 in Pac1 cells (**Figure 1A**). The phosphorylation of ATF2 was measured in response to UII treatment of Pac1 cells and observed through Immunoblot analysis using the ATF2 phospho-specific antibody (**Figure 1B**).

**FIGURE 2: UII induces time dependent expression of hypertrophy associated transcription factors and SMC marker genes.** Pac1 cells transiently transfected with enhancer and promoter reporter constructs were treated with UII in a time dependent manner. Enhancer reporter assays demonstrate that UII induces the time dependent increase in GATA (**Figure 2A**), SRF (**Figure 2B**), and MEF2 (**Figure 2C**) reporter activation. Promoter reporter assays demonstrate that UII induces a time dependent increase in SM- $\alpha$  Actin (**Figure 2D**), SM-MHC (**Figure 2E**), and SM-22  $\alpha$  (**Figure 2F**). (n = 4) \*  $p \leq 0.05$

**FIGURE 3: UII-induced activation of stress activated kinases are CaMKK dependent.** Western blot analysis was performed in the presence and absence of UII and the STO609 (CaMKK inhibitor). Phospho-specific antibodies to P38 and ATF2 were used to measure the phosphorylation status. CaMKK inhibitor blocks the UII-induced phosphorylation of P38 MAPK (**Figure 3A**). STO609 blocks the UII-induced phosphorylation of ATF2 (**Figure 3B**).

**FIGURE 4: UII-induced expression of stress associated transcription factors and SMC marker genes are CaMKK dependent.** Vectors containing SRF, MEF2, and GATA were transfected in Pac1 cells. 24 hours later cells were treated with UII in the presence and absence of CaMKK inhibitor (STO609). UII-induces the expression of stress associated transcription factors SRF (**Figure 4A1**), MEF (**Figure 4B1**), and GATA (**Figure 4C1**). A second set of reporter assays compared reporter activity of TGF-beta (positive control) to UII. UII induces the expression of transcription factors SRF (**Figure 4A2**), MEF2 (**Figure 4B2**), and GATA (**Figure 4C2**) when compared to TGF-Beta induced expression.

Transfected Pac1 cells with luciferase vectors containing the promoter sequences of SM- $\alpha$  actin, SM-MHC, and SM-22  $\alpha$  were treated subsequently (within 24hours) with UII in the presence and absence of STO609. UII induced expression of SMC marker genes; SM-MHC (**Figure 4D1**), SM- $\alpha$  actin (**Figure 4E1**), and SM-22  $\alpha$  (**Figure 4F1**) are CaMKK dependent. TGF-beta and UII stimulated cells were measured via promoter reporter assay, results demonstrate that UII-induces SM-MHC (**Figure 4D2**), SM- $\alpha$  actin (**Figure 4E2**), and SM-22  $\alpha$  (**Figure 4F2**) gene expression. (n = 4) \*  $p \leq 0.005$

**FIGURE 5: UII-induced phosphorylation of ATF2 is P38 dependent.** Pac1 cells treated in the presence and absence of UII and SB203580 were analyzed using the phospho-specific ATF2 antibody via western blot analysis. P38 inhibitor (SB203580) blocks the UII-induced phosphorylation of ATF2 (**Figure 5**).

**FIGURE 6: UII-induced expression of stress associated transcription factors and SMC marker genes are P38 dependent.** Transfected Pac1 cells with luciferase vectors containing the enhancer and promoter sequences of SRF, SM- $\alpha$  actin and SM-MHC were measured by

relative luminescence in turner units. P38 inhibitor (SB203580) blocks the UII-induced expression of SRF (**Figure 6A**), SM- $\alpha$  actin (**Figure 6B**), and SM-MHC (**Figure 6C**). (n = 4) \* p  $\leq 0.05$

FIGURE 1.

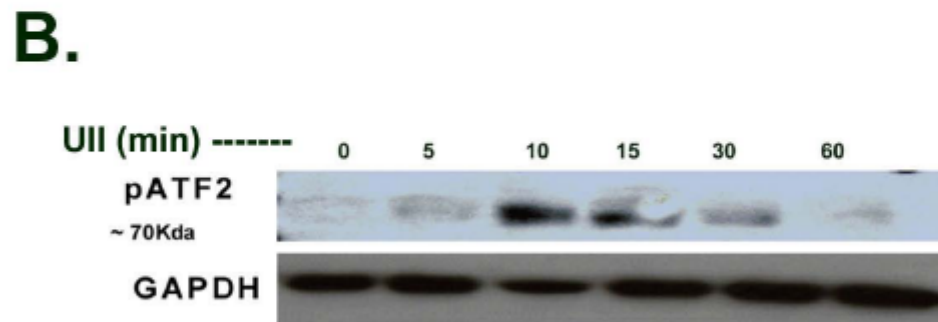
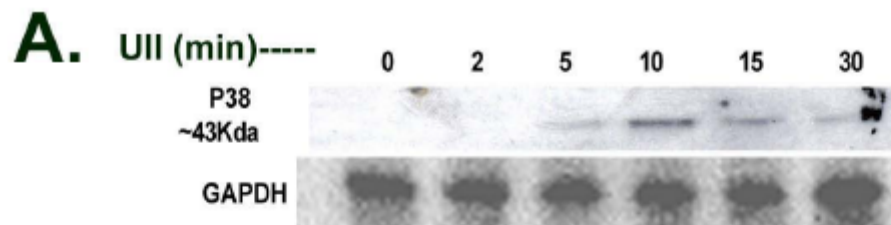


FIGURE 2.

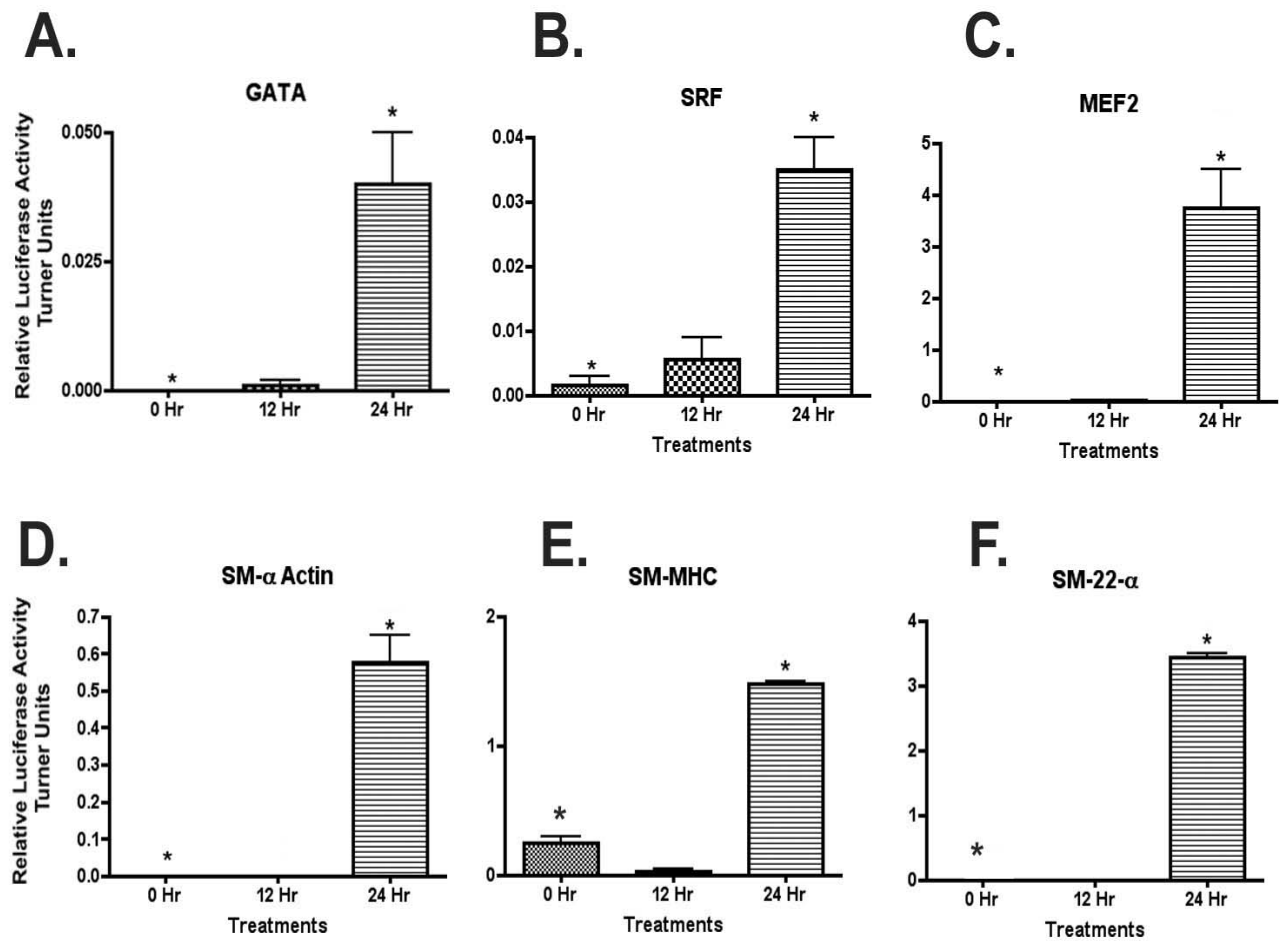
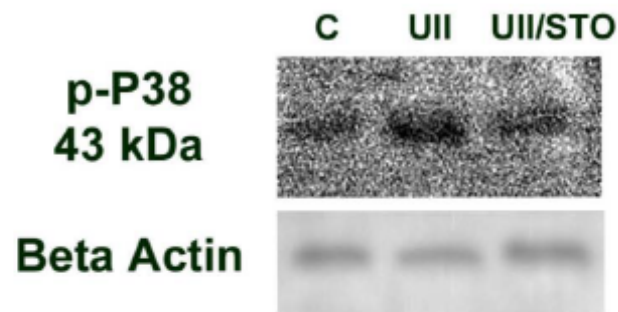


FIGURE 3.

**A.**



**B.**

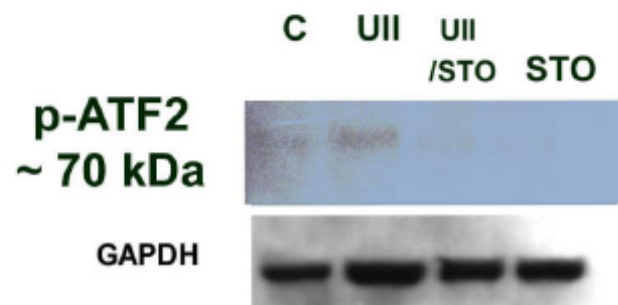




FIGURE 4.

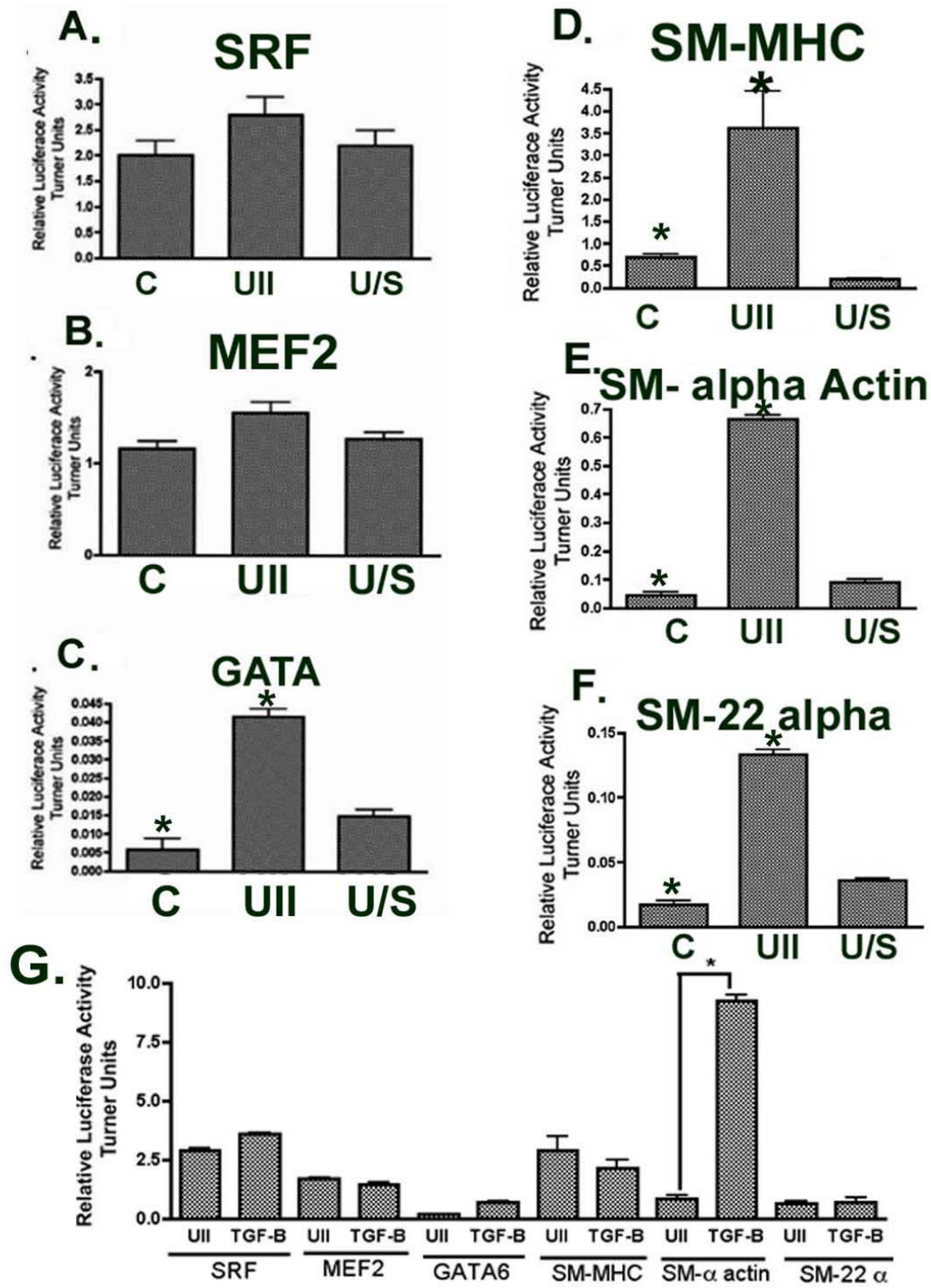
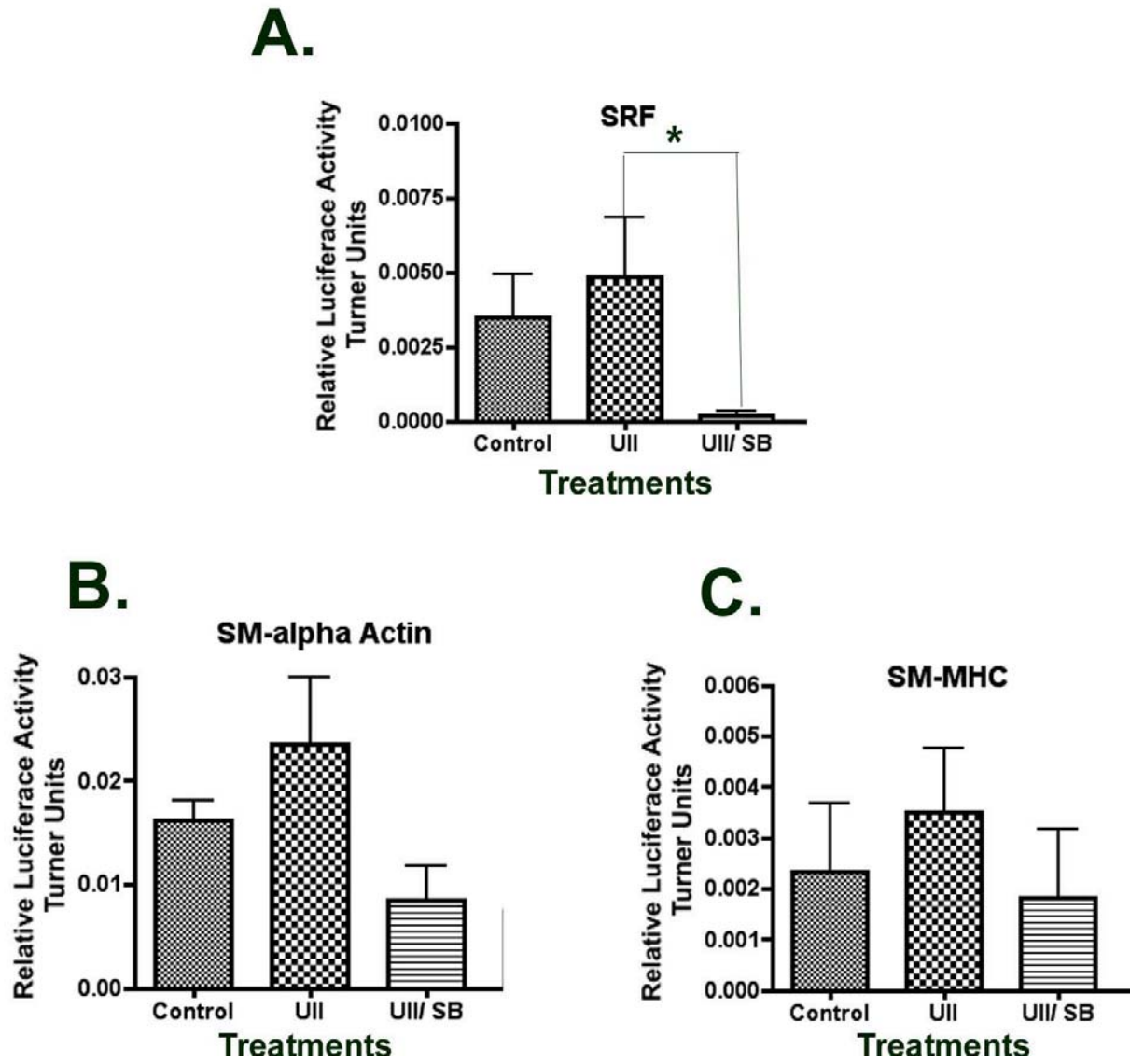


FIGURE 5.

**A.**



FIGURE 6.



## CHAPTER IV

### Angiotensin II Up-regulates Urotensin II Receptor Expression

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Process of Submission to

Biochemical Biophysical Research Communications (BBRC)

## ABSTRACT:

Vasoactive factors such as Angiotensin II (A2) and Urotensin II (UII) have important implications in various disease states pertaining vascular smooth muscle (VSM) cells. Insight into the nature of A2 receptor (ATR1) and UII receptor (UIIR) signaling may provide a target between the two signaling mechanisms under pathological conditions. Previous studies have not shown that A2 upregulates UII. We hypothesize that A2-induced up-regulation of UIIR in pulmonary arterial vascular smooth muscle (Pac1) cells is ERK dependent. RT-PCR and western blot studies identified that A2 up-regulates UIIR mRNA and protein levels. In Pac1 cells we demonstrate that ATR1 antagonist (losartan) and MEK inhibitors (PD98059 & U0126) blocked the A2-induced up-regulation of UIIR via immunoblot analysis. Therefore these results suggests that A2-induced up-regulation of UIIR is ERK dependent.

## INTRODUCTION:

Un-abated vascular proliferation following vascular injury, as in the case of arterial restenosis following angioplasty, often results in hypertrophy of VSM cells leading to both the stiffening and narrowing of the vessel wall (1, 2). Recent evidence implicated A2 and UII, in pathogenesis of a variety of diseases including hypertrophy and hypertension (3)(4). VSM cell proliferation is recognized as a key pathological event causing vascular hypertrophy (5). ERK has been identified as the primary MAP kinase involved in cellular proliferation (6). Cellular proliferation stimulated by A2-induced ERK phosphorylation has been shown in renal smooth muscle (SM) and VSM cells through Gq coupled signaling (7, 8)(9). Gq coupled signaling mechanisms lead to the activation of second messengers IP3, DAG, and more importantly  $\text{Ca}^{2+}$  (10, 11, 11). Increases in intracellular  $\text{Ca}^{2+}$ , and activation of calmodulin (CaM) dependent

kinases have been shown to induce the activation of ERK (12, 13). ATR1 is one such Gq coupled GPCR that induces the release of intracellular  $\text{Ca}^{2+}$  and has been shown to activate  $\text{Ca}^{2+}$  dependent kinases, such as CaMKII (14). A2 binding ATR1 in previous studies has been shown to induce synergism and the up-regulation of receptors, such as beta adrenergic receptors (15, 16).

Synergism studies have identified that A2 and UII elicit additive effects during vascular pathological conditions (17-19). UII binds to UIIR, which is also a Gq coupled GPCR that leads (17-19) to downstream signaling pathways resulting in vascular remodeling (20). Functional similarities between UII and A2 have been identified; such as both peptides are dual regulators of both the cardio-vascular system and vascular tone such as inducing vasoconstriction (18, 19, 21). To this date, the signaling mechanisms that link A2 and UIIR are not clearly understood.

These studies have identified that A2 treatment of Pac1 cells induced the elevated expression of UIIR mRNA and protein. These results demonstrate that A2-induced up-regulation of UIIR is ERK dependent. This study presents the identification of ERK as a target for blocking A2 induced up-regulation of UIIR, may provide potential pharmacological agents that could block the progression of vascular remodeling, fibrosis of blood vessels, cellular proliferation, and hypertrophy of the vascular system.

#### METHODS:

*Cell Culture- PAC1:* Established cell line, rat pulmonary arterial smooth muscle cells were cultured in 199 media (Invitrogen, Carlsbad, CA) supplemented with FBS (Atlanta Biologicals, Atlanta, GA) based on a previously described protocol (22) with gentamycin (Fisher, St. Louis,

MO). The cells were carried only through passages 3 to 15. At passage 15, Pac1 cells lose their normal phenotypic properties.

*Immunostaining:* Pac1 cells and primary aortic vascular smooth muscle cells were cultured on glass coverslips and were washed two times with 1x PBS and fixed for 15 minutes with paraformaldehyde at room temperature. Cells were blocked for 1 hour in block solution 3% BSA in 1x PBS, and then incubated for 1 hour primary antibody GPR14R IgG (Alpha Diagnostic Intl. Inc.) diluted 1:200 with blocking solution. Cells were then washed twice with 1x PBS. The secondary fluorescent labeling was incubated with cells using Alexa 488 goat/ anti-rabbit (Molecular Probes, Eugene, OR) diluted 1:1000 in blocking solution. The cells were washed three times with PBS. Using DAPI containing mounting solution, coverslips were mounted on slides with (Fluorosave – CalBioChem, Gibbstown, NJ). Visualization of fixed and mounted cells occurred using (LW Scientific Observer III – OB3MF) fluorescent microscopy with a 40X objective.

*Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):* Pac1 cell were seeded at a density of  $4 \times 10^4$  and cultured till cells reached 80% confluency in 100mm<sup>3</sup> dishes. Post-plating cells were serum reduced to 0.1% FBS + 199 media in gentamycin. After serum reduction cells were treated with A2 (Sigma), for 24hours. Total RNA was isolated using Trizol <sup>(r)</sup> reagent (Invitrogen) cDNA was synthesized using Super-Script <sup>TM-III</sup> (Invitrogen) as instructed by established protocol. Using specific gene –primers for UIIR [sense: 5'-CTGTGACTGAGCTGCCTGGTGAC-3' and antisense 5'-GGTGGCTATGATGAAGGGAAT-3'] Loading was verified with GAPDH [sense 5'GTGTGAACGGATTTGGCCGTATGG-3' and antisense 5'-TCATACTTGGCAGGTTTCTCCAGG-3']. Standardization of total RNA was

quantified with a spectrophotometer. mRNA expression and analysis detected running a 1.5% agarose gel – northern blot with ethidium bromide and a UV lamp. Normalization relative to GAPDH was performed using Image J with the Digital blot photograph – in a TIF format. Samples of total RNA were stored in -80 degrees Celsius and cDNA (after reverse transcription) samples were stored in -20 degrees Celsius.

*Western Blot Analysis:* Pac1 cells were seeded at a density  $4 \times 10^4$  and cultured till cells were 80% confluent in 100mm<sup>3</sup> dishes. The day prior to treatment of the cells, the cells were serum reduced to 0.1% FBS in 199 media with gentamycin. 24 hours after serum reduction cells were treated with UII (Sigma Aldrich, St. Louis, MO) AngII (Sigma Aldrich– A9525), TGF- $\beta$  (Sigma Aldrich 59553), STO609 (CalbioChem – Ann Arbor, MI), PD98059 (Cayman Chem.), and UO126 (Sigma Aldrich- 572314946). Inhibitors were incubated 30 minutes prior to Gq –agonist treatments. Dose response with UII was performed using doses ranging from 0nm to 200 nm. Time course experiments involved the use of 100nM UII for time points ranging from 0 minutes to 60 minutes. Detection of the following phosphorylated proteins were analyzed using antibodies; p-ERK1/2 (Cell Signaling- 9101) and GPR14R (Antibody for Total UIIR – Alpha Diagnostic Intl. Inc.). Total protein levels were determined using Beta Actin (Santa Cruz Antibodies, Santa Cruz, CA) Cell lysates were collected using RIPA buffer and protease inhibitor cocktail (Sigma). Lysates were stored in -80 degrees Celsius.

*Inhibitor Studies:* Based on several preliminary studies we identified losartan was most effective at blocking the ATR1 receptor. 30 minutes losartan pretreatment for A2 induced ERK phosphorylation studies was the optimal treatment time used. Although for A2 induced UIIR up-regulation studies in the presence of losartan, Pac1 cells were treated with losartan and A2 for 24



hours. For the U0126 and PD98059 inhibitor studies Pac1 cells were treated with A2 and MEK inhibitors for 15 minutes and inhibition ERK phosphorylation was measured. MEK inhibitors U0126 and PD98059 were used in the presence of A2 for 24 hours to measure inhibition of UIIR up-regulation.

*Statistical Analysis:* RT-PCR densitometry values were normalized and evaluated relative to control. Densitometry, data was subjected to ANOVA (GraphPad – Prism, San Diego, CA) the t-test with pair-wise comparisons of the means was analyzed. Statistical significance was indicated with at least a  $p \leq 0.05$ .

## RESULTS:

***Pac1 exposure to A2 induces UIIR up-regulation:*** A2 has been shown in prior experiments to induce the up-regulation of other growth factors as well as, receptors of endogenous agents (15, 16). As a result, Pac1 cell up-regulation of A2 was tested. Previous studies observed synergism through A2 and ATR1 receptor signaling (17). These studies identified that A2 stimulation leads to increased expression and up regulation of beta-adrenergic receptors (15, 16). Although, to our knowledge few A2 studies have reported that A2-induces the up-regulation of other endogenous vaso-active factors or their receptors. Therefore, to determine if A2-induces an increase in UIIR expression, Pac1 cells were with 100 nM A2 and RT-PCR was performed using primers for UIIR. UIIR mRNA expression was measured in response to 24 hour A2 treatment. [These results in Figure 1A & 1B, demonstrate that A2 treatment increases the mRNA expression of UIIR.](#)

UIIR protein expression on the surface of the plasma membrane plays a more substantial role in signaling, compared to the simple discovery of increased UIIR mRNA expression.

Therefore, this prompted our laboratory to measure A2 up regulation of UIIR protein levels through western blot analysis and immuno-cytochemistry. Pac1 cells treated with A2 in a time and dose dependent manner were analyzed via immunoblot analysis and immuno-cytochemistry. Results in **Figure 1C & 1D** identified that A2 induces maximal UIIR up-regulation within 24 hours of Pac1 cells exposure to A2. Dose dependent studies in **Figure 1E & 1F** identified that A2-induces maximal UIIR up-regulation with 100 nM A2 treatment. The A2-induced up-regulation of UIIR is overall highlighted by the similarities that have been recognized between the two peptides and their signaling pathways (17, 19). Therefore, these results in **Figure 1** establish that A2-induces an increase in UIIR mRNA expression and up-regulates UIIR.

***A2-induces ERK phosphorylation in Pac1 cell:*** Recent ATR1 studies in cardio-myocyte cultures established that activation of ATR1 results in Gq-dependent ERK phosphorylation signaling mechanisms (8, 9, 23). These studies have identified ERK as a pertinent target in the ATR1 signaling pathway(9, 23). Therefore, in continuation of determining the signaling mechanisms involved in the A2-induced up regulation of UIIR, a series of western blot studies were conducted. Measuring A2 in a time and dose dependent manner, using a phospho-specific ERK antibody, A2-induced ERK phosphorylation, was observed. Results in **Figure 2B** show that A2-induces maximal ERK phosphorylation within 15 minutes. In dose dependent studies shown in **Figure 2A**, these results demonstrated that 100nM A2-induces the phosphorylation of ERK.

To determine if 100 nM A2 through ATR1 induced the phosphorylation of ERK, western blot analysis was performed using 10 uM of a specific ATR1 antagonist (losartan). **Figure 2C** reveals that losartan blocks A2-induced ERK phosphorylation. Therefore, these results in

**Figure 2** identify that A2-induced ERK phosphorylation occurs through an ATR1 signaling mechanism.

***ATR1 signaling up-regulates UIIR via ERK 1 /2 dependent mechanisms:*** Studies have suggested that A2, through ATR1 receptor signaling pathway, induces the up-regulation of growth factors such as TGF-beta and VEGF(15, 16). Therefore, to determine if UIIR up-regulation occurs through the ATR1 receptor, western blot studies were conducted using Pac1 cells. Through immunoblot analysis, we measured the UIIR up-regulation using the GPR-14R antibody and 100 nM A2 in the presence of 1 uM and 10 uM concentrations of Losartan. [These results in Figure 3A demonstrated that losartan blocks the A2-induced up regulation of UIIR.](#)

Recent observations as well as these findings have revealed that activated ERK is triggered by ATR1 signaling mechanisms (9, 18). These studies have prompted the analysis of ERK inhibition using MEK inhibitors that block the A2-induced up-regulation of UIIR. To identify if MEK inhibitors (1  $\mu$ M and 10  $\mu$ M- PD98059 and U0126) block the A2-induced up regulation of UIIR, western blot analysis was performed and UIIR protein levels were measured using GPR-14R antibody. [Results in Figure 3B reveal that 10  \$\mu\$ M PD98059 and 10  \$\mu\$ M U0126 blocks the A2-induced up-regulation of UIIR. The data in Figure 3C demonstrates that 10  \$\mu\$ M U0126 and 10 \$\mu\$ M PD98059 inhibits the A2-induced up-regulation of UIIR.](#) Taken together these results in **Figure 3** identify that A2-induced up-regulation of UIIR is ERK dependent.

## DISCUSSION:

A2 signaling through ATR1 plays an essential role in vascular remodeling during vascular pathology (24, 25). There are several recent observations as well as evidence reporting

that ERK regulates vascular remodeling and hypertrophy/ hypertension during vascular disease (4, 24). In this present study we demonstrate in gene expression and protein studies that A2 induces an up-regulation of UIIR. We reveal ERK kinases are involved in the ATR1 cascade that leads to the UIIR up-regulation.

We show that A2 with UIIR signaling may act synergistically by A2-induced up-regulation of UIIR. The recent prediction that A2 may interact with other endogenous vaso-active and vasopressor agents evolved from the identification of shared similarities and a possibility of vaso-active synergism that was identified between A2 and specific growth factors (19, 21, 26). We identified that A2 induces an increase in UIIR mRNA levels (Figure 1A & 1B). However, an increase in mRNA levels was not pertinent evidence for our signaling studies; therefore we measured UIIR protein levels. We show A2 in dose (Figure 1C & 1D) and time dependent (Figure 1E & 1F) studies induces UIIR. Therefore Pac1 exposure to A2 up-regulates UIIR.

In this present study we demonstrate that A2 induces ERK phosphorylation in Pac1 cells. It has been well established that A2 through the ATR1 signaling mechanisms activates ERK by a discrete G-protein-dependent pathway. Here we demonstrate that A2 induces ERK phosphorylation in a dose and time dependent manner (Figure 2A & 2B) in Pac1 cells. Previous studies have identified that A2-induced ERK phosphorylation occurs directly via  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$ /CaM dependent kinases such as CaMKII through Gq coupled signaling (27-30). In this present study we did not examine  $\text{Ca}^{2+}$ /CaM dependent kinase activation of ERK (27, 29-35), nor did we test if CaMKKII activates ERK phosphorylation through the ATR1 signaling pathway. Moreover the signaling mechanisms have not been completely delineated, but in this

present study we did identify that ERK phosphorylation is triggered through A2 activation of ATR1. This was demonstrated with the use of the ATR1 antagonist in the presence of A2, which blocked A2-induced ERK phosphorylation (Figure 2C). These observations are consistent with the previous findings which established that A2 stimulation of ATR1 induces ERK phosphorylation.

Despite the increasing awareness that the mechanisms by which UIIR is up-regulated is crucial for understanding Gq coupling and signaling synergism (17, 19, 21), little evidence has emerged identifying up-regulation of other receptors nor the signaling mechanisms. Having demonstrated that A2 induces UIIR up-regulation, we also used an ATR1 inhibitor (Losartan) to confirm that this up-regulation is occurring through a seven trans-membrane Gq coupled receptor. Our results show that A2 through the ATR1 receptor up-regulates UIIR (Figure 3A). These observations that A2 induces ERK phosphorylation via the ATR1 pathway, suggests that ERK is a pertinent target in the Gq signaling mechanism that up-regulates UIIR. We also demonstrated that A2 induces UIIR up-regulation in an ERK dependent manner, using MEK inhibitors U0126 and PD98059 (Figure 3B & 3C). In these studies, we reported that A2 stimulation of ATR1 signaling up-regulated UIIR through ERK dependent mechanisms.

In conclusion, the present study demonstrates that activation of ATR1 by A2 in Pac1 cells leads to UIIR up-regulation. The A2-induced up-regulation of UIIR is ERK dependent, and this contributes to synergism in hypertrophy/ hypertension and associated vascular pathologies. Identifying that ERK kinases inhibition in the AT1R signaling pathway blocks the Angiotensin II induced UIIR up-regulation highlights, as well as identifies, the importance of targeting synergistic signaling and proteins that promotes the progression of vascular pathology.

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## FIGURE LEGEND:

**Fig 1. A2 up-regulates UIIR in Pac1.** (A) RT-PCR data demonstrates mRNA levels (B) mRNA data normalized to loading control and graphically plotted. (\*  $P < 0.05$ ) (n = 2)(C) Immunoblot analysis and (D) Immunocytochemistry with 100 nM A2 treatment of Pac1 cells in a time dependent manner up-regulates UIIR. (E) Western blot analysis and (F) immunocytochemistry results demonstrate that 100 nM A2 treatment results in the time dependent up-regulation of UIIR. (UIIR – Urotensin II Receptor, AngII or A2 – Angiotensin II)

**Fig 2. A2 exposure to Pac1 cells induces ERK phosphorylation.** (A) Immunoblot analysis demonstrates that A2 treatment in Pac1 cells induces ERK phosphorylation in a dose dependent manner. (B) Time dependent exposure to A2 in Pac1 cells induces ERK phosphorylation, as shown through western blot analysis. (Figure 2C) Immunoblot data demonstrates the pre-treatment of (1  $\mu$ M & 10  $\mu$ M) losartan for 24 hours, (C) in the presence of 100 nM A2, blocks the phosphorylation of ERK in Pac1 cells. (AngII – Angiotensin II, ARB – Angiotensin II Receptor Blocker Losartan)

**Fig 3. ATR1 signaling up-regulates UIIR via ERK dependent mechanisms. (Figure 3A)** Pac1 cells treated with 100 nM A2 and (1  $\mu$ M & 10  $\mu$ M) losartan for 24 hours and inhibition of UIIR up-regulation was observed via immunoblot analysis. (A) Western blot analysis demonstrates that losartan blocks the up-regulation of UIIR. (Figure 3B) Pac1 cell 15 minute treatment with MEK inhibitors in the presence of A2 (1  $\mu$ M & 10  $\mu$ M) U0126 and (10  $\mu$ M) PD98059 were examined using western blot analysis using a phosphor-specific ERK antibody. (B) Immunoblot analysis reveals that MEK inhibitors block A2 induced ERK phosphorylation.

**(Figure 3C)** Pac1 cell 24 hour exposure to (10  $\mu$ M) PD98059 in the presence of A2 were analyzed through western blot using the GPR14R antibody. **(C)** Immunoblot analysis shows that MEK inhibitors block the A2 induce up-regulation of UIIR. (AngII – Angiotensin II, UIIR – Urotensin II Receptor, ARB – Angiotensin II Receptor Blocker Losartan, U0126 & PD98059 – MEK inhibitors)

FIGURE 1.

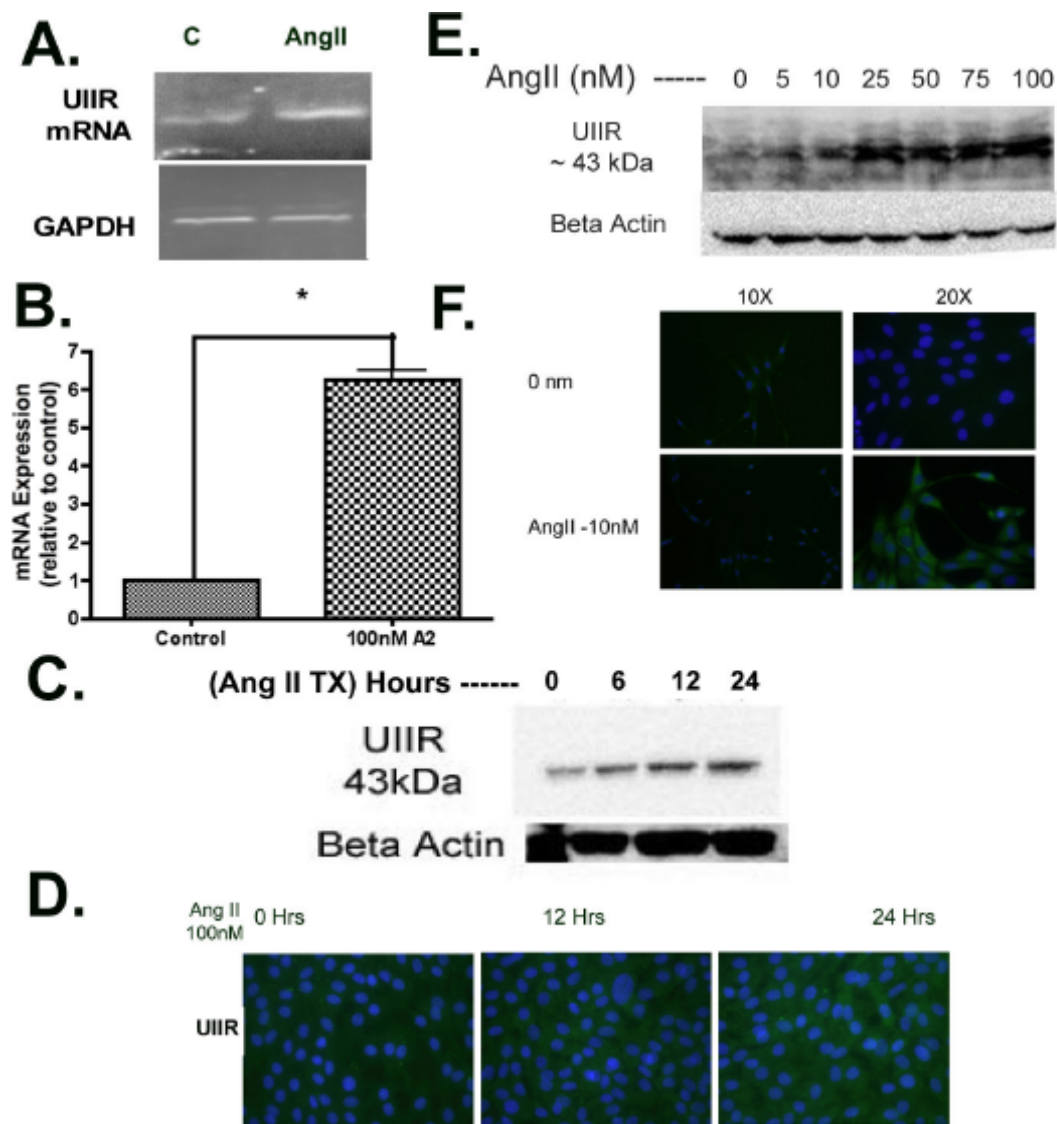


FIGURE 2.

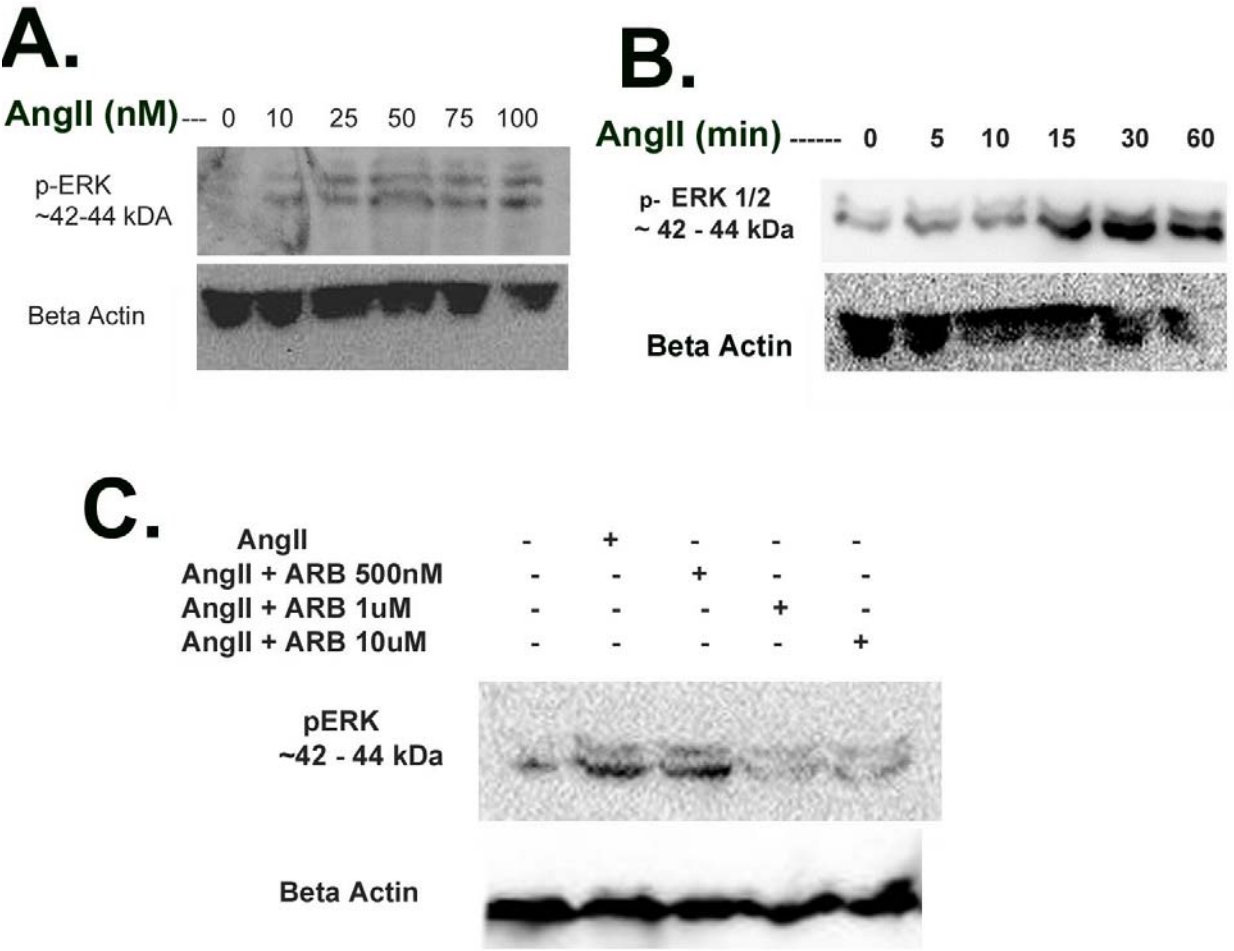
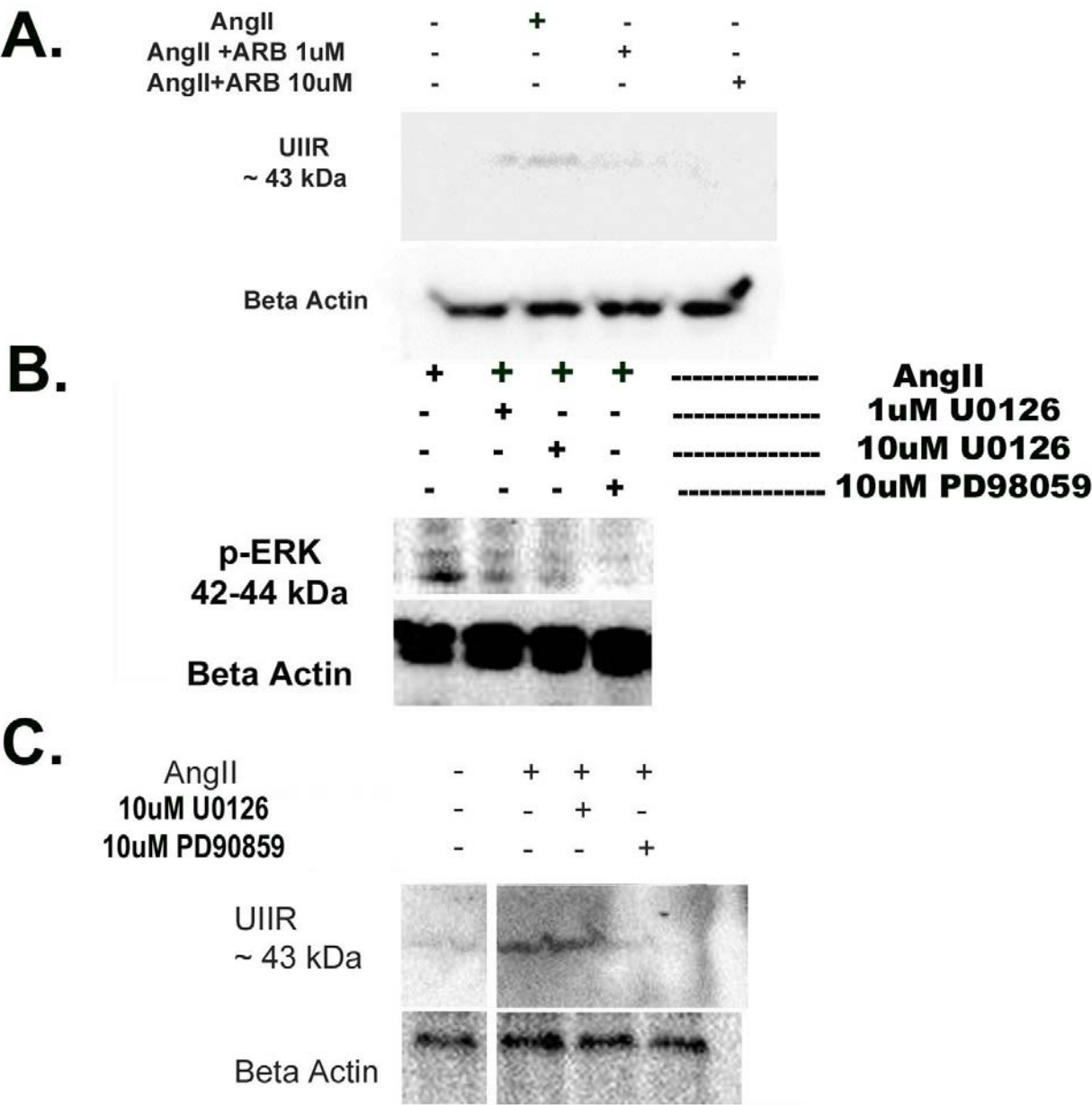


FIGURE 3.



## CHAPTER V

### CONCLUSION & FUTURE DIRECTIONS:

In the present study, the data presented demonstrates that UII exposure to cells triggers UIIR signaling which leads to the activation of  $\text{Ca}^{2+}$  / CaM dependent kinases & stress activated kinases. These studies also reveal that UII stimulates gene expression of transcription factors, and initiates new gene induction, all of which play an important role in VSM cell proliferation and hypertrophy. Recent work from our lab in cardiac pathogenesis demonstrated that UIIR coupled Gq signaling in Pac1 cells activates both  $\text{Ca}^{2+}$  dependent kinases and stress-activated kinases. The present study revealed that UIIR signaling in VSM cells results in the immediate activation and phosphorylation of  $\text{Ca}^{2+}$  dependent kinases. This data demonstrates that UII-induced activation of  $\text{Ca}^{2+}$  dependent kinases subsequently results in cellular proliferation and stimulation of SMC specific differentiation genetic markers. Using a combination of pharmacological inhibitors in the presence and absence of UII treatment, we have revealed that UII treatment induces an increase in CaMKK/ CaMKI phosphorylation. These results demonstrate that UIIR signaling in Pac1 and PAVSMC cells activates CaMKK/ CaMKI as a primary downstream target. The data presented in this study identifies that UII-induces the phosphorylation of PKD, ERK, P38, and ATF2. Using inhibitors of CaMKK in the presence and absence of UII, this data suggest that UII-induced PKD, ERK, P38, and ATF2 are CaMKK dependent. Western blot analysis using the inhibitor to CaMKK, imply that CaMKK is pertinent target for blocking UII-induced downstream phosphorylation of PKD, ERK, P38, and ATF2. This data reveals that CaMKK/ CaMKI plays a direct role in the initiation of cellular

proliferation, stimulating stress activated kinases, and leads to the induction of specific transcription factors that modulate new gene expression. We have also demonstrated that A2 induces the up-regulation of UIIR, and that UIIR up-regulation occurs in an ERK dependent manner leading to VSM cell hypertrophy. Determining that A2 induces UIIR up-regulation was a pertinent finding, although identifying the signaling mechanisms by which A2 induces this up-regulation will become a focus for future studies. In addition to identifying the direct ATR1 and UIIR signaling mechanisms, the use of a two transgenic (Tg) mouse models will be incorporated, with one being a transgenic mouse that over-expresses UIIR and the other being dominant negative ERK 1 / 2 mouse. Generating and using Tg mice models, and developing studies in in-vivo systems, will help elucidate the role of UII/ UIIR signaling mechanisms. Also, this will assist in identifying the role of UIIR downstream targets in vascular pathology.

***Dominant Negative ERK 1/ 2 Transgenic Mice:*** One way of determining the possible A2-induced signaling mechanisms that lead to UIIR up-regulation, was by identifying the pertinent signaling molecules in the ATR1 signaling pathway. ERK 1/ 2 is one pertinent MAP kinase that has been linked to the up-regulation of several proteins and new gene induction in the ATR1 signaling pathway.

Therefore, in the present study we used MEK inhibitors to argue that ERK 1/ 2 was one of the kinases involved in the A2-induced UIIR up-regulation. Although, ruling out the involvement of other MAP kinases is not possible unless our studies can specifically target ERK 1/ 2 in an in-vivo study, using an in-vivo system genetically targeting ERK 1/ 2 would determine its role of in the A2-induced up-regulation of UIIR. The in-vivo model would involve the use of a dominant negative ERK 1/ 2 transgenic mouse. In these future studies, a conditional transgene

vector containing a floxed transcriptional and translational “stop” cassette would be inserted downstream of a smooth muscle specific promoter (SM- $\alpha$ -actin). A dominant neg (dn)- ERK 1/2 cDNA bearing a mutation at K97M (pMCL-dn ERK 1/2 – obtained from N. Ahn) will be used. Smooth muscle specific promoter (SM- $\alpha$ -actin) will be PCR amplified, and inserted in the eukaryotic expression vector in front of the cDNA containing K97M in the ERK coding sequence. The expression vector with the dominant negative gene will result in the expression of a non-functional mutated ERK 1/2 utilized in Tg mice. Tg- dn- ERK 1/2 mice will be generated at UT-Southwestern, using the expression vector carrying the dn-ERK 1/2. Using the Tg mouse, UIIR expression levels will be compared in response to A2 treatment. Animal studies will be conducted measuring the A2 and UII induced proliferation in both the ATR1 and UIIR pathways. The incidence of hypertrophy, or the progression of hypertension, will be analyzed in these dn-ERK1/2 -Tg mice. Various hemodynamic measurements will be conducted along with immunohistochemistry analysis of the vascular tissue. Overall, these studies using dn-ERK 1/2-Tg mice will delineate and identify the importance of ERK in both the ATR1 and UIIR signaling pathway.

***CaMKI or CaMKII using Phospho-specific Antibodies:*** The focus of this present study was to identify the proteins involved in the ATR1 pathway that lead to up-regulation of UIIR. The ATR1 signaling pathways involve Gq coupled signaling that activates a few important second messengers including  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  signaling has been linked to the up-regulation of several proteins and new gene induction. In this study we identified that ERK kinase is involved in the A2- triggered UIIR up-regulation pathway, although we did not identify the direct signaling mechanisms that lead to ERK activation and subsequent gene modulation resulting in UIIR up-regulation. Many studies identified various kinases which phosphorylate ERK through a Gq



coupled pathway. The kinases reported to stimulate ERK activation include: Ras/ Raf signaling (known as the traditional pathway), GPCR signaling through DAG and PKC, or GPCR signaling through  $\text{Ca}^{2+}$  /CaM dependent kinases (CaMKI or CaMKII). ERK is commonly recognized as a  $\text{Ca}^{2+}$  dependent kinase. Some studies have shown that ERK requires the activation of  $\text{Ca}^{2+}$  / CaM dependent kinases for the stimulation and phosphorylation of ERK. Many VSM cell and cardiomyocyte studies have acknowledged that CaMKII is the downstream CaM dependent kinase which is activated in response to ATR1 stimulation. Although, studies conducted in our laboratory in cardiomyocytes suggest that CaMKK and CaMKI are activated by A2 binding ATR1. Using both the phospho-specific CaMKI antibody and CaMKII antibody, we will identify which CaM kinases are activated upon A2 treatment. A series of western blots with A2 treatment in a time and dose dependent manner will identify if A2 induces the phosphorylation of CaMKI or CaMKII. However it is also possible that both CaM kinases will be activated. Therefore if this situation occurs, the emphasis will then be to determine which CaM kinase is involved with the A2-induced up-regulation of UIIR. Thus, defining the  $\text{Ca}^{2+}$ / CaM dependent kinases involved in the A2-induced up-regulation of UIIR could provide insight to a possible mechanism of therapeutically targeting kinase activation using pharmacological inhibitors.

***Transcription Factors involved in New Gene Induction via UIIR Promoter Studies:*** In the “A2 induced up-regulation of UIIR” study we identified that A2 induces the up-regulation of UIIR via ERK. In western blot and RT-PCR studies we determined that A2- induces an increase in UIIR expression. The data presented here also reveals that A2-induces ERK dependent UIIR up-regulation, although the signaling mechanism that leads to UIIR gene expression has not been completely elucidated. Transcription factors play an important role in determining the expression of genes, such as UIIR. Delineating the transcription factors that regulate UIIR

expression, either through gene activation or repression, would be important for understanding the signaling mechanisms that lead to UIIR up-regulation. Bio-informatic analysis of the promoter region, and the identifying DNA sequences bound by transcription factors that regulate gene expression could provide insight. Elucidating the transcription factors involved in the signaling of UIIR up-regulation may enhance the general understanding of the signaling mechanisms that occur during vascular hypertrophy. Up-regulation of key proteins requires the detection of pertinent transcription factors at the promoter region of the gene of interest. The binding of transcription factors to the promoter sequences mediate gene expression at a transcriptional level. Up-regulation of UIIR requires the activation of transcription factors that interact with the promoter region of the UIIR gene, which have to date not been identified. Therefore, future studies should include promoter analysis of the 5' region of UIIR and establishing the key transcription factors that mediate the A2- induced up-regulation of UIIR. Delineating transcription factors that mediate A2-induced UIIR up-regulation could help identify therapeutic targets to halt A2/ UIIR synergism during vascular pathological conditions.

***Studies in Primary Cells:*** These studies identifying that “A2-induces UIIR up-regulation” and “UII induces stress activated signaling” were performed in Pac1 cells. Pac1 cells do not accurately represent the characteristics observed in primary cells. Pac1 cells are rapidly dividing cells that are serum dependent. The presence of serum in cultured media induces Pac1 cells to proliferate and grow. Therefore, Pac1 cells are not ideal for identifying proteins involved in the A2 and UII signaling pathways. The use of primary cells was used in our “UII-induced proliferation” study, and was identified as a more suitable model for understanding the proteins involved in UIIR signaling. Future studies will involve delineating the overall signaling mechanisms involved in ATR1 and UIIR up-regulation in primary cells. Using primary cells as a

model would make this data more convincing. The UII studies identifying the kinases involved and new gene induction should be conducted in primary cells, as well. Using primary cells to perform UII induced kinase activation would be optimal because Pac1 cells are rapidly dividing cells, and identified as truly differentiated cells already in the contractile state. Pac1 do not accurately represent the characteristics observed in normal VSM cells. Thus, the incorporation of primary cells to identify UIIR signaling mechanisms would theoretically produce more convincing data.

***UIIR antagonist:*** In our studies measuring “UII-induced Proliferation” we measured the signaling mechanisms that lead to cellular proliferation. Inhibitors to various kinases were used to block downstream signaling events. Proliferation was measured in the presence of CaMKK, MEK, and PKC inhibitors. These chemical agents all blocked UII-induced cellular proliferation. However, in addition to the experiments conducted, many aspects of this study have not been completely delineated. Our studies indirectly identify that  $\text{Ca}^{2+}$  / CaM kinases are target kinases in the UII-UIIR pathway which lead to cellular proliferation. Future studies are needed to elucidate UIIR signaling mechanisms using a UIIR- specific antagonist. Utilizing a UIIR antagonist will allow us to determine if CaMKI, ERK, and PKD phosphorylation occurs directly through the UIIR signaling pathway and is not a product of crosstalk. The UIIR antagonist may also indicate if cellular proliferation is occurring through the UIIR signaling pathway, and not via UII binding non-specifically to other receptors expressed in VSM cells. These studies will determine the direct involvement of  $\text{Ca}^{2+}$  / CaM kinases in the UII-induced proliferation pathways of VSM cells.

In the experiments conducted in “UII-induces Stress activated Signaling” manuscript we measured P38, ATF2 phosphorylation, and new gene induction. Our results demonstrated that UII-exposure to Pac1 cells induced stress activated pathways, which were revealed in phosphorylation studies of P38 and ATF2. Using the UIIR antagonist would also be useful in the “UII-induces stress activated signaling” manuscript, because these results did not clearly demonstrate that the UII-induction was through the UIIR signaling pathway. Therefore, the use of a UIIR antagonist would determine if this were occurring through UIIR signaling pathway. New gene induction will be measured by UII exposure to Pac1 cells transfected with promoter reporters in the presence and absence of UIIR antagonist. Relative luminescence will be recorded as a measurement of UII-induced gene transcription. This data will demonstrate that UII-induces SMC marker gene expression, and with the use of the UIIR antagonist we will delineate if UII induction is occurring through the UIIR signaling pathway. Therefore, the use of a UIIR antagonist would identify if UIIR signaling results in (P38/ ATF2/ PKD/ ERK/ & CaMKI) phosphorylation, cellular proliferation, and new gene expression, or if this was an indirect result of crosstalk.

***UIIR Expression Vector:*** In the “UII-induced cellular proliferation” and the “UII-induced stress activated signaling” manuscripts, kinase activation, proliferation, and new gene induction were revealed. Map kinases such as CaMKI, ERK, PKD, P38 and ATF2 were identified as the kinases that were phosphorylated upon UII exposure. Although over-expression of UIIR in a Tg- mouse would identify the effects of elevated UIIR expression, neither Pac1 cell lines nor primary cells (PAVSMC) accurately represent the conditions seen in-vivo. Therefore, to strengthen the results from our studies conducted in cells, animal studies must also be used. Currently, only a UIIR knockout animal exists. Even through, studies incorporating animal models, such as a transgenic

mouse model that over-expresses UIIR cDNA, could be a useful tool for understanding UIIR signaling in an in-vivo system. A Tg model over-expressing UIIR has not been generated yet. Though, using a conditional transgene eukaryotic expression vector pCLSL, that contains a floxed transcriptional and translational “stop” cassette inserted downstream of the promoter sequence could be generated. The promoter sequences will consist of a smooth muscle specific promoter (SM- $\alpha$  actin), which will be up-stream of the UIIR cDNA in the eukaryotic expression vector. The SM- $\alpha$  actin promoter to the UIIR expression vector will provide tissue specificity, so that UIIR is only overexpressed in differentiated VSM cells. The expression vector carrying UIIR cDNA will result in over-expression of the UIIR, which will then determine the role of UIIR overexpression in an animal model. Immunohistochemistry studies will be performed identifying the effects of UIIR overexpression in vascular tissue. Elucidating the role that UIIR plays in hypertrophy and the progression of hypertension will be the primary goal of these animal studies.

In conclusion, data presented in this dissertation clearly demonstrates a pertinent role for A2 and UII. Observed data shows that A2 induces the up-regulation of UIIR. These studies have identified key  $\text{Ca}^{2+}$ / CaM dependent protein kinases are involved in the UIIR signaling pathway. UII-induced cellular proliferation was measured in the presence and absence of  $\text{Ca}^{2+}$ / CaM dependent kinase activity. This work was the first to demonstrate UIIR signaling stimulates the activation of CaM-dependent protein kinases, cellular proliferation, as well as stress activated pathways. We also revealed that new gene induction of specific SMC marker genes and transcription factors were expressed in response to UII exposure to Pac1 cells. Taken together,

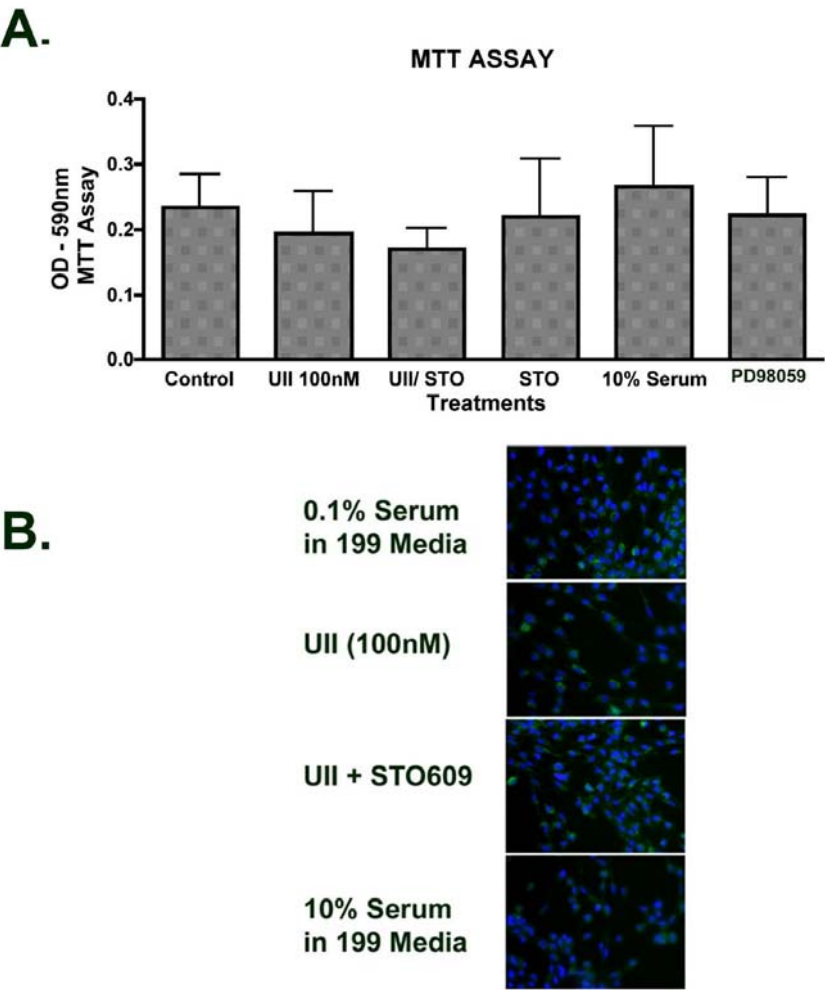
this data establishes, for the first time, the existence of a novel UIIR signaling pathway that induces VSM cell hypertrophy.

## CHAPTER V

## APPENDIX

**Appendix Figure 1. CaMKK and MEK Inhibitors Do Not Affect Cell Viability.** Pac 1 cells were cultured in 24 well plates and MTT assay was performed to measure cell viability. (A) Data graphically represented reveals that CaMKK and MEK inhibitors do not alter cell viability. (B) Cells were cultured on coverslips in media containing 0.2% FBS media containing 100 nM UII in the presence and absence of 250 nM CaMKK and 10  $\mu$ M MEK inhibitors. Following treatment, calcien staining was visualized and captured using the Nikon fluorescent microscope.

APPENDIX FIGURE 1.



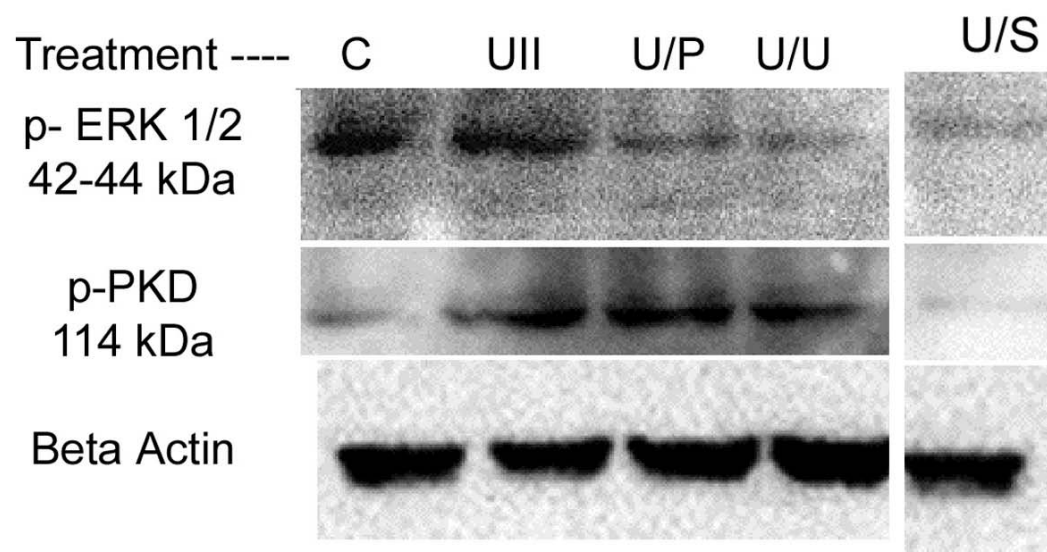
**Appendix Figure 2. Inhibitors which block UII-Induced phosphorylation of ERK and PKD.**

**in Pac1 cells.** Recent studies have identified that DAG and PKC kinase activation are involved in PKD and ERK phosphorylation. Therefore to delineate which signaling pathways are involved in the phosphorylation of ERK and PKD various inhibitors such as 10  $\mu$ M MEK inhibitors (PD90859 & UO126), PKC Inhibitor (Chelentrine Chloride), and 250 nM CaMKK inhibitor (STO609) were exposed to Pac1 cells in the presence and absence of 100 nM UII. The UII-induced phosphorylation status of ERK and PKD were measured in the presence of the inhibitors via western blot analysis.



APPENDIX FIGURE 2.

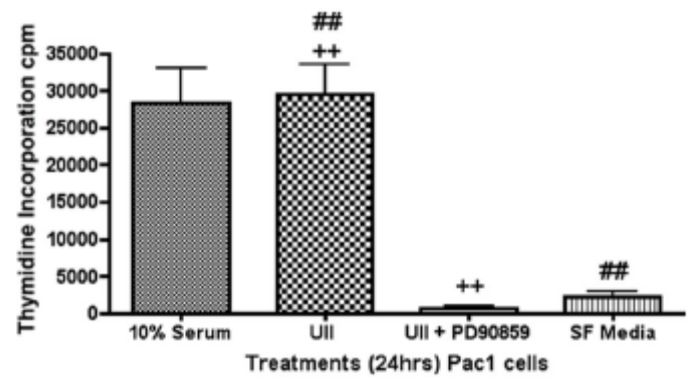
**A.**



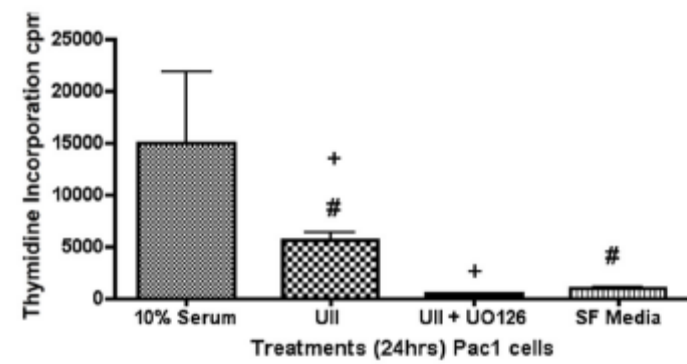
**Appendix Figure 3. CaMKK, MEK, and PKC inhibitors all block UII-induced cellular proliferation.** Studies suggest that PKC is the primary kinase that stimulates ERK and PKD phosphorylation and subsequently leads to cellular proliferation. ERK phosphorylation depends on MEK activation. In order to test these findings, PKC, and 10 $\mu$ M MEK, inhibitors were used in the presence of UII and were compared to 250 nM CaMKK inhibition through  $^3$ H –thymidine incorporation. We found that STO609 in the presence of UII reduced cellular proliferation as compared to MEK and PKC inhibitors in the presence of UII.

APPENDIX FIGURE 3.

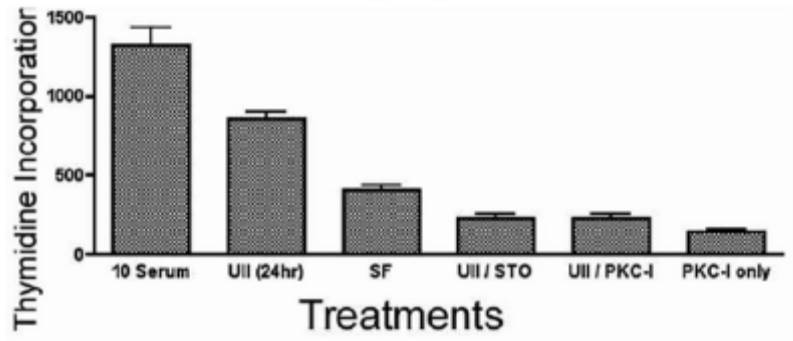
A.



B.



C.



## CHAPTER VI

### THE ECONOMIC BURDEN AND SOCIOECONOMIC IMPLICATIONS OF VASCULAR DISEASES

There is a greater prevalence of vascular disease in western countries compared to any other country in the world (1, 2). It has been estimated that vascular diseases such as hypertension, atherosclerosis, and restenosis due to angioplasty affect a quarter of the US population (2, 3). The actual number of people that are impacted by vascular disease such as hypertension may actually affect more people than what is reported (3). The fact that many of the vascular diseases are considered “silent killers” is a major factor for both the delayed or undiagnosed patients that are untreated (3). In general, later disease intervention results in prolonged progression of the diseases (3, 4).

Within the last decade there has been a decrease in the economic national budget for health care, which has indirectly affected the treatment of vascular disease conditions such as hypertension and atherosclerosis (5-7). The national health care budget, which provides funds for both Medicare and Medicaid programs, has recently suffered from monetary constraints, leading to the increased incidence of cardio-vascular morbidity and mortality (3, 5). The economic volatility that has overcome the nations economy has led to a heightened sense of distress for several patients diagnosed with cardio-vascular diseases (5).

The national economic burden for managing hypertension and its complications has drastically increased, due to the lack of prevention programs and the growing unhealthy culture

of today's society (7, 8). Few patients are able to receive treatment for their diseases; the lower socio-economic population bracket typically relies on emergency room (ER) visits as a source of medical intervention/ treatment (5, 7). Of the individuals that are economically privileged and have access to covered health care, it has been estimated that roughly half of these patients that receive treatment are unable to halt the progression of these vascular diseases after receiving treatment (7). This provides evidence that the current treatment strategies are not successful at preventing the progression of vascular diseases (7). The overall cost of current treatments for vascular diseases are increasing annually, from the high fees of procedures such as angioplasty for atherosclerotic plaques, to the cost of pharmacological treatment with anti-hypertensive medications (3, 9).

In the majority of western countries the cost of vascular diseases absorbs a large portion of the overall health budget (3). Treatment of vascular diseases is estimated to cost the US \$37.2 billion dollars a year, as shown in studies from 2003 (3). This is understandable considering that roughly 50 to 60 million people in the US seek treatment for various vascular diseases. This takes into account cost accruals with hospital expenditures, physician costs, surgery fees, and drug prices (7). Antihypertensive drugs alone cost the US roughly \$17.8 billion dollars annually (9). Obesity and poverty, due to poor diet, and lack of exercise, are two of the high-risk populations that are likely to suffer from vascular diseases (3, 7, 8). Government and insurance costs are depleting and more of the healthcare costs have become an individual burden rather than a government subsidized burden (3, 7, 8).

The medical, economic, and overall costs of untreated patients or inadequate control of vascular diseases such as hypertension in patients are enormous (5, 7, 8). Adequate management

of vascular disease and controlled blood pressure can be hampered by the inadequacies in delayed diagnosis, poor treatment options, and the rapid progression of the disease due to the lack of health care (3, 5, 7). Health care providers, medical providers, pharmaceutical companies, and scientific researchers face many obstacles in achieving and providing sufficient care for patients suffering from many types of vascular diseases (4). Currently the optimal treatments available involve multi-drug regimens. Although, multi-drug therapy results in a patient's reluctance to comply with therapy, mainly for the reason that taking several pills at various times in the day for a single disease condition, often proves to be more complicated for patients. Plus the costs for a multi-drug therapy are far greater than mono-therapy (9). Presently fewer drugs and treatments are available, along with limited funding for innovative research to develop novel medical approaches for treating the disease (5, 8). Moreover, the treatment and therapeutic strategies do not suffice (9). It is critical that research achieves a point in which pharmacological targets are identified and drugs are designed with the nations economic status and healthcare demands taken into consideration.

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