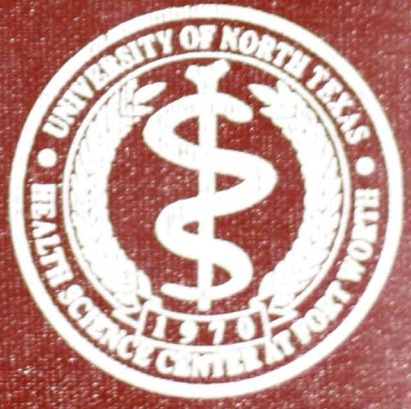
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Cardiac hypertrophy arises from various forms of physical stress that result in an increased workload and decreased cardiac output. Therefore, cardiac hypertrophy is the common compensatory mechanism employed by the heart to maintain a normal cardiac output. Gq-coupled receptors such as the angiotensin II receptor (AngIIR) and the endothelin-1 receptor (ET-1R) are capable of activating the CaMK and MAPK cascades and are involved in cardiac hypertrophy. Mechanical stress has been shown to result in the release of both AngII and ET-1 from the heart leading to an autocrine stimulation of myocyte hypertrophy. The Urotensin II receptor (UIIR) is coupled to Gq and is expressed in the healthy adult heart at low levels and becomes over-expressed under pathological conditions that lead to hypertrophy. UII is capable of inducing hypertrophy in cardiomyocytes only when sufficient receptor is expressed. In this study, the mechanism by which UII becomes expressed was examined as was the mechanisms through which UII induces hypertrophy of cardiomyocytes. Data described in this dissertation demonstrated that PE and AngII stimulation of cardiomyocytes results in the up-regulation of UIIR message and protein. UII was able to stimulate the promoter activity of ANF and SkA and the transcriptional activity of MEF2 in a CaMKK-dependent manner. UII stimulation of ANF, BNP, βMHC and SkA gene expression was dependent on CaMKK. UII stimulation caused the CaMKK-dependent activation of CaMKI.

CaMKI completely rescued UII stimulation of ANF and SkA promoter activities as well as MEF2 activity with CaMKK pharmacologically inhibited. We demonstrated that the UII-induced activation of p38 and ERK1/2 MAP kinases was dependent on CaMKK suggesting a novel cross-talk mechanism not previously described in cardiomyocytes. Both UII- and CaMKI-mediated induction of ANF, SkA and MEF2 reporter activities was dependent on p38 and ERK1/2. Taken together, these data identify CaMKK as a central mediator in Gq activation of hypertrophy by UII.

UROTENSIN II-MEDIATED CARDIAC

HYPERTROPHIC GENE INDUCTION

REQUIRES CAM KINASE KINASE

Thomas G. Valencia, M.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

University Member

Chair, Department of Biomedical Sciences

Dean, Graduate School of Biomedical Sciences

UROTENSIN II-MEDIATED CARDIAC HYPERTROPHIC GENE INDUCTION REQUIRES CAM KINASE KINASE.

DISSERTATION

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CHAPTER I

INTRODUCTION

Preface

Heart disease is the leading cause of mortality in western societies and is directly associated with poor diet and exercise as well as poor stress management. Cardiac hypertrophy is linked with a considerable increase in the risk of heart failure, dilated cardiomyopathy, ischemic heart disease, and sudden death, leading to increased incidence of mortality[1-5]. In the adult myocardium, cardiac hypertrophy results from various forms of physical stress (chronic hypertension, severe mechanical load, volume overload, myocardial stress from disease or stress from infarction or coronary insufficiency). These stresses to the heart nearly always result in an increased workload and decreased cardiac output. Therefore, cardiac hypertrophy is the common compensatory mechanism employed by the heart to maintain a normal cardiac output. The overall initial response of the heart is to increase ventricular mass to restore its pumping ability. Since cardiomyocytes in the adult heart no longer retain the ability to divide, the hypertrophic response is characterized by an increase in cardiomyocyte size rather than number. Eventually, chronic cardiac hypertrophy resulting from sustained myocardial stress leads from an initial compensatory mechanism to myocardial decompensation and failure. All

of these events arise from a modification of signal transduction mechanisms that results in changes of cardiomyocyte gene expression.

Recent work has led to substantial increases in our understanding of the molecular mechanisms underlying the hypertrophic response of the heart. Physiological cardiac hypertrophy results directly from athletic conditioning and is generally considered healthy. This type of hypertrophy does not result from altered myocyte protein content or changes in gene expression. Normal fetal and postnatal cardiac growth can also be described as hypertrophic, but is necessary for heart development. The fetal heart expresses a genetic program distinct from the adult heart; however, with pathological cardiac hypertrophy, there is a re-emergence of the fetal gene expression pattern. The switch from the adult gene expression pattern to the fetal pattern involves both the calcium/calmodulin-dependent protein kinases (CaMKs) as well as the mitogen activated protein kinases (MAPKs). Signaling from these two distinct kinase cascades converge on an important transcription factor named the myocyte enhancer factor 2 (MEF2). MEF2 is a critical factor in both heart development and cardiac hypertrophy.

G-protein coupled receptors (GPCRs) that couple to Gq, such as the angiotensin II receptor (AngIIR) and the endothelin-1 receptor (ET-1R) are capable of activating the CaMK and MAPK cascades and are involved in cardiac hypertrophy[6-9]. Mechanical stress has been shown to result in the release of both AngII and ET-1 from the heart leading to an autocrine stimulation of myocytes[10-15]. Moreover, AngII stimulates the expression of ET-1_BR in cardiomyocytes[16]. More important, signaling through Gq-coupled receptors have been implicated in the development of compensated cardiac

hypertrophy and ultimately, decompensated hypertrophy that leads to failure. Therefore, a major question that arises is: how does the initial adaptive hypertrophy response degrade toward maladaptive decompensated hypertrophy and failure? The answer to this question seems to be, in part, that Gq signaling events in the cardiomyocyte are the initial trigger for compensatory hypertrophy but as mechanical stresses persist, the increased Gq signaling events lead to changes characteristic of decompensated hypertrophy (Figure 1). Indeed, transgenic models of Gq over-expression have shown that moderate degrees of Gq signaling stimulate adaptive hypertrophy[17-19], whereas high degrees of Gq signaling result in maladaptive cardiomyocyte apoptosis[20-23].

The Urotensin II receptor (UIIR) is a recently de-orphanized GPCR that is coupled to Gq and is activated by its peptide ligand, Urotensin II (UII). UII and its receptor are expressed in the healthy adult heart at low levels and become over-expressed under pathological conditions that lead to hypertrophy [39, 137, 141, 149]. By itself, UII is capable of inducing the hypertrophic phenotype in cultured cardiomyocytes only when sufficient receptor is expressed[78].

Because UIIR is a newly discovered Gq coupled receptor, many questions still remain concerning UIIR expression during cardiac disease states as well as its role in downstream signaling mechanisms that contribute to the hypertrophic phenotype. Therefore, the focus of this dissertation is to i) identify whether known hypertrophic agonists lead to expression of UII in cardiomyocytes and ii) further delineate the signaling mechanisms through which UII induces hypertrophic gene expression. The section that follows will describe our current knowledge of cardiac hypertrophy and will

lay the foundation for the studies conducted in this dissertation.

The Cardiomyocyte

The adult mammalian heart consists of several cell types, of which, the cardiomyocyte is the most prominent. Normal cardiomyocytes express a genetic program that enables them to function as a contractile cell. This program includes the expression of all of the proteins necessary to form a working contractile apparatus in the sarcomere. Adult cardiomyocytes are multi-nucleated and are rich in mitochondria that are necessary to meet the high-energy demands of the heart. Neighboring cardiomyocytes fuse together to form a functional syncytium thereby allowing information to flow freely from cell to cell. In a practical sense, the syncytium facilitates the beat-to-beat coordination of the cardiomyocytes.

Adult cardiomyocytes are terminally differentiated; therefore, they have lost the ability to enter mitosis. As such, any post-natal growth of the heart is due to an increase in the size of individual cardiomyocytes rather than an increase in total cell number[24]. The increase in the size of cardiomyocytes is termed cardiomyocyte hypertrophy.

On the cardiomyocyte level, the hallmarks of hypertrophy are an increase in cell size, increased protein synthesis, and sarcomeric reorganization[25, 26]. The hypertrophic changes in cardiomyocyte phenotype are preceded by the re-expression of a fetal gene program in the left ventricle. Most notably, there is a shift in the expression from the adult α-myosin heavy chain (αMHC) to fetal β-myosin heavy chain (βMHC) and increased expression of skeletal α-actin (SkA), atrial natriuretic factor (ANF) and brain

natriuretic peptide (BNP)[27-30]. All of these have been accepted as markers for cardiac hypertrophy. Of these; however, only ANF, BNP and SkA share similar expression profiles during hypertrophy between the rodent and man. βMHC re-expression during cardiac hypertrophy occurs specifically in the rodent heart[31]. The switch from αMHC to βMHC and increased expression of SkA has a direct effect on myocyte contraction and relaxation that is initially compensatory; however, prolonged hypertrophy results in myocardial remodeling that interacts poorly with the fetal isoforms[32]. The overall compensatory effects of both ANF and BNP are manifested in their ability to reduce blood volume thereby decreasing hemodynamic load[33]. Thus, the ventricular expression of ANF and BNP in hypertrophy are strictly compensatory and probably do not modify contractile function.

In addition to βMHC, SkA, ANF and BNP, there are also changes in the expression of proteins that are involved with calcium management that occur during hypertrophy. Most notably, in both the rodent and human, the calcium ATPase (SERCA) is down-regulated in hypertrophied cardiomyocytes[34]. In the normal cardiomyocyte, SERCA functions as a calcium re-uptake channel to modulate the contraction-relaxation cycle[35]. A decrease in the expression of SERCA results in the inability of the cardiomyocyte to correctly modulate intracellular calcium concentrations ($[Ca^{2+}]_i$) resulting in a net increase in basal $[Ca^{2+}]_i$. One of the major consequences of signaling through Gq-coupled receptors is the IP3-dependent release of calcium from the sarcoplasmic reticulum (SR) of cardiomyocytes. As noted above, increased Gq signaling is a major factor contributing to cardiac hypertrophy. Increased Gq signaling combined with

downregulation of SERCA contributes to the overall mismanagement of $[Ca^{2+}]_i$. The mismanagement of $[Ca^{2+}]_i$ not only leads to impaired contraction-relaxation cycles, but also activates calcium/calmodulin-dependent protein kinases (CaMKs), which are known to influence the expression of hypertrophy-sensitive genes[36-38]. As a major part of the current work, the CaMKs will be discussed in detail below.

Signaling Mechanisms Leading to Hypertrophic Gene Expression

As discussed above, one of the major causes of cardiac hypertrophy is mechanical stress induced by pressure or volume overload. *In vivo*, mechanical stress manifests as stretch on the cardiomyocyte level. Cardiomyocytes experimentally stretched in culture release AngII[11, 12, 14] and ET-1[10, 13, 15]. Both AngII and ET-1 stimulate the hypertrophic phenotype of cardiomyocytes through Gq. The recently discovered UIIR is also Gq-coupled, its expression in cardiomyocytes increases during hypertrophy[39], and its ligand (UII) is also expressed in cardiomyocytes[40].

Several signaling pathways have been implicated in cardiac hypertrophy. Among these are the CaMK, MAPK, protein kinase C (PKC) and calcineurin pathways. All of these pathways are activated as a result of Gq signaling. Signaling through hypertrophic pathways ultimately leads to the activation of transcription factors such as MEF2, NFAT, GATA and NF-kB. It is through the activation of these transcription factors that the expression of hypertrophy-sensitive marker genes is increased. Of these, MEF2 has emerged as the major transcription factor controlling hypertrophic gene expression.

a. The CaM Kinase Signaling Pathway

In nearly all cell types, calcium is a major second messenger important for not only regulating contraction in myocytes, but also as a regulator of kinase activity. The basal $[Ca^{2+}]_i$ for a cardiomyocyte is approximately 10^{-7}M. The concentration of calcium in the SR is 10,000 times greater at 10^{-3}M. Signaling mechanisms that cause the release of SR calcium are controlled by release and re-uptake mechanisms in order to maintain the correct $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ by itself is not sufficient to activate CaMKs. Therefore, rises in $[Ca^{2+}]_i$ are coupled to CaMK activity through a calcium binding protein called Calmodulin (CaM). This is accomplished by Ca^{2+}/CaM binding of CaM kinase kinase (CaMKK) followed by CaMKK-dependent phosphorylation and activation of downstream effector kinases (either CaM kinase I or IV (CaMKI, CaMKIV) or AKT/PKB) (Figure 2A).

Calmodulin

Calmodulin is a small (148 amino acids) protein classified as a "calcium sensor" and undergoes a conformational change as a result of calcium binding[41]. Since CaM binds calcium with an affinity similar to that of basal $[Ca^{2+}]_i$ ($K_d = 5 \times 10^{-7}$ to 5×10^{-6} M), it is well suited as a sensor of transient increases in $[Ca^{2+}]_i$[42]. The classification of calcium sensor proteins, under which calmodulin falls, is distinguished by a structural motif known as the E-F hand. The E-F hand motif consists of an N-terminal helix (E helix) followed by a calcium coordinating loop and a C-terminal helix (F helix)[43]. Calmodulin contains four E-F hand motifs and is therefore able to coordinate four

calcium ions[43]. E-F hands 1 and 2 are separated from E-F hands 3 and 4 by a short flexible linker region. Upon binding calcium, the E-F hands change conformation from a closed globular structure to a more open structure exposing a methionine-rich hydrophobic region[41] (Figure 2B). It is this hydrophobic region that interacts with the calmodulin binding domain of the CaMKs[44], resulting in a conformational change in the kinases that relieves autoinhibition of the substrate binding site[45].

CaM kinase kinase

The pinnacle member in the CaMK signaling cascade is CaM kinase kinase (CaMKK). To date, two isoforms of CaMKK have been discovered (CaMKKα and CaMKKβ) and are products of two distinct genes[46, 47]. CaMKKα is 505 amino acids and CaMKKβ is 587 amino acids in length. Each is composed of a catalytic domain, an autoinhibitory domain and calmodulin binding domain (Figure 2C). Upon binding of Ca^{2+}/CaM, CaMKK undergoes a conformational change resulting in relief of autoinhibition allowing substrate access to the catalytic domain. Thus far, only three substrates have been described for CaMKK (CaMKI, CaMKIV and AKT/PKB). CaMKK phosphorylates CaMKI at Thr180[48], CaMKIV at Thr196[49] and AKT/PKB at Thr308[50]. Phosphorylation at these sites results in activation of the respective kinases. Although phosphorylation by CaMKK is required for full activation of kinase activity, CaMKI and CaMKIV also require Ca^{2+}/CaM while AKT/PKB does not.

Only one pharmacological inhibitor of CaMKK has been described to date[51]. 7H-Benz[de]benzimidazo[2,1-a]isoquinoline-7-one-3-carboxylic Acid, Acetate (STO-609)

inhibits CaMKKα (IC_{50} = 120 ng/mL) and CaMKKβ (IC_{50} = 40 ng/mL). Other kinases are inhibited by STO-609, but only at concentrations greater than 10 μg/mL. STO-609 inhibits CaMKK activity by competing with ATP.

Only CaMKI and AKT/PKB are expressed in the heart. Though the importance of CaMKI has been demonstrated in cardiac hypertrophy, full CaMK cascade activation through Gq coupled receptor signaling has not been adequately studied in cardiomyocytes. In fact, only one study to date has demonstrated an intact CaMKK cascade in heart[52]. The authors successfully purified both CaMKI and CaMKK from the porcine heart and concluded, "the CaMK cascade should be taken into account in any consideration of Ca^{2+} signal transduction[52]." Others circumvented CaMKK regulation of effector kinases by either heart targeted transgenic expression of active CaMKIV (not relevant to heart)[37] or transient transfection of constitutively active CaMKI or CaMKIV[53].

CaM kinase I

A major downstream effector of CaMKK is CaMKI. There have been three isoforms of CaMKI discovered to date (α, β and γ) of which, CaMKIα predominates in the heart[54]. CaMKIα is 332 amino acids in length (~42 kDa) and consists of an N-terminal catalytic domain and a C-terminal autoregulatory domain[55] (Figure 2C). The binding of Ca^{2+}/CaM to the autoregulatory domain causes a conformational change in CaMKI resulting in partial kinase activity. For full activation, CaMKI requires phosphorylation (Thr180) by CaMKK. Incubation of CaMKI with Ca^{2+}/CaM results in a slight activation of kinase activity; however, the presence of CaMKK and Ca^{2+}/CaM results in full activity, while CaMKK alone cannot activate CaMKI[47]. There are no specific

pharmacological inhibitors for CaMKI at present.

The first substrate identified for CaMKI was synapsin-1 in brain[56]. Subsequently, others demonstrated that cAMP response element binding protein (CREB) and activating transcription factor-1 (ATF-1) are substrates for CaMKI[57]. More important, the CaMK cascade cross-talks with MAPKs in neurons[58]. It is well known that signaling through Gq-coupled receptors in cardiomyocytes leads to the activation of the MAPKs. Whether or not activation of MAPKs in cardiomyocytes is dependent on Ca^{2+} (as in neurons) remains to be demonstrated. It is interesting to note; however, that AngII-dependent transactivation of the EGF receptor is dependent on Ca^{2+}/CaM and leads to the activation of MAPKs in cardiac fibroblasts[59]. Whether or not the CaMK cascade is involved with AngII-dependent transactivation of the EGF receptor remains to be explored.

Plenty of data exists demonstrating the importance of CaMKs in the regulation of hypertrophic gene expression in cardiomyocytes. However, there has been a lack of data connecting extracellular signaling through Gq-coupled receptors resulting in the activation of the endogenous CaMK cascade in cardiomyocytes. Moreover, the discovery of CaMK to MAPK crosstalk in neurons leads one to question whether the same mechanism exists in other excitable cells such as myocytes. The importance of CaMK to MAPK crosstalk becomes apparent when considering the activation of MEF2. CaMKI, a cyotplasmic kinase, activates MEF2, which is a nuclear transcription factor. The result of MEF2 activation in cardiomyocytes is the increased expression of hypertrophy-sensitive genes. The mechanism of MEF2 activation will be discussed in detail below; however, it is important to note here that activation of MEF2 also requires

the MAPK p38. CaMK crosstalk with MAPKs provides a mechanism that may explain the ability of CaMKI to activate MEF2.

b. Mitogen-activated Protein Kinase (MAPK) Signaling

Extracellular signals integrated through receptor tyrosine kinases (RTKs) culminate in the activation of MAPKs. MAPKs, in turn, phosphorylate and activate numerous transcription factors that modulate gene expression (Figure 3). All MAPK pathways are organized in three tiers, each leading to the activation of the next: i) MEK kinases (MEKKs), ii) MAP/ERK kinases (MEKs) and iii) MAPKs. The MEKKs are Ser/Thr kinases that activate downstream MEKs by dual phosphorylation on a conserved Ser and Ser/Thr residue lying within a Ser-X-X-X-Ser/Thr motif[60].
Activation of MAPKs by MEKs require dual phosphorylation on a Thr and Tyr residue lying within a Thr-X-Tyr motif[61]. The MAPKs are proline-directed kinases and therefore phosphorylate substrates at the motif Pro-X-X-X-Ser/Thr-Pro[62]. The three best characterized MAPK cascades are: i) the extracellular signal-regulated kinases (ERKs); ii) the p38 MAPK cascade; and iii) the c-Jun N-terminal kinases (JNKs). The MAPKs can be distinguished by differences in the X portion of their phosphorylation motifs (for EKRs X = Glu, for p38 X = Gly and for JNKs X = Pro)[63].

In the heart, growth factor signaling through RTKs results in cardiac hypertrophy, mainly through the activation of p38 and ERK1/2 MAPKs. In addition, MAPKs are activated in cardiomyocytes as a result of stretch-induced release of AngII and ET-1.

ERK MAPKs

The ERKs constitute a family of MAPKs comprised of six members, of which ERK1/2 are the best characterized[64]. ERK1 (44 kDa) and ERK2 (42 kDa) share 85% homology and are encoded by separate genes[64]. The classical mode of activation for ERK1/2 is through a kinase cascade whose activation originates from RTKs; however, signaling through Gq-coupled receptors also activates ERK1/2.

There are two widely used pharmacological inhibitors of ERK1/2; however, they do not inhibit the kinases directly. Rather, they inhibit the activation of MEK1. The first, 2'-amino-3'-methoxyflavone (PD98059), binds to the inactive form of MEK1 and prevents phosphorylation by upstream activators[65]. The second, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126), also blocks the activation of MEK1 from upstream kinases[66]. Both result in the inability of MEK1 to phosphorylate and activate ERK1/2.

Signaling through ERK1/2 MAPKs has been implicated in cardiac hypertrophy. In cultured cardiomyocytes, ERK1/2 become activated in response to either agonist stimulation or cell stretching[67-70]. ANF promoter-reporter activity is increased in cardiomyocytes transfected with constitutively active MEK1[71]. Recall that MEK is the immediate upstream activator of ERK1/2. Others have demonstrated that ERK1/2 is required for agonist induced sarcomeric reorganization in cardiomyocytes[72]. *In vivo*, aortic banding in rodent models that induces pressure overload of the left ventricle results in the activation of ERK1/2[73, 74]. It is interesting to note that most of the "hypertrophic

agonists" used in the above studies signal through Gq-coupled receptors and mechanical stretch of cardiomyocytes releases Gq-coupled receptor agonists. Indeed, transfection of dominant negative MEK1inhibits both the ET-1 and PE-induced hypertrophic response in cardiomyocytes[75, 76]. One major mechanism of Gq-dependent activation of MAPKs is through PKC[77]. Recently, signaling through the UIIR was shown to result in the activation of ERK1/2 in cardiomyocytes; however, inhibition of PKC had no effect on UII induced ERK1/2 activation, thereby suggesting an alternative mechanism[78].

ERK1/2 modulate gene expression through the phosphorylation and activation of several protein targets in the heart. One of the major manifestations of hypertrophy in cardiomyocytes is an increase in overall protein content and an increase in gene transcription. ERK1/2 have been shown to phosphorylate p70 S6 kinase resulting in increased protein translation efficiency and protein accumulation[79]. In addition, ERK1/2 activates upstream binding factor (UBF), which aids in the expression of ribosomal RNA[80]. ERK1/2 regulates cardiac gene expression mainly through phosphorylation of the transcription factor GATA4[81]. GATA4 is critical for the expression of hypertrophy-responsive genes such as ANF and BNP[82, 83].

p38 MAPKs

The p38 (38 kDa) MAPKs are a family of kinases with four isoforms discovered to date (α, β, γ and δ)[84-86]. Of these, p38α and β are ubiquitously expressed and are the best characterized because there are pharmacological inhibitors available[87]. The major p38 isoforms expressed in heart are p38α and β[72]. p38 MAPKs are activated by dual

phosphorylation of the motif Tyr-Gly-Thr by the upstream kinases MKK3 and MKK6[88] (Figure 3). Upstream activators of MKK3/6 include TAK-1, MTK1/MEKK-4, TAO, and ASK-1, all of which are induced by cell stress[89-92]. Of these, ASK-1 has been implicated in hypertrophy signaling[93, 94].

As mentioned, there are pharmacological inhibitors available for p38. The most commonly used is 4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1H-imidazole (SB203580)[95]. SB203580 functions as an ATP competitive inhibitor and is specific for p38α and β[96].

Recently, p38 was shown to be involved with mechanical stress-induced cardiac hypertrophy. In a rodent model of pressure overload, the activity of p38 is increased[97]. Likewise, stretching of cultured cardiomyocytes results in activation of p38[98]. In models of ischemia-reperfusion injury, inhibition of p38 by SB203580 resulted in increased cardioprotection as well as reduced hypertrophy[99, 100]. In addition, transfection of cardiomyocytes with MKK6 (upstream activator of p38) results in hypertrophy[97]. Current data reveal that the overall hypertrophic response attributed to p38 includes an increase in cardiomyocyte surface area, sarcomeric reorganization and increased expression of ANF[101]. Since mechanical stress induces p38 activity in cardiomyocytes and Gq-coupled agonists such as AngII and ET-1 have been shown to transduce stretch signaling, one would expect that Gq signaling could also lead to the activation of p38. Indeed, stimulation of cardiomyocytes with AngII, ET-1 and PE results in the activation of p38[72, 102, 103]. Likewise, recent work demonstrated that UII stimulation of cultured cardiomyocytes results in p38 activation; however, the exact mechanism remains

unknown[78].

p38 contributes to hypertrophic signaling in cardiomyocytes mainly through the activation of transcription factors. These include ELK-1, Ets-1, MAX, MEF-2A, MEF-2C, NF-[kappa]B[104-109]. Of these, MEF2 proteins have emerged as major factors contributing to cardiac hypertrophy.

c. Myocyte Enhancer Factor 2 (MEF2) and its Regulation

The MEF2 proteins are constituents of the MADs-box family of transcription factors that have all been defined on the basis of sequence homology across many species. The MADS-box is a conserved motif found within the DNA-binding domain of MEF2 that confers binding ability to an A/T rich sequence ($CTA(A/T)_4TAA/G$) in the promoters of various genes[110]. MEF2 proteins are expressed in developing skeletal, smooth and cardiac muscle cells[111]. Though first discovered as a factor necessary for the development of muscle, MEF2 is a critical factor in cardiac hypertrophy[37, 112]. The regulation of MEF2 is complicated and requires direct phosphorylation, dissociation from class II histone deacetylases (HDACs) and partnering with other transcription factors such as GATA4 and NFAT[113-115]. In fact, the CaMK cascade and the MAPK cascade converge on MEF2 and influence its activation status.

Regulation of MEF2 by Class II Histone Deacetylases (HDACs)

HDACs are nuclear proteins that are capable of deacetylating core histones

thereby facilitating the condensation of chromatin. When chromatin is in the condensed state, access of the transcriptional machinery to the promoter regions of genes is restricted thereby inhibiting gene expression. On the other hand, when histone acetyltransferases (HATs) are recruited to the promoter region, the core histones become acetylated. Acetyl groups on histones carry a net negative charge that repels the negatively charged sugar-phosphate backbone of the associated DNA resulting in the de-condensation of the chromatin. It is when chromatin is de-condensed that the transcriptional machinery is able to gain access to the promoter thereby facilitating gene expression[116, 117]. There are three classes of HDACs[118]. Classes I and III are capable of binding DNA directly and are expressed ubiquitously. The expression of class II HDACs seems to be restricted to brain, T-cells and muscle[119]. Interestingly, unlike class I and III HDACs, class II HDACs must partner with a protein capable of binding DNA in order to direct HDAC activity to histones[118]. One such protein is MEF2.

In the heart, four class II HDACs are expressed: HDAC4, HDAC5, HDAC7 and HDAC9. Of these, HDAC4 and HDAC5 are the best characterized partners of MEF2 and are critical regulators of MEF2-dependent gene expression[53, 120-122]. When bound to MEF2, HDAC4/5 deacetylate local histones thereby inhibiting gene expression. In order to remove HDAC-directed repression of MEF2-sensitive gene expression, HDAC4/5 must be phosphorylated. Upon phosphorylation, HDAC4/5 become substrates for the phosphomotif binding protein 14-3-3β[123]. Once HDAC4/5 are bound to 14-3-3β, the complex is translocated to the cytoplasm through the nuclear pore complex via a Crm1-dependent mechanism. In cardiomyocytes, the net result of HDAC4/5 removal from the

nucleus is an increase in MEF2-dependent gene transcription and thus, MEF2-driven hypertrophy[124]. To date, only two mechanisms have been proposed that result in HDAC4/5 phosphorylation and translocation to the cytoplasm. The first is through the CaMK pathway and the second is through protein kinase D (PKD).

Activation of MEF2 by CaMKs and Protein Kinase D (PKD)

Signaling through members of the CaMK cascade stimulates MEF2 activity by disrupting the MEF2/HDAC complex. This is accomplished through the phosphorylation of Ser_{259} and Ser_{498} of HDAC5[125]. Likewise, activation of the α1-AR by PE results in the CaM-dependent activation of MEF2[53]. We have also demonstrated that transfection of cardiomyocytes with constitutively-active CaMKI or CaMKIV results in activation of MEF2[37]. In vascular smooth muscle cells, stimulation with PE or transfection of active CaMKI or active CaMKIV result in the activation of MEF2 as well as the 14-3-3β-dependent nuclear export of HDAC4/5[126]. As mentioned above, CaMKIV is not an endogenous kinase in the heart and CaMKI does not have access to the nucleus. This led us as well as others to speculate that there must be an effector kinase downstream of CaMKI that has access to the nucleus and can phosphorylate HDAC4/5. To date, there has been no kinase discovered downstream of CaMKI to provide this function. Recently; however, it was demonstrated that PKD phosphoryates HDAC4/5 leading to their nuclear export and activation of MEF2 in cardiomyocytes[127].

PKD was initially classified as a member of the PKC family. Since PKD only shares homology with PKCs in the DAG binding domain and low homology in the kinase

domain, the classification was called into question[128]. Since then, it has been shown that the kinase domain of PKD shares considerable homology with the CaMKs; therefore, it has been re-classified as a novel subgroup of the CaMKs[129]. PKD is able to induce hypertrophy of cardiomyocytes through the phosphorylation of HDAC5[127]. In fact, PKD phosphorylates the same serine residues as CaMK. It is important to note that PKD-dependent activation of MEF2 has been described as a parallel pathway to CaMKI. It is also worth mentioning that the inhibitor used in this study for PKD (Gö6976; IC_{50} = 20 nM) was used at a concentration of 10 μM, 500 times the IC_{50} for the inhibitor. At this concentration, members of the PKC family are also inhibited[130].

Activation of MEF2 by p38 MAPK

Like other transcription factors, the transactivation activity of MEF2 is regulated by phosphorylation[131]. In addition to loss of repression by HDAC4/5 removal, MEF2 requires phosphorylation in its C-terminal activation domain. This is accomplished by the MAPK p38[104]. As mentioned above, p38 is involved with the regulation of hypertrophy through Gq-coupled receptors. Likewise, p38 has been implicated in *in vivo* models of hypertrophy using pressure overload as a model.

Urotensin II and Its Receptor

Urotensin II (UII) is a peptide hormone first discovered in fish brain and characterized by Bern et al. in 1967[132]. Subsequently, the cDNA for UII was identified in other organisms including man[133-135]. Like other peptide hormones, UII is first expressed

as a pre-pro peptide, and then cleaved for biological activity. Once fully processed, human UII is a cyclic dodecapeptide with a conserved cysteine-linked motif (CFWKYC)[136]. This cyclic motif is 100% conserved throughout evolution from fish to man suggesting an important physiological role. UII is predominantly expressed in brain; however, UII is expressed in other areas including cardiomyocytes, vascular endothelial cells and vascular smooth muscle cells[40, 133, 135, 137-139]. Initial studies regarding the physiological role of UII suggests that the peptide hormone is a potent vasoconstrictor[140]. In addition, data has demonstrated that UII is profibrogenic and can regulate hypertrophic signaling as well as apoptosis in cardiomyocytes[141]. These roles of UII are interesting in that all are characteristic in the transition from compensated to decompensated hypertrophy.

The Urotensin II receptor (UIIR) was originally an orphan receptor named GPR14 that was cloned from rat. UIIR is a 389 amino acid 7-transmembrane domain receptor that is coupled to the small G-protein G_q. GPR14 was de-orphaned in a set of experiments where cloned human GPR14 was transfected into HEK293 cells which were then used to screen a set of 400 ligands to determine which one could activate GPR14 as evidenced by an increase in intracellular calcium. Only one ligand activated GPR14 (fish UII); therefore, GPR14 was de-orphanized and named UIIR[133].

UII and its receptor are co-expressed in cardiomyocytes and up-regulated in cardiac disease states [39, 137, 141, 149]. In addition, we looked at microarray data at NCBI's GEO database and found that Gq-coupled receptor stimulation of cardiomyocytes resulted in increased expression of UIIR (Figure 4). Using adenoviral up-regulation of

UIIR in cardiomyocytes, Onan et al. showed that UII induced the hypertrophic phenotype as evidenced in enlargement of cardiomyocytes, sarcomeric reorganization as well as activating ERK 1/2 and p38 MAP kinases[78]. Interestingly, MAP kinase activation and hypertrophy was shown to be independent of PKC activity and the authors suggested that UIIR may transactivate EGFR, which would account for the MAP kinase activation. Though the signaling mechanism by which UII induces hypertrophy is beginning to be delineated, much work needs to be done to fully understand the extent of UII signaling.

Project Hypothesis and Specific Aims

As mentioned above, cardiac hypertrophy arises in response to mechanical stress. Though initially compensatory, sustained cardiac hypertrophy leads to decompensation and failure. The initial response to stretch of cardiomyocytes is the release of Gq-coupled receptor agonists such as AngII and ET-1. Gq-coupled receptor signaling is a strong activator of hypertrophy through calcium mediated events. In addition, moderate levels of Gq activation lead to a compensatory hypertrophic phenotype while high levels of Gq activation lead to cardiomyocyte death—a hallmark of decompensation.

UIIR is a newly discovered Gq-coupled receptor that is only expressed in cardiomyocytes after mechanical stress. UII stimulation of cardiomyocytes results in hypertrophy through the activation of the MAPKs ERK1/2 and p38 in a PKC-independent manner. Since UII stimulation results in increased $[Ca^{2+}]_i$, it is likely that the CaMK cascade is active under these conditions. Therefore, we hypothesize that the hypertrophic up-regulation of UIIR and the resultant increase in G_q signaling and

intracellular calcium will activate CaMKK resulting in the downstream activation of CaMKI. CaMKI, once active, could regulate the activities of ERK1/2 and p38, which can account for the PKC-independent hypertrophic phenotype. Because UIIR is a newly discovered G_q coupled receptor, many questions still remain concerning UIIR expression during cardiac disease states as well as its role in downstream signaling mechanisms that contribute to the hypertrophic phenotype. Therefore, we proposed the following specific aims:

a. Specific Aim 1: To test the hypothesis that hypertrophic stimuli can up-regulate the expression of UIIR in primary cardiomyocytes.

The expression of UIIR is undetectable to slight in healthy myocardium. It is only in states of myocardial disease or dysfunction that UIIR expression becomes markedly up-regulated. It is possible that at some point during the etiology of heart failure, available hypertrophic agonists stimulate the expression of UIIR over the necessary threshold for UII to elicit its biological activity thereby contributing to a downward spiral toward dilated cardiomyopathy and failure. Therefore; the purpose of this aim was to determine whether known inducers of cardiac hypertrophy are capable of up-regulating UIIR in cardiomyocytes. Cardiomyocytes were cultured in the presence of either AngII or PE. We determined the expression of UIIR mRNA by RT-PCR and the expression of UIIR protein by western blot.

b. Specific Aim 2: To test the hypothesis that UII signaling involves activation of CaMKK and CaMKI which cross talks with members of the MAPK signaling pathway.

One of the major consequences of signaling through Gq-coupled receptors is the IP3-dependent release of calcium from the sarcoplasmic reticulum (SR) of cardiomyocytes. The increase of $[Ca^{2+}]_i$ activates CaMKs, which are known to influence the expression of hypertrophy-sensitive genes. The complete signaling pathway through UIIR leading to hypertrophy has not been fully delineated; however, others have shown that UII stimulation activates members of the MAP kinase pathway. In order to study UII signaling, cardiomyocyte cultures were infected with an adenoviral vector that expressed UIIR. Cardiomyocytes were then stimulated with UII and examined for changes indicative of hypertrophy. Specifically we monitored; i) ANF, SkA and MEF2 reporter activity and ANF, BNP, SkA and bMHC gene expression, ii) CaMKI, ERK1/2, p38 and PKD activation, and iii) HDAC/14-3-3β association. To determine whether UII-dependent hypertrophy required CaMKK we assessed the ability of a specific inhibitor of CaMKK to block UII effects.

Significance of the Project

Gq coupled receptors are important regulators of myocyte hypertrophy and some of their agonists are regulated by mechanical stress in the heart. UIIR itself is G_q coupled and contributes to the hypertrophic phenotype. It would be advantageous to lessen the overall G_q contribution to cardiac disease states as increases in Gq signaling result in

decompensated hypertrophy. Therefore, gaining insight into how UIIR is regulated in the etiology of cardiac hypertrophy may provide insights into developing novel therapies to either decrease UIIR expression, or antagonize the receptor during the disease state. In addition, delineating the UIIR signaling pathway will provide insights into the mechanisms by which Gq-coupled receptors activate both the CaMK cascade and MAPKs resulting in hypertrophy.

Figure 1. Increased Gq signaling events lead to changes characteristic of decompensated hypertrophy. Mechanical stress in the form of hemodynamic overload (*in vivo*) or cardiomyocyte stretch (*in vitro*) result in the release of Gq-coupled receptor agonists such as AngII and ET-1, which initiate compensated hypertrophy. In addition, Gq signaling results in the expression of other Gq-coupled receptors in cardiomyocytes such as ET-1_B receptor and now, UIIR. The resultant increases in Gq signaling can cause decompensated hypertrophy since moderate levels of Gq signaling is known to induce compensation whereas high levels of Gq signaling results in decompensation.

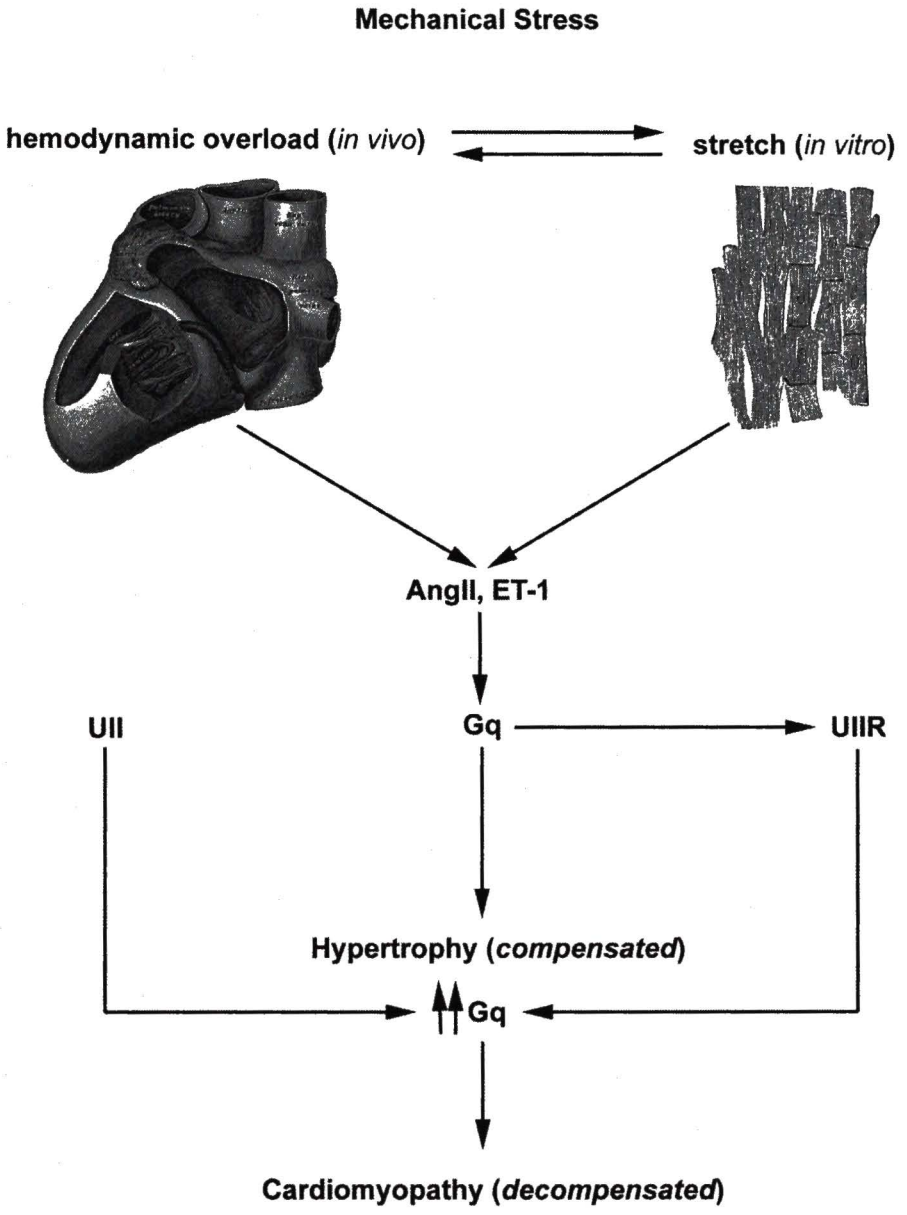
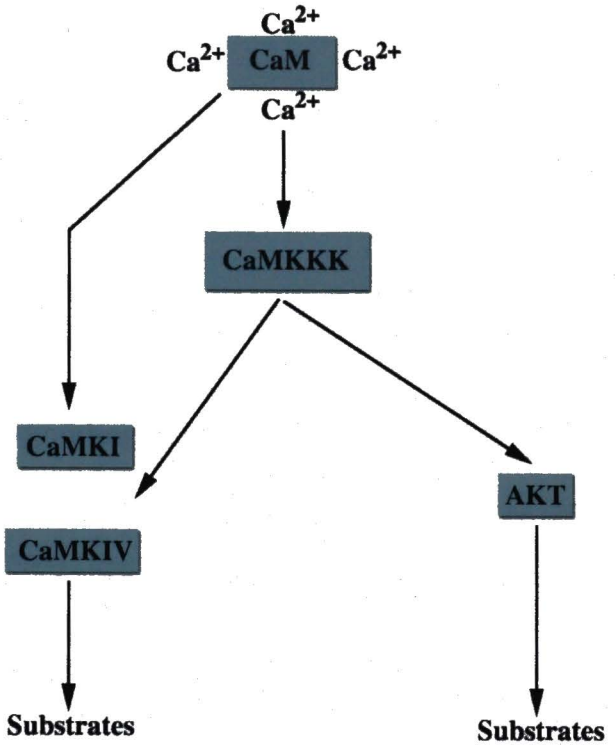
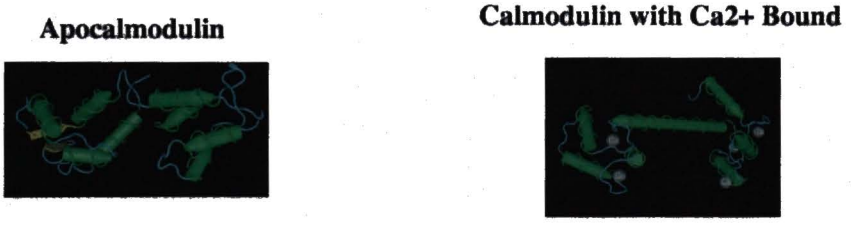

Mechanical Stress
hemodynamic overload (in vivo)
stretch (in vitro)
AngII, ET-1
UII
Gq
UIIR
Hypertrophy (compensated)
Gq
Cardiomyopathy (decompensated)

Figure 2. The CaM kinase signaling pathway. A) Calcium/Calmodulin activates CaMKK by interacting with its calmodulin binding domain and altering its conformation such that CaMKK's catalytic domain has access to its substrates. There are only three know substrates of CaMKK: CaMKI, CaMKIV and AKT. Of these, only AKT does not also require Calcium/Calmodulin. B) Calmodulin without calcium bound (apocalmodulin) is globular. Once calcium binds; however, calmodulin assumes a conformational change that resembles a dumbbell with hydrophobic domains exposed. These hydrophobic domains interact with hydrophobic residues in the calmodulin binding domains of CaMKK, CaMKI and CaMKIV. Structures were downloaded from NCBI: Apocalmodulin (PDB: 1CFC), Calmodulin with calcium bound (PDB: 1EXR). C) Schematic of CaMKK and CaMKI primary structure. AID/CBD is autoinhibitory domain/calmodulin binding domain (Adapted from: Soderling TR. The Ca-calmodulin-dependent protein kinase cascade. Trends Biochem Sci. 1999 Jun;24(6):232-6.)

A.


Ca2+
Ca2+
CaM
Ca2+
Ca2+
CaMKKK
CaMKI
AKT
CaMKIV
Substrates
Substrates

B.


Apocalmodulin
Calmodulin with Ca2+ Bound

C.

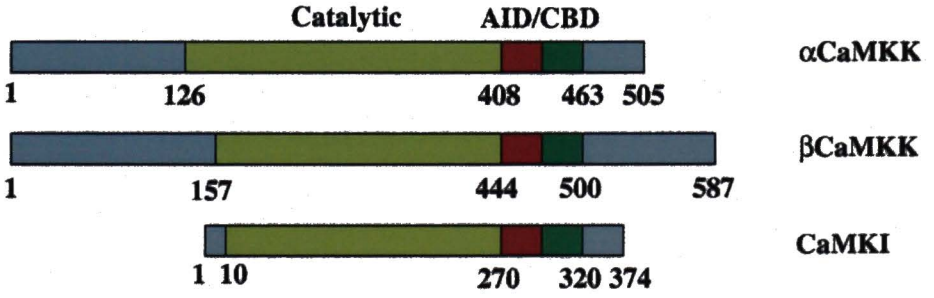

Catalytic
AID/CBD
αCaMKK
1
126
408
463
505
βCaMKK
1
157
444
500
587
CaMKI
1
10
270
320
374

Figure 3. The MAP kinase signaling pathway. All MAPK pathways are organized in three tiers, each leading to the activation of the next: i) MEK kinases (MEKKs), ii) MAP/ERK kinases (MEKs) and iii) MAPKs. The MEKKs are Ser/Thr kinases that activate downstream MEKs by dual phosphorylation on a conserved Ser and Ser/Thr residue lying within a Ser-X-X-X-Ser/Thr motif. Activation of MAPKs by MEKs require dual phosphorylation on a Thr and Tyr residu lying within a Thr-X-Tyr motif. The MAPKs are proline-directed kinases and therefore phosphorylate substrates at the motif Pro-X-X-X-Ser/Thr-Pro. The best characterized MAPK cascades are: i) the extracellular signal-regulated kinases (ERKs); ii) the p38 MAPK cascade. ERK1/2 and p38 contribute to the hypertrophic response by increasing protein sysnthesis and activating transcription factors that interact to drive gene expression. Figure adapted from: Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, Cobb MH. MAP kinases. Chem Rev. 2001 Aug;101(8):2449-76.

Tier

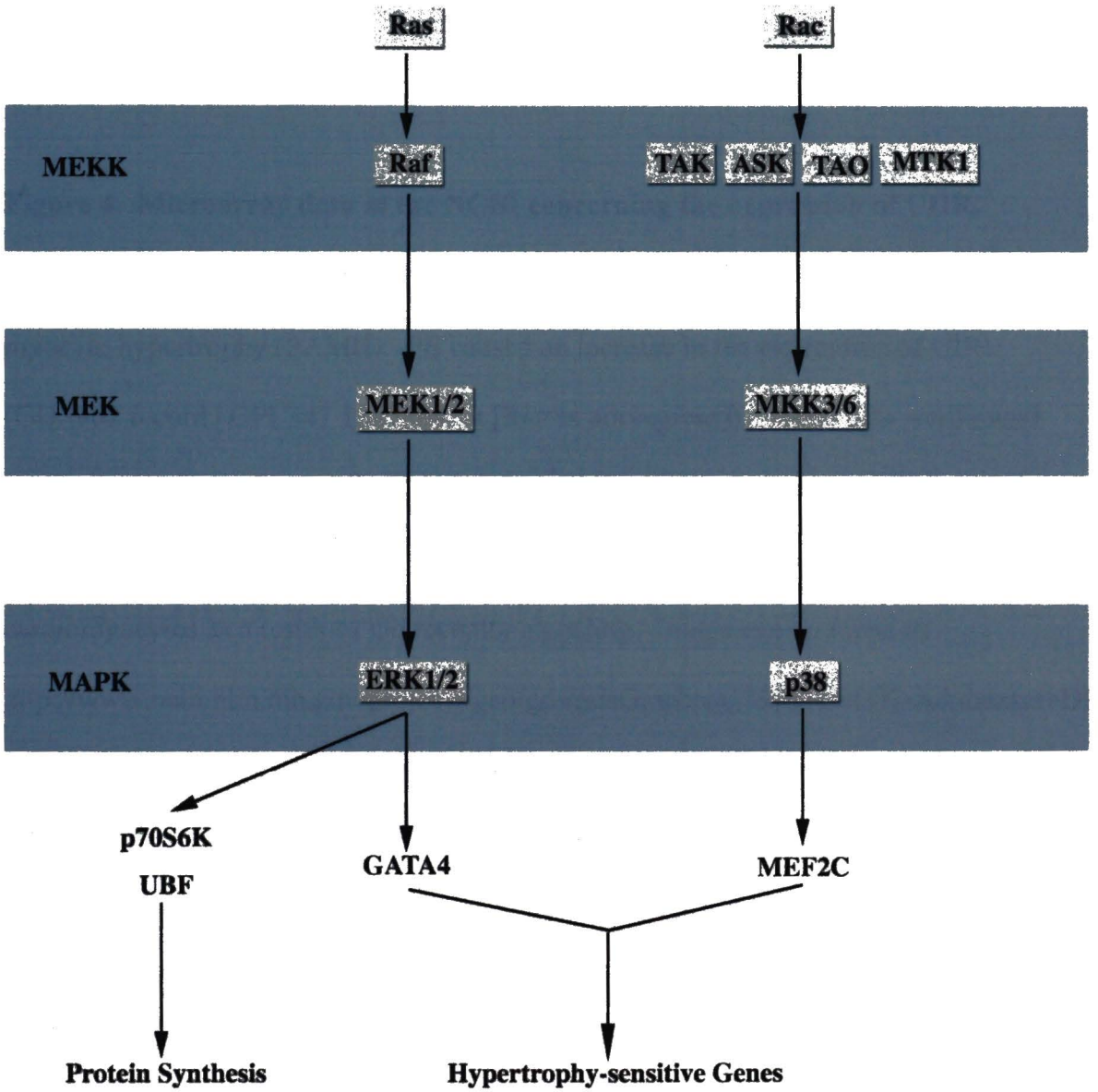
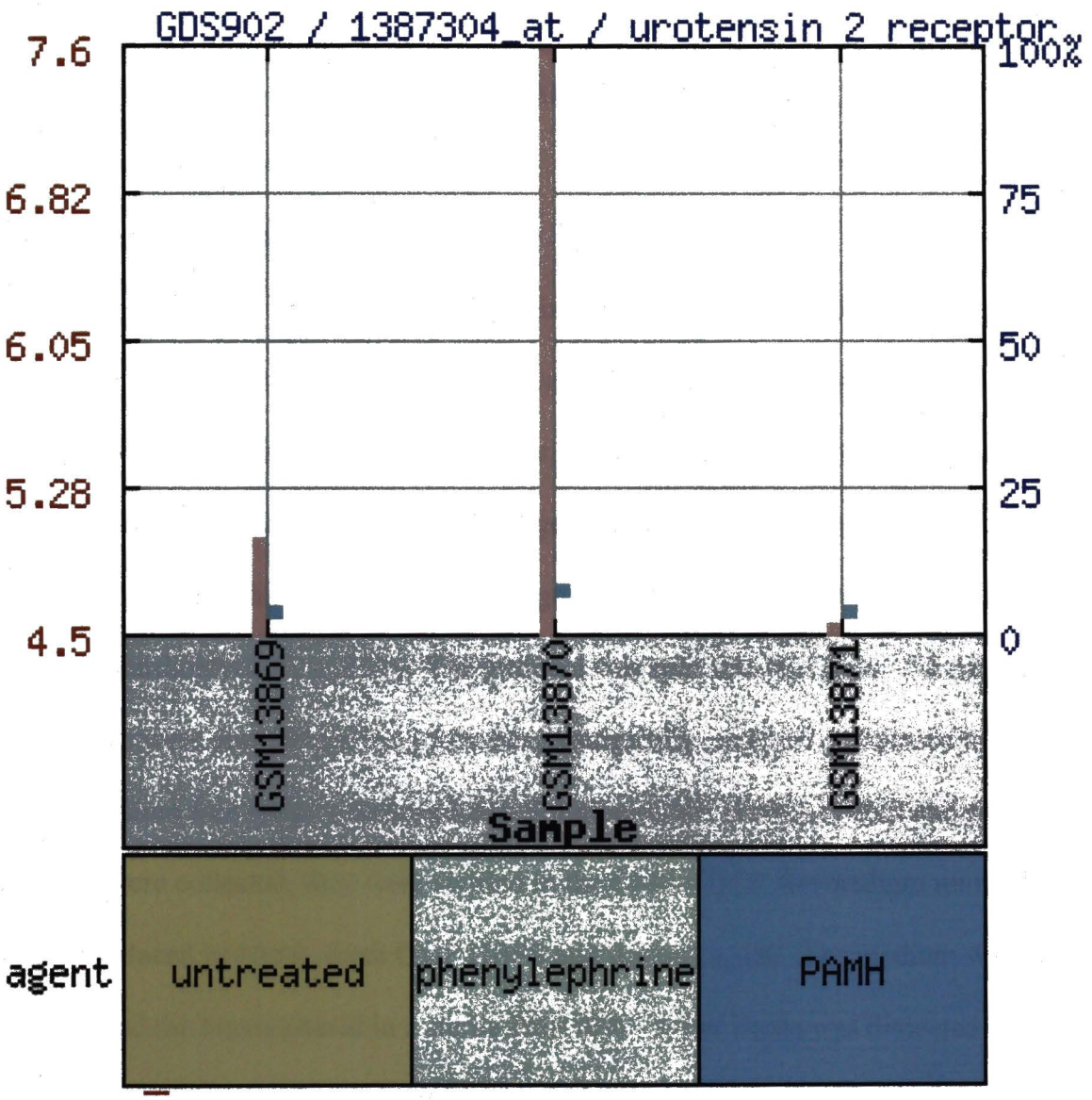

Ras
Rac
MEKK
Raf
TAK
ASK
TAO
MTK1
MEK
MEK1/2
MKK3/6
MAPK
ERK1/2
p38
p70S6K
UBF
GATA4
MEF2C
Protein Synthesis
Hypertrophy-sensitive Genes

Figure 4. Microarray data at the NCBI concerning the expression of UIIR. Cardiomyocyte cultures were stimulated with PE (10 μM) or pyridine activator of myocyte hypertrophy (PAMH). PE caused an increase in the expression of UIIR (GDS902 record | GPL341 1387304_at [Rattus norvegicus]). These data were found within the GEO database (NCBI) and the results were not reported anywhere in the literature. These data demonstrate the possibility that UIIR is up-regulated in cardiomyocytes as a result of Gq receptor signaling. Image can be found at: http://www.ncbi.nlm.nih.gov/projects/geo/gds/gdsGraph.cgi?&dataset=KzA&dataset=DFD$&labels=13869p1p1&group=13869:13870:13871&grouplabel=agent&gmax=7.600000&gmin=4.500000&title=GDS902+/+1387304_at%20/%20urotensin%202%20receptor&absc=13869p1p1.


GDS902 / 1387304_at / urotensin 2 receptor
7.6
6.82
6.05
5.28
4.5
100%
75
50
25
0
GSM13869
GSM13870
GSM13871
Sample
agent
untreated
phenylephrine
PAMH

CHAPTER II

MATERIALS AND METHODS

Cell Culture and Reagents

In the past, we used a crude and partially purified enzyme preparation (Viokase) to isolate cardiomyocytes from ventricular tissue[38]. In order to increase the yield and purity of our primary cardiomyocyte cultures, used the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical). The digestive enzymes provided with this system were purified rather than crude and Worthington verified each lot for the ability to yield consistent cardiomyocyte cultures. The protocol was split into two days. On day one, neonate rats were anesthetized and their hearts surgically removed and placed in calcium and magnesium free Hank's Balanced Salt Solution (CMF HBSS) to chill and rinse. After all hearts were collected, they were swirled in the CMF HBSS, the medium removed, and the hearts placed in 10 mL fresh CMF HBSS and washed again. The medium was then removed and the hearts placed in a sterile Petri dish. Atrial tissue was dissected away and the ventricular tissue was minced with scissors and then finely minced ($\leq 1 mm^3$) with a sterile scalpel. After mincing, 9.0 mL of CMF HBSS was added to the tissue to which 1.0 mL of reconstituted trypsin (50mg/mL final) was added. The tissue was incubated for 16-20 hours at 4°C. After overnight trypsin digestion, the tissue was

pipetted into a sterile 50 mL tube to which 1.0 mL of trypsin inhibitor was added (2 mg in CMF-HBSS). Oxygen was then passed over the medium for 1 minute and then incubated at 37° C for 5 minutes. When the tissue reached 37° C, 5.0 mL of collagenase (1500 units diluted in 5.0 mL of Leibovitz L-15 medium) was added and incubated for 45 minutes at 37° C shaking at 4 rpm. After incubation, the digested tissue was triturated 10X at a rate of 3.0 mL/sec with a 10 mL serological pipette to fully disperse the cells. After trituration, a sterile cell strainer was rinsed with 2.0 mL of fresh L-15 medium and the cell suspension was passed through it, without disturbing the settled tissue. An additional 5.0 mL of L-15 medium was added to the remaining tissue, triturated 10X and passed through the cell strainer. An additional 2.0 mL of fresh L-15 medium was used for a final rinse of the cell strainer. The cell suspension was then oxygenated for one minute and remained undisturbed at room temperature for 20 minutes. The cells were then sedimented at 100 x g for 5 minutes. The medium was then discarded and the cell pellet suspended in medium 199 supplemented with 10% FBS and gentamycin. At the time of plating, we included 10 μM Cytosine β-D-arabinofuranoside (AraC) to prevent the proliferation of any cardiac fibroblasts that sedimented with the cardiomyocytes. The cells were plated on Falcon 12-well, 6-well or 100 mm tissue culture dishes and incubated at 37° C in 95% humidity with a 5% CO_2 atmosphere and left undisturbed for 16-20 hours. The cell cultures were then washed 2X with fresh medium 199 and re-fed with medium 199 supplemented with 1% FBS. After 24 hours, the cultures were then washed and re-fed in medium 199 and 0.2% FBS. PE (10 μM), AngII (100 nM), ET-1 (10 nM), UII (as indicated), STO-609 (250 ng/mL), SB203580 (10 μM), and U0126 (10

μM) were purchased from Sigma Aldrich (St. Louis, MO) and AKT inhibitor (5 μM) was purchased from EMD Biosciences (San Diego, CA).

Plasmids and Adenoviral Constructs

In order to quantify hypertrophy-sensitive promoter activation, two luciferase-based promoter-reporter plasmid constructs were used; i) A 700 bp fragment (NP337) of the ANF promoter (received from Mona Nemer, University of Montreal, Quebec, Canada) which was described previously[142] and ii) a 400 bp fragment of the SkA promoter (received from Robert J Schwartz and Michael D. Schneider, Baylor College of Medicine, Houston, Texas), also described previously[143]. In order to quantify the activation of MEF2, we used a luciferase-based MEF2 enhancer-reporter plasmid that contained three MADS box repeats immediately upstream from a minimal promoter and luciferase structural gene (received from Eric Olson, University of Texas Southwestern Medical Center, Dallas, TX) and was previously described[144]. The plasmid vector encoding CaMKI was obtained from Eric Olson (University of Texas Southwestern Medical Center, Dallas, TX)[124]. The plasmid vector encoding dominant negative p38 ($p38_{AF}$) was obtained from J. Han (Scripps Research Institute, La Jolla, CA) and were previously described[86, 145, 146]. The adenoviral vectors that express UIIR were received from Walter Thomas (Baker Heart Institute, Melbourne, Australia) and were described previously[78, 141].

Infection of Cardiomyocytes

E1A positive HEK293 cells were infected with AdUIIR-GFP in order to amplify the virus. Another culture was infected in parallel in order to amplify the control virus as well. To obtain a high titer viral stock, a T-175 flask was infected and after approximately 24 hours, the cells and media were collected and were freeze-thawed five times, alternating in dry ice and a 37° water bath. The crude viral supernatant was then purified with a commercially-available kit (ViraBind Adenovirus Purification Kit, Cell Biolabs, Inc.) and stored in liquid nitrogen. Primary cardiomyocytes were infected with the crude supernatant at a dilution of 10^{-3}. This concentration of viral supernatant was sufficient to infect approximately 95% of the cardiomyocyte culture (Figure 5).

Transient Transfection and Luciferase Assay

For transfections, cardiomyocytes were cultured in 12-well tissue culture plates as described. Once cultures were incubated in medium 199 with 0.2% serum for 24 hours, cultures were either infected with AdUIIR as described or were just transfected. Figure 6 describes the overall scheme of infection and/or transfection. Depending on the experiment, cardiomyocytes were transfected with either control empty vector (pSG5), ANF promoter-reporter (50 ng/well), SkA promoter-reporter (50 ng/well), MEF2 enhancer-reporter (250 ng/well), CaMKI (10 ng/well) or p38aAF (100 ng/well) using LipofectAMINE™ Plus reagent (Invitrogen, Carlsbad, CA) per manufacturer's protocol. Total DNA per well was normalized with empty vector (pSG5). Three hours after transfection, the cultures were washed and re-fed with medium 199 and 0.2% FBS.

Depending on experiment, 24 hours post transfection, cells were stimulated with UII (concentrations as indicated); cultures that were to receive pharmacological inhibitors were incubated with them 45 minutes prior to UII stimulation at the indicated doses. Cardiomyocyte cultures were harvested 24-72 hours post treatment (per experiment) and luciferase activity was determined by luminometry (Model TD 20/20 Luminometer, Turner Designs, Sunnyvale, CA) using a commercially available kit (Luciferase Substrate, Promega, Madison, WI). The data shown represent the mean ± SEM of three transfections. The data were graphed and analyzed by one-way ANOVA using the GraphPad Prism software package and *post-hoc* comparisons were analyzed by Bonferroni's all pairwise comparisons test.

Total RNA isolation

Total RNA was extracted from rat cardiomyocyte cultures treated as indicated. Culture medium was removed from cell cultures and lysed with 1 mL of Trizol Reagent, placed in 1.5 mL microcentrifuge tubes and incubated at room temperature for 5 minutes. After incubation, 200.0 µl of chloroform was added to the tubes and incubated at room temperature for 3 minutes. The suspensions was then centrifuged at 12,000 x g for 15 minutes at 4°C and the upper phases transferred to 1.5 ml microcentrifuge tubes to which 500.0 µl of isopropanol was added to precipitate the RNA. The tubes were then incubated at room temperature for 15 minutes and then centrifuged at 12,000 x g for 15 minutes to pellet the RNA. The supernatants were discarded and the pellets re-suspended in 75% ethanol. The tubes were centrifuged at 7500 x g for 5 minutes at 4°C and the

supernatants discarded. The RNA pellets were re-suspended in 20.0 μl of nuclease free water. The total RNA was treated with DNase to remove any genomic DNA contamination and then filter purified. The concentrations of the suspended RNA was measured by UV spectrophotometry.

Reverse Transcription and PCR

Two micrograms of total RNA collected from each treatment was used as template for generation of cDNA. The total RNA was incubated in a reaction containing 1.0 μl of random hexamer primers (100ng), 1.0 μl of 10mM dNTPs and nuclease free water to a final volume of 13.0 μl. This reaction was heated to 65°C for 5 minutes and then incubated on ice to allow the primers to anneal to the RNA template. Following the annealing reaction, 4.0 μl of 5X reverse strand buffer, 1.0 μl of 0.1 M DTT, 1.0 μl of RNAseOUT™, and 1.0 μl of Superscript III (Invitrogen) reverse transcriptase (200 units/μl) was added and incubated at 25°C for 5 minutes, and then immediately incubated at 50° C for 1 hour. After 1 hour, the reactions were terminated by heating to 70° C for 15 minutes.

The cDNA generated from the previous step was used as template for PCR reactions. To a total of 1.0 μl of cDNA, 1.0 μl each of specific forward and reverse primers were used to amplify either UIIR, ANF, BNP, βMHC or SkA (Table 1). Nuclease free water was used to bring the template/primer mixture volume to a total of 15.0 μl, to which, 10.0 μl of 2.5X PCR Master Mix was added (Eppendorf). For loading control, GAPDH was also amplified to ensure equal loading of samples. PCR reactions

were carried our on a MasterCycler Gradient thermal cycler (Eppendorf) in standard 35 cycle reactions. Annealing temperatures used were dependent on the specific primer pair used (Table 1). PCR products were electrophoresed on a 2.0% agarose gel, stained with ethidium bromide and visualized under UV light.

Western Blot Analysis

Total protein was isolated from cardiomyocyte cultures treated as indicated. Protein concentration was determined by BCA assay (Pierce). Twenty micrograms of total protein collected from each treatment was used for western blot analysis. Kaleidoscope pre-stained protein marker (BioRad) was used to visualize electrophoresis and Broad range biotinylated protein marker (Cell Signaling Technology) was used for visualization of standard on blot. Samples were incubated at 95° C for 5 minutes in laemmli sample buffer (1X final concentration) and then loaded on a Tris-glycine SDS-PAGE gel (12% polyacrylamide) and electrophoresed for 2.5 hours at 120 volts. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotting for 2 hours at 35 volts. Membranes were then blocked in 1X Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) plus 5% nonfat milk overnight at 4° C. After blocking, the membrane was incubated with 1X TBST and 5% milk containing a 1:1000 dilution of a primary antibody (Table 2) as indicated for 3 hours at room temperature. Following primary antibody incubation, the membranes were washed for 10 minutes 3X in 1X TBST. After washes, the membrane was incubated with 1X TBST and 5% milk containing the necessary HRP-conjugated secondary antibody (Table 2) diluted 1:1000

and an HRP-conjugated anti-biotin antibody (Cat#7075, Cell Signaling Technology) diluted 1:2000 for one hour at room temperature. Following secondary antibody incubation, the membranes were washed for 10 minutes 3X in 1X TBST. After the wash, the membranes were incubated with 5.0 mL 1X LumiGLo reagent (Cat#7003, Cell Signaling Technology) for one minute. Following incubation with LumiGlo, films were exposed from the membranes and developed. The membranes were then stripped and re-probed for GAPDH as control.

Immunoprecipitation

Total cell lysate was isolated from cardiomyocyte cultures treated as indicated. 14-3-3β was immunoprecipitated using a commercially available kit (Protein G Immunoprecipitation Kit, Sigma, St. Louis, MO). Briefly, whole cell lysate was incubated with 14-3-3β antibody overnight at 4°C. The lysate/antibody mixture was then incubated with protein G beads overnight at 4°C after which, the mixture was transferred to a spin column. The immune complex was immobilized on the spin column by centrifugation at 12,000 x g for 30 seconds at 4°C. The immune complex was then washed 5X in 1X IP buffer, each time centrifuged at 12,000 x g for 30 sseconds at 4°C. After the washes, the immune complex was resuspended in 1X laemelli sample buffer and heated at 95°C for 5 minutes. After heating, the samples were immediately loaded onto a 12% polyacrylamide gel and electrophoresed. The protein was then transferred to a nitrocellulose membrane and probed for HDAC5 using a specific antibody. Antibodies

used are listed in Table 2. The membranes were then stripped and reprobed for input (14-3-3β) to ensure equal loading.

Figure 5. Infection of cardiomyocyte culture with AdUIIR. AdUIIR expresses rat UIIR from a CMV driven promoter and GFP from an independent CMV promoter. Crude viral supernatant was added to medium 199 supplemented with 0.2% FBS at a dilution of 10^{-3}. The cultures were infected for 24 hours and then washed and re-fed with medium 199 and 0.2% FBS. The expression of GFP was visualized 24 hours later by fluorescent microscopy.

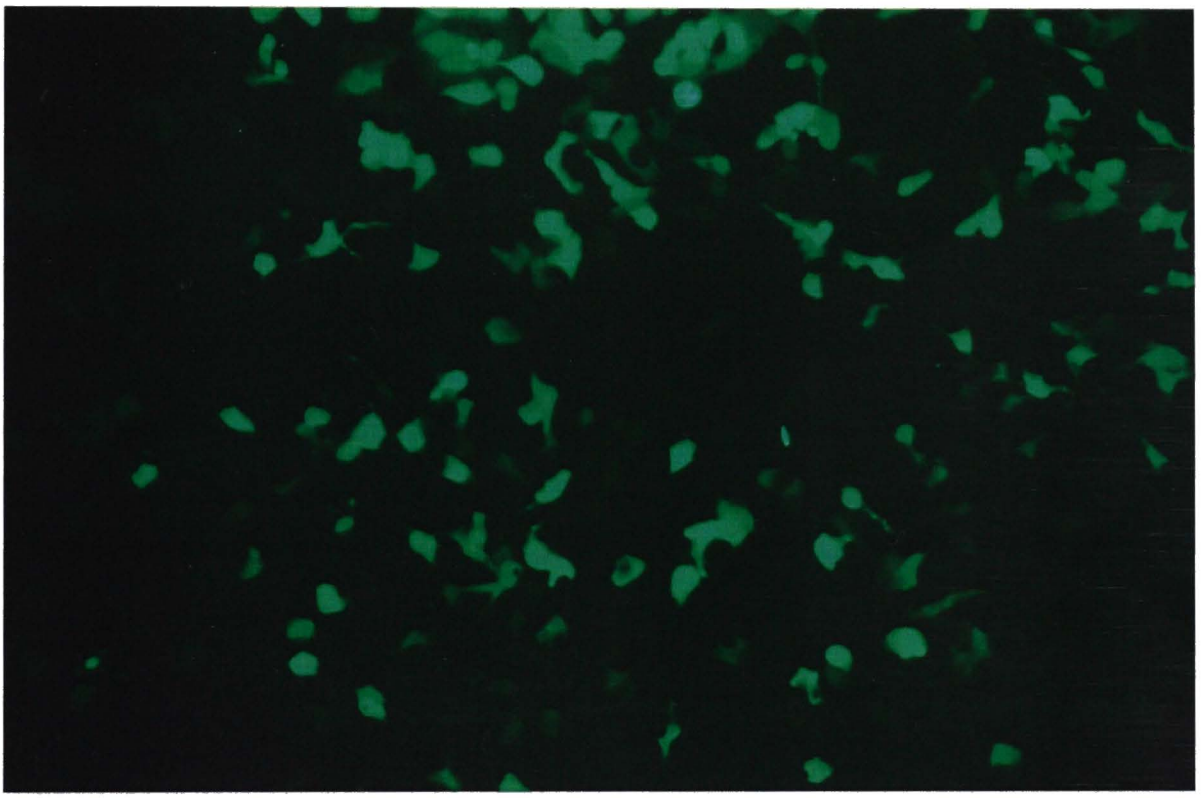


Figure 6. Overall scheme for infection/transfection experiments. Cardiomyocytes were cultured as described. Total serum concentration was reduced in increments from 10% down to 0.2% to avoid serum-induced hypertrophy. Transfections were done prior to infections due to mild toxicity of transfection reagent and infection with virus. During transfection, the serum was reduced to 0%, after which, cultures were re-fed with 0.2% serum medium in which they remained until assay end-point.

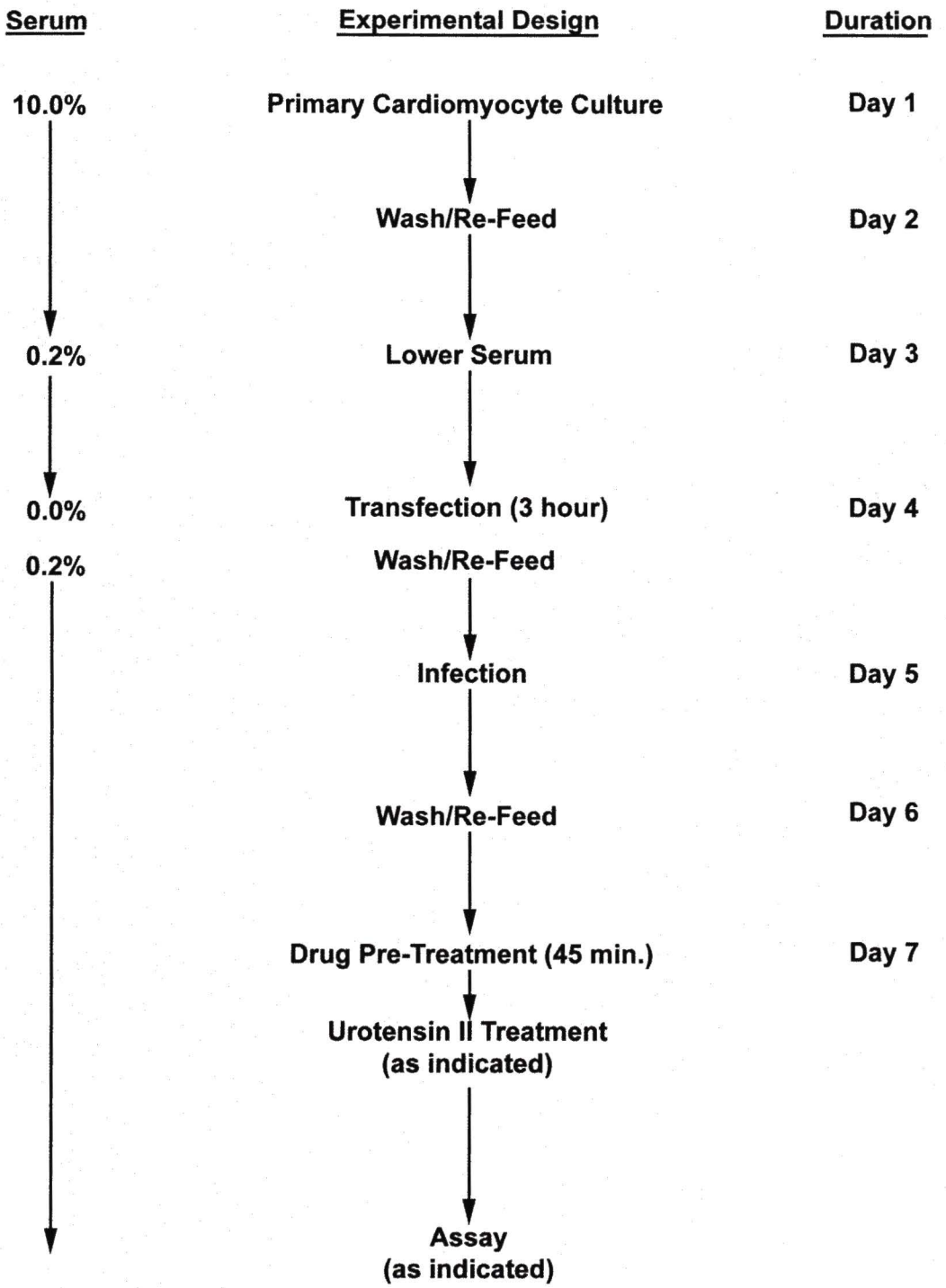

Serum
Experimental Design
Duration
10.0%
Primary Cardiomyocyte Culture
Day 1
Wash/Re-Feed
Day 2
0.2%
Lower Serum
Day 3
0.0%
Transfection (3 hour)
Day 4
0.2%
Wash/Re-Feed
Infection
Day 5
Wash/Re-Feed
Day 6
Drug Pre-Treatment (45 min.)
Day 7
Urotensin II Treatment
(as indicated)
Assay
(as indicated)

Table 1. Sequence of primers used for RT-PCR amplification. PCR primers were designed using PrimerQuest^{SM} (©2006 Integrated DNA Technologies). Primers were chosen based on the following criteria: i) minimum length of 20-25 base pairs (bp), ii) a theoretical melting temperature (Tm) between 55-62°C and iii) a GC content of 50% or less with minimal primer-dimer and internal secondary structure. The primers designed by PrimerQuest^{SM} were then submitted for synthesis by Integrated DNA Technologies (Coralville, IA). Prior to experimental amplification, actual melting temperature (annealing temperature) was determined empirically by setting up standard PCR reactions with a ± 10°C gradient around the theoretical Tm. Tm values reported in Table 1 are actual annealing temperatures used.

Gene Amplified	Primers	Tm/Product Size
UIIR		
Forward	5'-CTGTGACTGAGCTGCCTGGTGAC-3'	61°C/296bp
Reverse	5'-GGTGGCTATGATGAAGGGAAT-3'	
ANF		
Forward	5'-TGCCGGTAGAAGATGAGGTC-3'	56°C/200bp
Reverse	5'-AGCCCTCAGTTTGCTTTTCA-3'	
BNP		
Forward	5'- GACGGGCTGAGGTTGTTTTA-3'	56°C/182bp
Reverse	5'- TTGTGCTGGAAGATAAGAAA-3'	
βMHC		
Forward	5'-CTCCCAAGGAGAGACGACTG-3'	56°C/303bp
Reverse	5'-CCCTTGGTGACGTACTCGTT-3'	
SkA		
Forward	5'- TGCCCATTTATGAGGGCTAC-3'	56°C/398bp
Reverse	5'- GGCATACAGGTCCTTCCTGA-3'	
GAPDH		
Forward	5'GTGTGAACGGATTTGGCCGTATGG-3'	56°C/746bp
Reverse	5'-TCATACTTGGCAGGTTTCTCCAGG-3'	

Table 2. Antibodies used for western blotting and immunoprecipitation. The following table lists the primary and secondary antibodies used for western blotting and immunoprecipitation along with their commercial sources.

Protein	Antibody	Source
UIIR		
1°	Rabbit anti-rat Urotensin II Receptor IgG	Alpha Diagnostics
2°	Anti-rabbit IgG, HRP-linked	Cell Signaling Technology
pCaMKI (Thr178)		
1°	Mouse anti rat phospho-CaMKI IgG	Thomas Soderling[1]
2°	Anti-mouse IgG, HRP-linked	Cell Signaling Technology
pERK1/2 (Thr202/Tyr204)		
1°	Mouse anti rat phospho-ERK1/2 IgG	Cell Signaling Technology
2°	Anti-mouse IgG, HRP-linked	Cell Signaling Technology
p-p38 (Thr180/Tyr182)		
1°	Mouse anti rat phospho-p38 IgG	Cell Signaling Technology
2°	Anti-mouse IgG, HRP-linked	Cell Signaling Technology
HDAC5		
1°	Rabbit anti-rat HDAC5 IgG	Cell Signaling Technology
2°	Anti-rabbit IgG, HRP-linked	Cell Signaling Technology
p-PKD (Ser744/748)		
1°	Rabbit anti-rat phospho-PKD	Cell Signaling Technology
2°	Anti-rabbit IgG, HRP-linked	Cell Signaling Technology
GAPDH		
1°	Mouse anti-rat GAPDH IgG	Abcam
2°	Anti-mouse IgG, HRP-linked	Cell Signaling Technology
14-3-3β (Immunoprecipitation)		
1°	Rabbit anti-rat 14-3-3β IgG	Abcam

[1] Thomas Soderling, Vollum Institute, Oregon Health and Sciences University, Portland, Oregon 97239

CHAPTER III

RESULTS

Preface

Many attempts have been put forth to isolate a cell line that exhibits the characteristics of primary cardiomyocytes and that responds to various stimuli in a manner consistent with that observed *in vivo*. Unfortunately, the putative cardiomyocyte-like cell lines do not behave exactly as differentiated cardiomyocytes. In order to approximate more faithfully the endogenous cardiomyocyte responses and gene expression patterns, we utilized a primary neonate ventricular cardiomyocyte cell model in the following studies.

The Gq coupled receptor, GPR14, was recently de-orphanized due to its sensitivity to stimulation by the peptide agonist, UII[133]. UII was originally isolated from the urophysis of fish and has since been recognized endogenously in mammals including man[132]. UII elicits a strong vasoconstrictive response and has been implicated in cardiac hypertrophy. Interestingly, the UII receptor becomes up-regulated in the heart under many of the same forms of stress that result in cardiac hypertrophy[39, 137, 141, 149]. Work has been done demonstrating that UII is capable of stimulating markers of cardiac hypertrophy in ventricular cardiomyocytes; however, the entire signal transduction mechanism through which this is accomplished has not been fully delineated.

The CaM kinase signaling pathway is initially activated by rises in intracellular calcium. The first member of the cascade, CaMKK, once active, phosphorylates and activates CaMKI. We have shown that CaMKI is capable of stimulating the expression of hypertrophy-sensitive genes as well as MEF2[38]. The MAP kinase p38 is a major activator of MEF2[104]. p38 was also shown to be activated by the CaM kinase pathway. In addition to activation by p38, MEF2 must be released from the negative control of class II HDACs. We have shown that CaMKI is capable of causing the translocation of HDAC5 from the nucleus to the cytoplasm, thus resulting in the activation of MEF2[126].

Gq coupled receptors activate PLC which acts to cleave PIP2 to IP3 and DAG. IP3 is capable of stimulating the release of calcium from the SR via the IP3 receptor. It is this increase in intracellular calcium that is translated to kinase activity by the CaMKs. Since the UIIR is coupled to Gq, we hypothesized that the stimulation of UIIR resulting in hypertrophic gene induction is dependent on CaMKK.

In order to further characterize the UII-dependent hypertrophic signaling mechanism, we first tested whether other known hypertrophic agonists could up-regulate UIIR in cardiomyocytes. We next determined whether CaMKK was necessary for the UII-dependent induction of ANF, SkA and MEF2. Effects of CaMKK inhibition on the UII-dependent activation of CaMKI were then determined as CaMKI is a major effector of CaMKK. Since UII is known to activate p38 and ERK1/2 MAP kinases, we next determined the effects of CaMKK inhibition on the UII-dependent stimulation of p38 and ERK1/2. We also assessed the effects of MAP kinase inhibition on UII stimulation of ANF, SkA and MEF2. The ability of UII to stimulate the nucleocytoplasmic shuttling of

HDAC5 was determined. In addition, we looked at the CaMKK dependence of UII stimulated HDAC5 localization.

The data presented herein are the first to demonstrate that: ii) UII-dependent hypertrophy gene induction is dependent on CaMKK, iii) UII stimulates CaMKI activation, iv) UII-dependent activation of p38 and ERK1/2 requires CaMKK, v) UII stimulates HDAC5 translocation in a CaMKK dependent manner and vi) UII is able to activate PKD

Database query of microarray expression data regarding UIIR

Very little is known about the expression of the UIIR in cardiomyocytes other than the receptor is expressed during cardiac disease states. Therefore we decided to query the GEO microarray database at the NCBI (http://www.ncbi.nlm.nih.gov/geo/). We found that Eric Olson (UT Southwestern) submitted array data based on experiments in neonate cardiomyocytes that were treated with phenylephrine (PE) and Purine Activator of Myocyte Hypertrophy (PAMH). Buried in the array data, we found that UIIR was up-regulated in cardiomyocytes treated with 10 μM PE, an α-1 adrenergic agonist (Figure 4). Though the array data was called as absent, this by no means invalidates the biological data, but rather should be looked at more carefully (Pat Cooke, Affymetrix; personal communication) in order to determine whether the biological data is informative. Since mechanical stress is know to increase the availability of humoral factors such as α-1 adrenergic agonists, we hypothesized that the onset of hypertrophy

may contribute to the expression of UII and its receptor thereby functioning in an autocrine mechanism maintaining the hypertrophic phenotype.

Expression of UIIR in myocytes treated with hypertrophic agonists.

In order to validate the array data, we designed PCR primers to amplify UIIR. Cardiomyocyte cultures were treated with 10 μM PE for 24 hours and total RNA was extracted following the Trizol protocol (Invitrogen). Total RNA was then used for template for reverse transcription. The cDNA generated from the RT reaction was then used as template to amplify UIIR and GAPDH as control. Treatment of cardiomyocytes with both PE and AngII for 24 hours up-regulated the UIIR messenger RNA (Figure 7). To our knowledge, this is the first evidence that demonstrates the ability of an α-1 adrenoreceptor agonist up-regulating UIIR in cardiomyocytes.

We next wanted to determine whether increased mRNA of UIIR correlated with an increase in UIIR protein. To this end, we stimulated cardiomyocyte cultures with AngII and PE as before and isolated total protein. The total protein was resolved by PAGE (12% acrylamide), transferred to nitrocellulose, and blotted for UIIR using a specific antibody (Table 2). Treatment of cardiomyocytes with both PE and AngII resulted in the up-regulation of UIIR with no change in the expression of GAPDH (Figure 8). These data demonstrate that along with an increase in mRNA, the protein for UIIR is up-regulated in cardiomyocytes in response to PE and AngII.

Infection of cardiomyocytes with AdUIIR-GFP

Preliminary experiments were done in order to determine whether UII could induce ANF gene expression in primary cardiomyocytes. In our hands, UII had no effect at a concentration of 100 nM (data not shown). There are some arguments in the literature as to whether or not UIIR is expressed in normal cardiomyocytes. As we have shown, the hypertrophic agonists PE and AngII are capable of inducing the expression of UIIR. Using PE to first induce UIIR expression in primary cardiomyocytes and then stimulating with UII would confuse any results because the α-1 adrenoreceptor and UIIR are both G_q coupled. To circumvent this issue, we obtained an adenovirus that expresses UIIR (AdUIIR-GFP) from Walter Thomas (Baker Heart Research Institute, Melbourne, Australia). We were also given a control adenovirus that expresses GFP only (AdGO-GFP). E1A positive HEK293 cells were infected with AdUIIR-GFP in order to amplify the virus. Another culture was infected in parallel in order to amplify the control virus as well. To obtain a high titer viral stock, a T-175 flask was infected and after approximately 24 hours, the cells and media were collected and were freeze-thawed five times, alternating in dry ice and a 37°C water bath. The crude viral supernatant was then purified with a commercially-available kit (ViraBind Adenovirus Purification Kit, Cell Biolabs, Inc.) and stored in liquid nitrogen. Primary cardiomyocytes were infected with the crude supernatant at a dilution of 10^{-3}. This concentration of viral supernatant was sufficient to infect approximately 95% of the cardiomyocyte culture (Figure 5).

UII stimulates ANF and SkA promoter-reporter activities in AdUIIR-GFP infected cardiomyocytes

We first wanted to determine the dose-dependency of UII stimulation of ANF and SkA. Each well received 50 ng of either the ANF or SkA promoter-reporters. After three hours, the cells were washed twice with serum free medium 199 and re-fed with medium 199 supplemented with 0.2% FBS. The cells were allowed to recover for 24 hours and were then infected with AdUIIR-GFP. After the recovery periods, cardiomyocytes were treated with varying doses of UII (0, 10, 25, 50, 75, 100, 250, and 500 nM) for a period of 72 hours, at which point, the cultures were lysed, harvested and assayed as described. UII was able to increase both ANF and SkA promoter reporter activities in a dose-dependent manner (Figures 9 and 11). Data represent the mean ± SEM with n=3.

In order to determine whether UII stimulation of ANF and SkA was specific for cultures infected with AdUIIR, primary rat cardiomyocytes were transfected as described above. The cells were allowed to recover for 24 hours and were then infected with either the control virus (AdGO-GFP) or AdUIIR-GFP. The cells were infected for 24 hours and then washed twice with serum free medium 199 and re-fed with medium 199 supplemented with 0.2% FBS. The cells were then allowed to recover from viral infection for 24 hours. After 24 hours, the cardiomyocyte cultures were washed once with serum free medium 199 and then stimulated with 100 nM UII in medium 199 with 0.2%serum. The cultures were stimulated with UII for 24 hours. After stimulation, cells were lysed and harvested by scraping. The cell lysate was then assayed for relative luciferase activity using a luminometer. Promoter reporter activities were significantly

increased in AdUIIR-GFP infected cardiomyocytes when compared with AdGO-GFP infection (ANF: $t=10.04$; $p<0.0001$; SkA: $t=3.858$, $p=0.0182$) (Figures 10 and 12). The data shown are mean ± SEM for $n=4$. The data were analyzed by a two-tailed t-test for significance.

UII stimulates MEF2 enhancer-reporter activity in AdUIIR-GFP infected cardiomyocytes

CaMKs are directly involved with the activation of the transcriptional enhancer MEF2[125]. MEF2 activation results in the up-regulation of hypertrophy-sensitive genes and may be a major point of control for hypertrophy. In the normal adult cardiomyocyte, MEF2 transcriptional activity is held inactive by class II histone deacetylases (HDACs) until the necessary signaling results in the phosphorylation of HDACs. This phosphorylation event causes the 14-3-3-dependent nuclear ejection of either HDAC 4 or 5 resulting in a release of negative regulation of MEF2. We have been able to study the activity of MEF2 by transfection experiments of an MEF2-dependent enhancer reporter.

We first wanted to determine the dose-dependency of UII stimulation of MEF2. Each well received 250 ng of the MEF2 enhancer-reporter. After 3 hours, the cells were washed twice with serum free medium 199 and re-fed with medium 199 supplemented with 0.2% FBS. The cells were allowed to recover for 24 hours and were then infected with AdUIIR-GFP. After the recovery periods, cardiomyocytes were treated with varying doses of UII (0, 10, 25, 50, 75, 100, 250, and 500 nM) for a period of 72 hours, at which point, the cultures were lysed, harvested and assayed as described. UII was able to

increase MEF2 enhancer-reporter activity in a dose-dependent manner (Figure 13). Data represent the mean ± SEM with n=3.

In order to determine whether UII stimulation of MEF2 was specific for cultures infected with AdUIIR, primary rat cardiomyocytes were transfected as described above. After transfection, the cells were allowed to recover for 24 hours and were then infected with either the control virus (AdGO-GFP) or AdUIIR-GFP. The cells were infected for 24 hours and then washed twice with serum free medium 199 and re-fed with medium 199 supplemented with 0.2% FBS. The cells were then allowed to recover from viral infection for 24 hours. After 24 hours, the cardiomyocyte cultures were washed once with serum free medium 199 and then stimulated with 100 nM UII in medium 199 with 0.2%serum. The cultures were stimulated with UII for 24 hours. After stimulation, cells were lysed with 1X reporter lysis buffer (Promega) and harvested by scraping. The cell lysate was then assayed for relative luciferase activity using a luminometer (Turner). MEF2 enhancer-reporter activity was significantly increased in AdUIIR-GFP infected cardiomyocytes when compared with AdGO-GFP infection (Figure 14; $t=7.594$; $df=6$; $p=0.0003$). The data shown are mean ± SEM for N=4. The data were analyzed by a two-tailed T-test for significance.

Specific CaMKK inhibition abolishes UII induced ANF and SkA induction

Since UIIR is G_q coupled, we hypothesized that the canonical rise in intracellular calcium resulting from UII stimulation would be sufficient to activate CaMKK thereby activating CaMKI. To test the dependency of UII signaling on CaMKK, we transfected

cardiomyocyte cultures with either the ANF promoter-reporter (50ng/well) or the SkA promoter-reporter (50 ng/well) for a period of three hours and were then washed with serum-free medium 199 and re-fed with medium 199 supplemented with 0.2% FBS. After a recovery period of 24 hours, the cultures were infected with AdUIIR-GFP in wells that were to receive UII. The infection was allowed to proceed for 24 hours at which point the cells were washed and re-fed as described. One hour prior to UII stimulation, designated wells were pre-treated with either 250 ng/mL STO-609 or DMSO as vehicle. After one hour of pre-treatment, designated wells were then stimulated with UII. UII stimulation proceeded for 24 hours at which point, cells were lysed and harvested for luciferase activity assays as described. As seen previously, UII elicited a significant increase in promoter-reporter activities (ANF: $t=9.427$, $p<0.001$; SkA: $t=6.465$, $p < 0.01$) when compared with control (Figures 15 and 16). Inhibition of CaMKK activity in UII stimulated cultures resulted in a complete loss of ANF and SkA promoter-reporter activities (ANF: $t=11.27$, $p<0.001$; SkA: $t=6.881$, $p<0.001$) indicating that UII hypertrophic signaling is dependent on functional CaMKK (Figures 15 and 16). Vehicle control (DMSO) had no effect on UII stimulated promoter-reporter activities (ANF: $t=1.993$, $p>0.05$; SkA: $t=1.512$, $p>0.05$). The data were first analyzed by one-way ANOVA and the between treatment variance was significantly different (ANF: $F=60.78$; $df=3, 8$; $p<0.0001$; SkA: $F=24.16$, $p=0.0002$); therefore, all pairwise comparisons were analyzed *post hoc* using Bonferroni's test for statistical significance. The data presented in Figures 15 and 16 are the mean +/- SEM with N=3. These results are the first to demonstrate that UII-dependent hypertrophy signaling involves the downstream

activation of CaMKK. The CaMKK inhibitor (STO-609) is a highly selective, potent, ATP-competitive inhibitor of CaMKK [IC_{50} = 120 ng/ml and 40 ng/ml for CaMKKα and CaMKKβ isoforms, respectively. Other kinases are inhibited by STO-609 (CaM-KII, MLCK (IC_{50} ~10 μg/ml), CaMKI, CaMKIV, PKA, PKC, and p42 MAP kinase (IC_{50} >10 μg/ml); however, they require a much higher concentration of STO-609—all at μg/mL amounts.

Specific CaMKK inhibition abolishes UII induced MEF2 activity

Since many hypertrophic gene expression events in cardiomyocytes require MEF2 activity, we wanted to determine whether UII stimulation could activate MEF2 in a CaMKK dependent manner. Primary cardiomyocyte cultures were transfected with the MEF2 enhancer-reporter (250ng/well) as described. The transfection was allowed to proceed for 3 hours at which point, the cultures were washed and re-fed in medium 199 supplemented with 0.2% FBS. The cells were allowed to recover for 24 hours and then designated wells infected with AdUIIR-GFP. The infection was allowed to proceed for 24 hours and the cultures were then washed and re-fed as described. One hour prior to UII stimulation, designated wells were pre-treated with either 250ng/mL STO-609 or DMSO as vehicle. After one hour of pre-treatment, designated wells were then stimulated with UII. UII stimulation proceeded for 72 hours at which point, cells were lysed and harvested for luciferase activity assays as described. As was expected, UII elicited a significant increase in MEF2 enhancer-reporter activity ($t=8.871$; $p<0.001$) when compared with control cultures (Figure 17). Inhibition of CaMKK activity in UII

stimulated cultures resulted in a complete loss of MEF2 enhancer-reporter activity ($t=9.548$, $p<0.001$) indicating that activation of MEF2 by UII is dependent on CaMKK activity. Vehicle control (DMSO) had no effect on UII stimulated MEF2 enhancer-reporter activity ($t=1.395$, $p>0.05$). The data were first analyzed by one-way ANOVA and the between treatment variance was significantly different ($F=49.10$; $df=3, 8$; $p<0.0001$); therefore, all pairwise comparisons were analyzed *post hoc* using Bonferroni's test for statistical significance. The data presented in Figure 17 are the mean ± SEM with $N=3$. These data strongly suggest that UII-dependent activation of MEF2 requires CaMKK.

UII stimulated expression of ANF, BNP, βMHC and SkA is dependent on CaMKK

We have determined that UII stimulation of cardiomyocytes results in an induction of hypertrophy-sensitive promoter activities. The stimulation of ANF and SkA promoter activities by UII was also determined to be dependent on CaMKK. We next wanted to determine whether UII was able to increase mRNA levels for ANF, BNP, βMHC and SkA. We also tested whether UII stimulation of these genes was dependent on CaMKK. Caardiomyocyte cultures were infected with AdUIIR, allowed to recover, and were then stimulated with UII for 48 hours with or without STO-609. After stimulation, total RNA was collected, reverse transcribed and ANF, BNP, bMHC and SkA were PCR amplified using gene-specific primers. Treatment of cardiomycoyte cultures with UII for 48 hours resulted in the up-regulation of ANF, BNP, βMHC and SkA (Figure 18). In the presence of STO-609, UII was not able to stimulate the

expression of these genes. These data confirm that in addition to promoter activation, UII is also able to induce the mRNAs of ANF, BNP, βMHC and SkA.

UII stimulation results in the activation of CaMKI

One of the major downstream effectors of CaMKK is CaMKI. In addition to calcium/calmodulin, CaMKI requires phosphorylation by CaMKK to be active. Next, we wanted to determine whether UII stimulation was able to activate CaMKI. We stimulated AdUIIR-infected cultures with 100nM UII for 0, 2, 5, 10, 30 and 60 minutes, collected whole cell lysate, and resolved 20μg of total protein on a 12% polyacrylamide gel. The protein was then transferred to a nitrocellulose membrane for blotting. The primary antibody that we used recognizes only CaMKI phosphorylated on Thr178 (active CaMKI). There was a robust UII-induced phosphorylation of CaMKI by 2 minutes with a maximum activation at 10 minutes (Figure 19). CaMKI phosphorylation persisted to the 60 minute time-point. The membrane was stripped and re-probed for GAPDH to ensure equal loading of lanes.

Since CaMKI requires CaMKK to be active, we next wanted to determine whether CaMKK inhibition with STO-609 would inhibit UII-dependent activation of CaMKI. We stimulated AdUIIR-infected cultures with 100 nM UII for 2 and 5 minutes in the absence or presence of STO-609 (250 ng/mL). As seen previously, UII stimulated the activation of CaMKI at 2 and 5 minutes; however, in the presence of STO-609, UII was not able to stimulate the phosphorylation of CaMKI (Figure 20). These results

demonstrate that UII-dependent activation of CaMKI is dependent on the activity of CaMKK.

Constitutively-active CaMKI rescues STO-609 inhibition of UII-stimulated ANF and SkA promoter-reporter activities

We have shown that STO-609 inhibition of CaMKK abolishes UII-stimulated ANF and SkA promoter-reporter activities. Since CaMKI is immediately downstream of CaMKK we hypothesized that constitutively active (not dependent on CaMKK or calcium/calmodulin) CaMKI will recapitulate UII stimulation of ANF and SkA promoter-reporter activities when CaMKK is inhibited by STO-609. We transfected cardiomyocyte cultures with either ANF (50 ng/well) or SkA (50ng/well) followed by infection with AdUIIR in designated wells. Some of the wells were co-transfected with constitutively-active CaMKI (10 ng/well). After infection with AdUIIR, designated wells were stimulated with UII (100 nM) for 72 hours in either the absence/presence of STO-609 (250 ng/mL). As we have previously shown, UII stimulation increased ANF and SkA promoter-reporter activities approximately 2-fold when compared with control (ANF: $t=4.780$, $p<0.01$; SkA: $t=8.595$, $p<0.001$) while CaMKK inhibition completely blocked UII stimulation of ANF and SkA as seen previously (Figures 21 and 22). More importantly, constitutively-active CaMKI was able to completely recapitulate UII stimulation of ANF and SkA in the presence of STO-609 (ANF: $t=13.07$, $p<0.001$; SkA: $t=10.93$, $p<0.001$). The ability of CaMKI to rescue UII stimulation of ANF and SkA

promoter activity further demonstrates the involvement of the CaM kinase pathway with the UII-mediated hypertrophy response in cardiomyocytes.

Constitutively-active CaMKI rescues STO-609 inhibition of UII-stimulated MEF2 enhancer-reporter activity

It is well know that MEF2 activity is critical for stimulation of hypertrophy marker genes in cardiomyocytes and is sensitive to stimulation by members of the CaM kinase pathway. We next wanted to determine whether constitutively-active CaMKI could also rescue MEF2 activity stimulated by UII when CaMKK was inhibited. We transfected cardiomyocytes with the MEF2 enhancer-reporter (250ng/well). Designated wells were co-transfected with constitutively-active CaMKI (10ng/well). Following transfection, the necessary wells were infected with AdUIIR. After infection, the cells were stimulated with UII (100 nM) in either the absence/presence of STO-609 for 72 hours. As seen previously, UII significantly stimulated the activity of MEF2 ($t=4.693$, $p<0.01$) when compared with control while STO-609 completely inhibited this stimulation ($t=6.822$, $p<0.001$) (Figure 23). However, expression of CaMKI completely rescued UII stimulation of MEF2 in the presence of STO-609 ($t=10.09$, $p<0.001$) (Figure 23). The data were first analyzed by one-way ANOVA and the between treatment variance was significantly different ($F=58.89$, $p<0.001$); therefore, all pairwise comparisons were analyzed *post hoc* using Bonferroni's test for statistical significance. The ability of CaMKI to rescue UII stimulation of MEF2 when CaMKK was inhibited

strengthens our argument that the CaM kinase pathway is necessary for hypertrophic gene induction by UII.

Inhibition of AKT/PKB has no effect on UII stimulation of ANF and SkA promoter activity

One of the reported downstream substrates of CaMKK is AKT/PKB. We have shown that UII stimulation is dependent on CaMKK; therefore, AKT could be involved with the hypertrophic response and may contribute to ANF and SkA promoter activation. To determine if AKT was involved with the UII-mediated stimulation of ANF and SkA promoters, we transfected cardiomyocytes with either 50ng/well of ANF (Figure 24) or 50ng/well of SkA (Figure 25). After transfection, designated wells were infected with AdUIIR and allowed to recover. UII stimulation (100 nM) proceeded for 72 hours alone or in the presence of 5 μM 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (AKTi), a potent and selective inhibitor of AKT/PKB. As seen previously, UII stimulation resulted in a significant increase in ANF promoter activity ($t=4.559$, $p<0.05$) (Figure 24) as well as SkA promoter activity ($t=4.618$, $p<0.05$) (Figure 25). In the presence of AKTi, UII-stimulation of ANF and SkA promoter activities was unchanged. These results show that AKT/PKB is not involved with the hypertrophic response mediated by UII and lends support to our hypothesis that CaMKI is the major downstream effector of UII stimulation of CaMKK.

Inhibition of AKT/PKB has no effect on UII stimulation of MEF2 activity

We next wanted to determine whether AKT/PKB inhibition had any effect on UII stimulation of MEF2 enhancer activity. In other cell types, it appears that AKT/PKB may play a role in the activation of MEF2[147]. To test this possibility, we transfected cardiomyocyte cultures with the MEF2 enhancer-reporter (250 ng/well), infected designated wells with AdUIIR and stimulated with UII (100 nM) for 72 hours alone or in the presence of 5 μM AKTi. UII stimulation resulted in a significant activation of MEF2 ($t=12.12$, $p<0.001$) (Figure 26). UII stimulation of MEF2 activity was not affected by inhibition of MEF2 ($t=1.759$, $p>0.05$) suggesting that UII-dependent activation of MEF2 is independent of AKT/PKB.

UII stimulates the activation of p38 and ERK1/2 MAP kinases

UII stimulation of the phosphorylation p38 and ERK1/2 was previously reported in primary neonate cardiomyocyte cell cultures[78]. We wanted to confirm these results in our laboratory in order to gain a better understanding of the effects of UII stimulation on our own cell culture model as well as to determine whether UII stimulation of the MAP kinases was dependent on CaMKK. To this end, we infected cardiomyocyte cultures with AdUIIR for 24 hours and then stimulated the cultures with 100 nM UII for a period of 0, 2, 5, 10, 30 and 60 minutes. After UII stimulation, we collected whole cell lysates and equally loaded 20μg of total protein on a 12% acrylamide gel, transferred to nitrocellulose and blotted for either active p38 (phospho-Thr_{180}/Tyr_{182}) or active ERK1/2 (phospho-Thr_{202}/Tyr_{204}). UII stimulation of cardiomyocytes resulted in the activation of

p38 after 2 minutes, reached a maximum at 5 minutes, and returned to near-control by 60 minutes (Figure 27). Likewise, ERK1/2 activation was obvious at 2 minutes, maximum at 5 minutes and returned to near-control by 60 minutes of UII stimulation (Figure 27). The membranes were stripped and re-probed for GAPDH to ensure equal loading of total protein. These data confirm that UII stimulation of our cardiomyocyte cell culture model result in a time-dependent activation of p38 and ERK1/2 MAP kinases.

UII-dependent activation of p38 and ERK1/2 requires CaMKK

It has been reported that in neuronal cells, the CaM kinase pathway is capable of activating p38 and ERK1/2[58]. Both p38 and ERK1/2 have been shown to contribute to the hypertrophic phenotype. In addition, p38 was shown to be a major activator of MEF2 activity. We have also shown that CaMKI was able to activate MEF2 as well as induce class II HDAC nucleocytoplasmic shuttling. We wanted to determine whether UII stimulation of p38 and ERK1/2 in cardiomyocyte cultures was dependent on CaMKK activity. Cardiomyocyte cultures were infected with AdUIIR and stimulated with UII (100nM) for 2 and 5 minutes alone or with 250ng/mL of STO-609. Whole cell lysate was collected and 20μg of total protein was resolved on a 12% acrylamide gel and subsequently transferred to a nitrocellulose membrane and blotted for either phospho-p38 or phsopho-ERK1/2. At 2 and 5 minutes, UII stimulated the phosphorylation of p38 and ERK1/2 as seen previously; however, inhibition of CaMKK completely blocked phosphorylation of p38 and ERK1/2 (Figure 28). The membranes were stripped and re-probed for GAPDH to ensure equal loading of total protein. The CaMKK-dependent

phosphorylation of p38 and ERK1/2 has not been previously reported in cardiomyocytes and these data have uncovered an important cross-talk mechanism between the CaM kinase pathway and the MAP kinase pathway.

MAP kinase inhibition results in the inability of UII to stimulate the ANF and SkA promoters

Signaling through $G\alpha_q$ results in the induction of hypertrophic gene expression in cardiomyocytes and is dependent on MAP kinase activation. Since UII is able to induce hypertrophic gene expression through a Gq mechanism, we next wanted to determine whether inhibition of p38 and ERK1/2 would result in the loss of UII-dependent ANF and SkA promoter activities. We transfected cardiomyocytes with either the ANF or SkA promoter-reporters (50ng/well) followed by infection with AdUIIR. We stimulated the cardiomyocytes with 100 nM UII alone or in the presence of either SB203580 (10 μM) or U0126 (10 μM) for 72 hours. UII stimulated the promoter-reporters as seen previously (ANF: $t=7.461$, $p<0.001$; SkA: $t=15.69$, $p<0.001$) (Figures 29 and 30). Inhibition of p38 with SB203580 resulted in a significant decrease in UII-dependent ANF promoter activity when compared with UII stimulation alone (ANF: $t=10.28$, $p<0.001$; SkA: $t=20.39$, $p<0.001$). Likewise, inhibition of ERK1/2 with U0126 resulted in a significant decrease in UII-dependent ANF promoter activity compared to UII stimulation alone (ANF: $t=12.78$, $p<0.001$; SkA: $t=20.63$, $p<0.001$). UII stimulation of the ANF promoter in the presence of

vehicle (DMSO) did not differ from UII stimulation alone (ANF: $t=1.694$, $p>0.05$; SkA: $t=3.493$, $p>0.05$). The inhibition of promoter activities by SB203580 and U0126 were similar to that observed when CaMKK was inhibited (ANF: $t=8.708$, $p<0.001$; SkA: $t=16.75$, $p<0.001$). These data demonstrate that UII activation of the ANF and SkA promoters requires p38 and ERK1/2 MAP kinases.

MAP kinase inhibition blocks UII activation of MEF2

Thus far, we are the first to show that UII is capable of stimulating the activity of MEF2. Since p38 is a major regulator of MEF2 activity, we wanted to determine whether UII stimulation of MEF2 was dependent on the activity of p38. There is some evidence that ERK may also be involved with the regulation of MEF2; therefore, we also wanted to determine whether UII stimulation of MEF2 was dependent on ERK1/2. We transfected cardiomyocytes cultures with the MEF2 enhancer-reporter (250 ng/well) followed by infection with AdUIIR. We next stimulated the cultures with UII (100 nM) alone or in the presence of either SB203580 or U0126. UII stimulation resulted in a significant increase in MEF2 activity when compared with control ($t=8.511$, $p<0.001$) (Figure 31). Inhibition of p38 with SB203580 resulted in a significant decrease in MEF2 activity compared with UII stimulation alone ($t=11.15$, $p<0.001$). Moreover, inhibition of ERK1/2 with U0126 also inhibited UII stimulation of MEF2 activity ($t=10.45$, $p<0.001$). Interestingly, these results are similar to the effect of CaMKK inhibition of UII stimulation ($t=10.68$, $p<0.001$). UII stimulation of MEF2 activity in the presence of vehicle (DMSO) did not differ from UII stimulation alone ($t=3.254$, $p>0.05$).

These data argue that MEF2 stimulation by UII requires p38 and ERK1/2 and that CaMKK may play an upstream role in their activation.

MAP kinase inhibition results in the inability of CaMKI to stimulate the ANF and SkA promoters

Since we had shown that CaMKK inhibition resulted in the inability of UII to stimulate the ANF and SkA promoters and that UII activation of p38 and ERK1/2 was dependent on CaMKK, we wanted to determine whether CaMKI stimulation of ANF and SkA was dependent on p38 and ERK1/2. To test this, we transfected cardiomyocytes with either ANF or SkA promoter-reporters (50 ng/well). Designated wells were also transfected with constitutively-active CaMKI (10 ng/well) alone or with either SB203580 (10 μM) or U0126 (10 μM). CaMKI significantly increased promoter activities (ANF: $t=7.750$, $p<0.001$; SkA: $t=5.459$, $p<0.01$) (Figures 32 and 33). In the presence of SB203580, CaMKI was not able to stimulate the promoters (ANF: $t=5.361$, $p<0.01$; SkA: $t=5.462$, $p<0.01$) when compared with CaMKI alone. Moreover, inhibition of ERK1/2 resulted in the inability of CaMKI to stimulate promoter activity (ANF: $t=5.044$, $p<0.01$; SkA: $t=5.822$, $p<0.01$) when compared with CaMKI alone. CaMKI in the presence of vehicle (DMSO) significantly increased ANF promoter activity compared to control (ANF: $t= 7.232$, $p<0.001$; SkA: $t=5.200$, $p<0.01$). These data are the first to argue that CaMKI activation of the ANF and SkA promoters depends on both p38 and ERK1/2 activity.

MAP kinase inhibition results in the inability of CaMKI to activate MEF2

We have shown that CaMKI activates MEF2. We next wanted to determine whether CaMKI activation of MEF2 was dependent on p38 and ERK1/2. Cardiomyocyte cultures were transfected with the MEF2 enhancer-reporter (250ng/well). Designated wells were co-transfected with constitutively-active CaMKI (10ng/well) alone, or in the presence of either SB203580 or U0126. CaMKI significantly increased ($t=7.834$, $p<0.001$) MEF2 activity compared with control (Figure 34). Treatment of cardiomyocytes with SB203580 completely inhibited CaM kinase stimulation of MEF2 ($t=10.26$, $p<0.001$). Additionally, inhibition of ERK1/2 resulted in a loss of CaMKI stimulation of MEF2 ($t=12.93$, $p<0.001$).

We have known for some time that CaMKI is capable of stimulating the activity of MEF2. What we had not been able to resolve is the apparent caveat of linking a cytosolic activity (CaMKI) with a nuclear event (activation of MEF2). These data are the first to begin to rectify this issue in that p38 and ERK1/2 reside in the cytoplasm until phosphorylated at which point, they are able to enter the nucleus. Our data suggest that CaMKI is able to translate cytosolic rises in intracellular calcium into kinase activity resulting in the activation of MEF2 in the nucleus. These data show that p38 and ERK1/2 are downstream of CaMKI and are necessary for the activation of MEF2.

Dominant negative p38 inhibits UII stimulation of MEF2

p38 is a known activator of MEF2. We next wanted to determine the effect of a dominant negative mutant p38 on UII stimulation of MEF2. We transfected

cardiomyocytes with the MEF2 enahancer-reporter (250ng/well) and designated wells were co-transfected with dominant negative p38 (100ng/well) followed by infection with AdUIIR. Assigned wells were then stimulated with UII for 48 hours. Dominant negative p38 had a modest effect ($t=4.060$, $p<0.05$) on unstimulated MEF2 activity compared with control (Figure 35). UII stimulation resulted in significant increase ($t=5.745$, $p<0.01$) in MEF2 activity while dominant negative p38 was able to significantly decrease ($t=11.79$, $p<0.001$) UII stimulation of MEF2.

Dominant negative p38 inhibits CaMKI stimulation of MEF2

We had previously shown that pharmacological inhibition of p38 resulted in the inability of CaMKI to activate MEF2. We therefore wanted to determine the effect of dominant negative p38 on CaMKI activation of MEF2. Cardiomyocytes were transfected with the MEF2 enhancer-reporter with designated wells also receiving dominant negative p38 and constitutively active CaMKI. There was no difference ($t=0.7726$, $p>0.05$) in MEF2 activity between un-stimultated control cultures and those that received dominant negative p38 (Figure 36). However, dominant negative p38 significantly inhibited CaMKI stimulation of MEF2 ($t=13.14$, $p<0.001$). These data show that the ability of CaMKI to stimulate MEF2 activity is dependent on p38.

UII stimulation induces association of HDAC5 with 14-3-3β

The activity of MEF2 is controlled, in part, by its association with class II HDACs in the nucleus. Class II HDACs repress the activity of MEF2-sensitive promoters by local deacetylation of nucleosomal histones causing the condensation of chromatin. The

repressive influence of HDACs on MEF2 can be relieved by CaMK-dependent phosphorylation of HDACs, which results in their dissociation from MEF2. The dissociation of HDACs from MEF2 is accompanied by 14-3-3-mediated nuclear export of the HDACs.

We have shown that UII stimulates the activity of MEF2; therefore, we wanted to determine whether UII stimulation resulted in the association of HDAC5 with 14-3-3β. Cardiomyocyte cultures were infected with AdUIIR, allowed to recover, and then stimulated with 100nM UII for 0, 5, 10, 30 and 60 minutes. After UII stimulation, whole cell lysates were collected and 14-3-3β was immunoprecipitated with protein G. The 14-3-3β immunoprecipitates were then resolved by electrophoresis, transferred to a nitrocellulose membrane and blotted for HDAC5. UII stimulation resulted in a time-dependent association of HDAC5 with 14-3-3β (Figure 37). In order to ensure equal loading, the membrane was stripped and re-probed for input (14-3-3β). The input 14-3-3β did not change over UII stimulation time. These data clearly demonstrate the ability of UII to stimulate the dissociation of HDAC5 from MEF2.

CaMKK and ERK1/2 inhibition diminishes the UII-induced interaction of HDAC5 with 14-3-3β

We next wanted to determine the effects of CaM kinase kinsase and MAP kinase inhibition on the UII-induced association of HDAC5 with 14-3-3β. We infected cardiomyocytes cultures with AdUIIR followed by a one-hour stimulation with UII (100nM) alone, or with STO-609, SB203580 or U0126. Whole cell lysates were collected and 14-3-3β was immunoprecipitated with protein G. The immunoprecipitate was resolved by electrophoresis, transferred to nitrocellulose and blotted for HDAC5.

Without UII stimulation, HDAC5 did not co-immunoprecipitate with 14-3-3β (Figure 38). One hour of UII stimulation resulted in a robust increase in the association of HDAC5 with 14-3-3β; however, inhibition of CaMKK almost completely prevented the HDAC5/14-3-3β interaction. Inhibition of p38 with SB203580 had no effect on UII-dependent HDAC5/14-3-3β co-immunoprecipitation while ERK1/2 inhibition resulted in a decreased HDAC5/14-3-3β association. In order to ensure equal loading, the membrane was stripped and re-probed for input (14-3-3β). The input 14-3-3β did not change over UII stimulation time. Importantly, these data demonstrate that UII-stimulated HDAC5/14-3-3β association is dependent on CaMKK.

p38 and CaMKI are known activators of MEF2. Additionally, we have shown that CaMKI was capable of stimulating the nucleocytoplasmic shuttling of HDAC5 by 14-3-3β. UII dependent activation of MEF2 as well as HDAC5/14-3-3β interaction were dependent on CaMKK. These data provide a mechanism whereby UII stimulation of CaMKK is sufficient to activate MEF2 (likely via phosphorylation by p38) and relieve HDAC5 association with MEF2—two events that are required for activation of MEF2.

UII activates PKD

We were well aware of a parallel pathway involving PKD that was shown to phosphorylate HDACs resulting in their 14-3-3-dependent translocation to the nucleus[127]. All of our previous work with the HDACs in cardiomyocytes employed active CaM kinases (I and IV) or phenylephrine to induce HDAC nucleocytoplasmic shuttling. We have shown that UII is able to induce the association of HDAC5 with 14-3-3β in a

CaMKK dependent manner. As was discussed previously, CaMKI is a cytoplasmic kinase and is activated by UII stimulation. We thought that it was possible that UII stimulation could activate PKD, which would account for the UII-induced HDAC5 translocation to the nucleus possibly working in conjunction with CaMKI. To test this, we infected cardiomyocytes cultures with AdUIIR, allowed them to recover, and then stimulated the cultures with UII for 0, 2, 5, 10, 30 and 60 minutes. After stimulation whole cell lysates were collected and a total of 20μg of each were resolved by polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. We next blotted the membrane for active PKD (Ser744/748). UII stimulation resulted in the phosphorylation of PKD by two minutes with maximum phosphorylation observed at 10 minutes (Figure 39). By 60 minutes, phosphorylation of PKD returned to near basal. The membrane was stripped and re-probed for GAPDH to ensure equal loading of protein. These data are the first to show that UII is capable of stimulating the activation of PKD and may account for the nucleocytoplasmic shuttling of HDAC5.

CaMKK inhibition blocks UII activation of PKD

We have now shown that UII stimulation of cardiomyocytes resulted in the activation of PKD. These data help explain the ability of UII to induce the association of HDAC5 with 14-3-3β. Since we had shown that UII induced association of HDAC5 with 14-3-3β was dependent on CaMKK, we next wanted to test whether the UII-dependent activation of PKD was also dependent on CaMKK. To test this, we infected cardiomyocytes with AdUIIR, allowed them to recover, and stimulated the cultures with

UII for 2 and 5 minutes either with or without STO-609. As seen previously, UII induced the time-dependent phosphorylation of PKD (Figure 40). However, when we inhibited the activity of CaMKK, the UII-dependent phosphorylation of PKD was inhibited. These results are the first to suggest that the activation of PKD may be dependent on CaMKK and that the CaMKI/PKD pathways which both lead to HDAC5 nucleocytoplasmic shuttling may not be parallel pathways, but rather, members of the same signaling pathway.

Figure 7. Hypertrophic agonists stimulate the expression of UIIR mRNA in cardiomyocytes. Cultures were stimulated with either PE (10 mM) or AngII (100 nM) for 24 hours. Total RNA was isolated and reverse transcribed. cDNA was used as template to amplify UIIR. Both PE and AngII stimulated the expression of UIIR mRNA in cardiomyocytes.

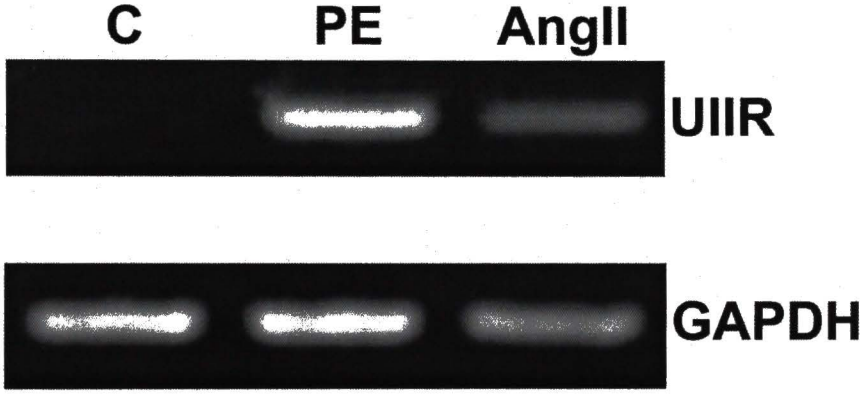

C
PE
AngII
UIIR
GAPDH

Figure 8. Hypertrophic agonists stimulate the expression of UIIR protein in cardiomyocytes. Cultures were stimulated with either PE (10 mM) or AngII (100 nM) for 24 hours. Total protein was isolated and resolved by PAGE. The resolved protein was transferred to nitrocellulose and blotted for UIIR using a specific antibody. Both PE and AngII stimulated the expression of UIIR protein in cardiomyocytes.

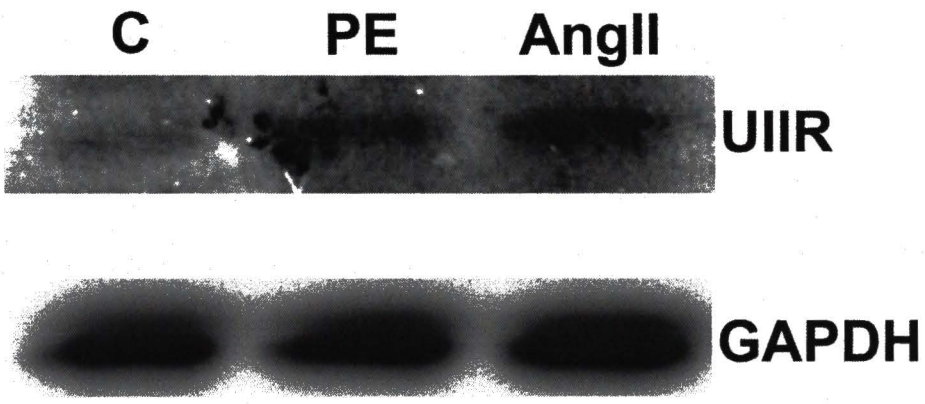

C
PE
AngII
UIIR
GAPDH

Figure 9. UII stimulates the ANF promoter in a dose-dependent manner.

Cardiomyocytes were transiently transfected with 50 ng/well of the ANF promoter-reporter using LipofectAMINE ™ Plus reagent. Three hours post-transfection, cells were re-fed with with medium 199 containing 0.2% FBS and allowed to recover for 24 hours. Cells were then infected with AdUIIR for 24 hours, washed and re-fed with medium 199 with 0.2% FBS. After 24 hours, cells were stimulated with the indicated doses of UII for 48 hours. After stimulation, luciferase activity was assayed by luminometry. The data represent the mean ± SEM for triplicate samples.

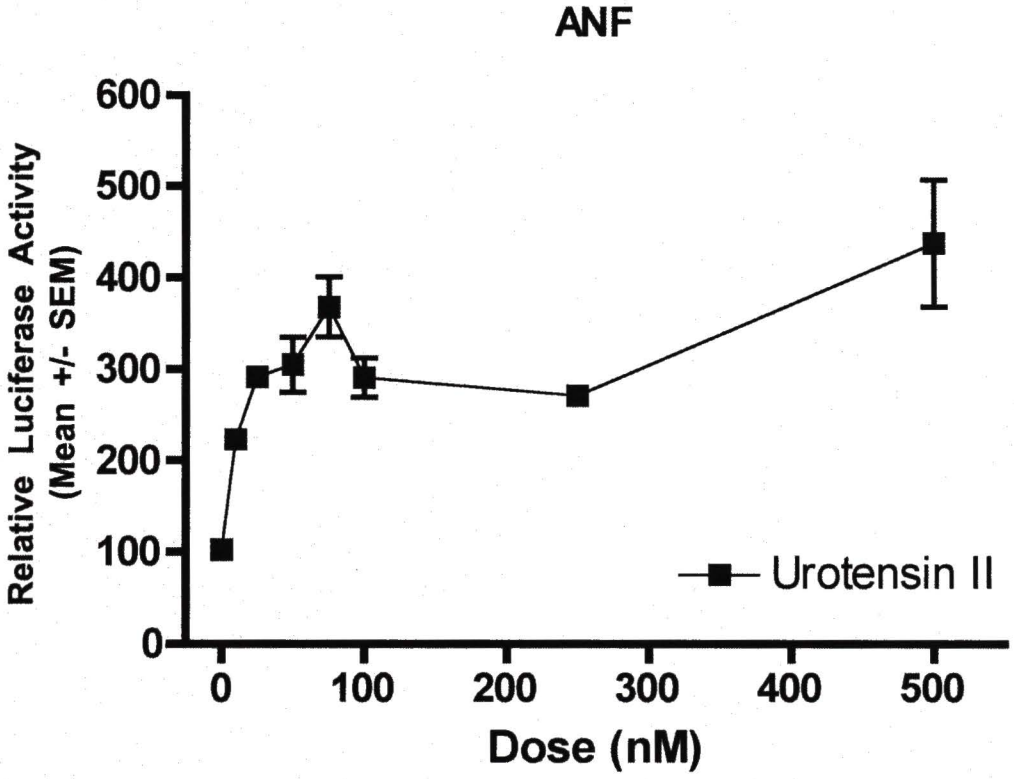

ANF
Relative Luciferase Activity
(Mean +/- SEM)
600
500
400
300
200
100
0
0
100
200
300
400
500
Dose (nM)
Urotensin II

Figure 10. UII specifically stimulates the ANF promoter in cardiomyocytes infected with an adenovirus containing UIIR. Cardiomyocytes were transiently transfected with 50ng/well of the ANF promoter-reporter for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. After recovery, cells were infected with AdUIIR or a control virus that expressed only GFP (AdGO-GFP) for 24 hours, washed and re-fed with medium 199 and 0.2% FBS, and allowed to recover for 24 hours. After recovery, cells were stimulated for 48 hours with UII (100 nM). After UII stimulation, luciferase activity was assayed by luminometry. The data represent the mean ± SE of six replicate samples per group. The data were analyzed for significance by Student's T test (*** indicates $p < 0.001$ compared with AdGO-GFP infection).

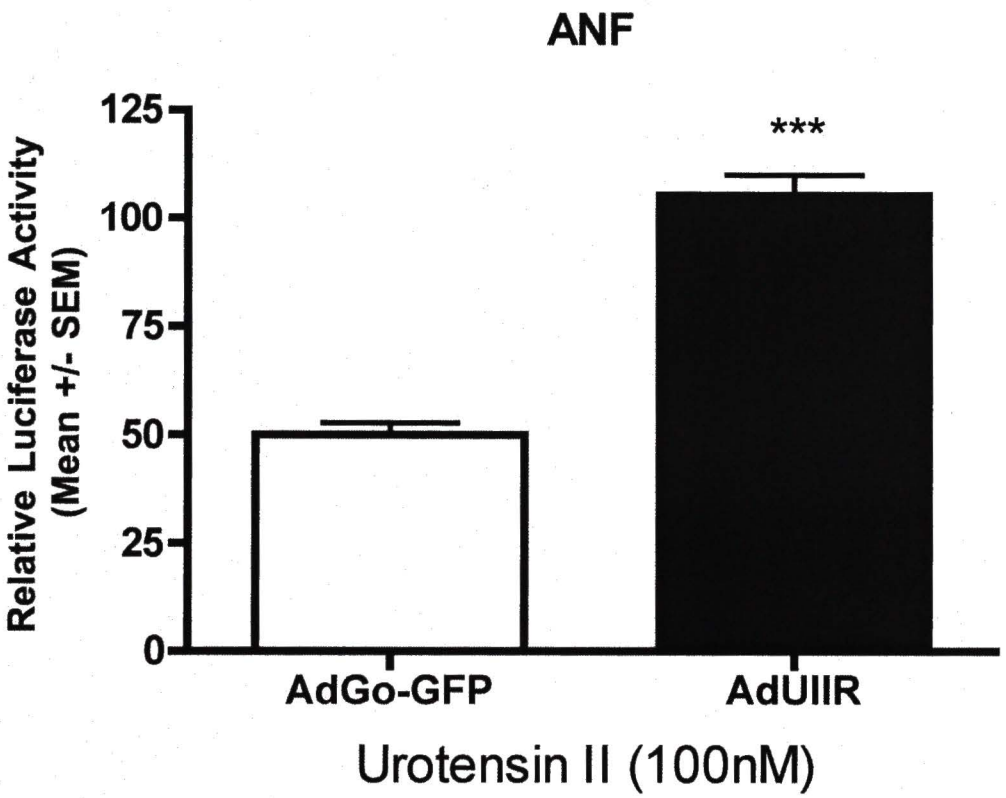

ANF
Relative Luciferase Activity
(Mean +/- SEM)
125
100
75
50
25
0
***
AdGo-GFP
AdUIIR
Urotensin II (100nM)

Figure 11. UII stimulates the SkA promoter in a dose-dependent manner.

Cardiomyocytes were transiently transfected with 50 ng/well of the SkA promoter-reporter using LipofectAMINE ™ Plus reagent. Three hours post-transfection, cells were re-fed with with medium 199 containing 0.2% FBS and allowed to recover for 24 hours. Cells were then infected with AdUIIR for 24 hours, washed and re-fed with medium 199 with 0.2% FBS. After 24 hours, cells were stimulated with the indicated doses of UII for 48 hours. After stimulation, luciferase activity was assayed by luminometry. The data represent the mean ± SEM for triplicate samples.

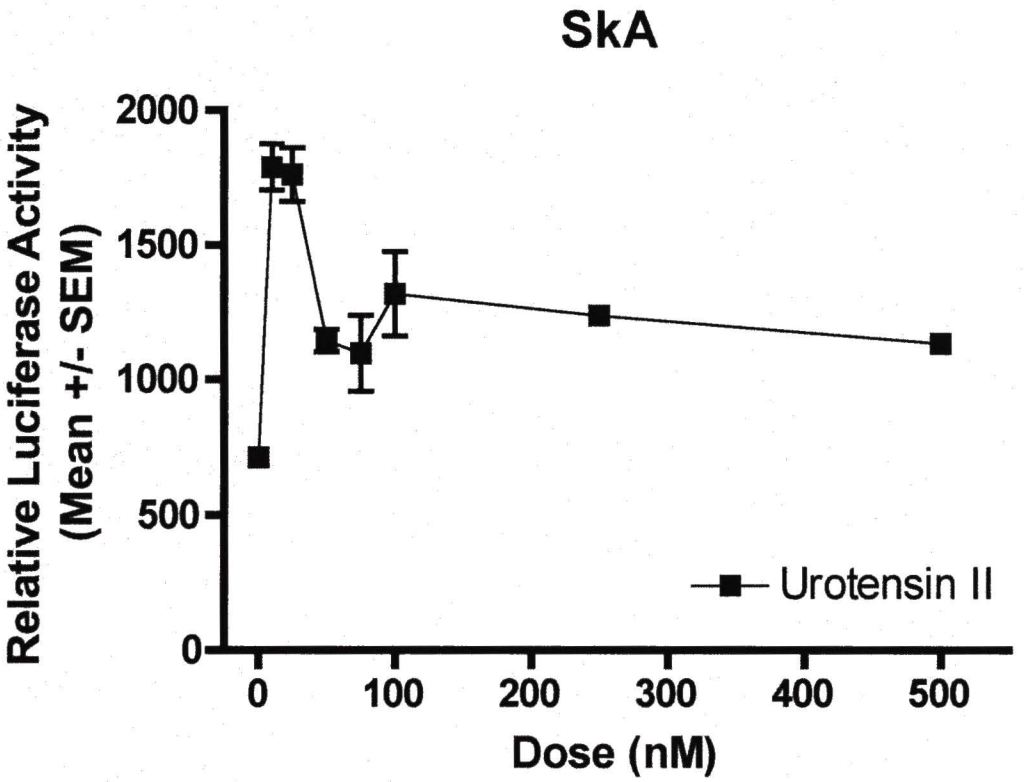

SkA
Relative Luciferase Activity
(Mean +/- SEM)
2000
1500
1000
500
0
0
100
200
300
400
500
Dose (nM)
Urotensin II

Figure 12. UII specifically stimulates the SkA promoter in cardiomyocytes infected with an adenovirus containing UIIR. Cardiomyocytes were transiently transfected with 50ng/well of the SkA promoter-reporter for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. After recovery, cells were infected with AdUIIR or a control virus that expressed only GFP (AdGO-GFP) for 24 hours, washed and re-fed with medium 199 and 0.2% FBS, and allowed to recover for 24 hours. After recovery, cells were stimulated for 48 hours with UII (100 nM). After UII stimulation, luciferase activity was assayed by luminometry. The data represent the mean ± SE of six replicate samples per group. The data were analyzed for significance by Student's T test (* indicates $p < 0.05$ compared with AdGO-GFP infection).

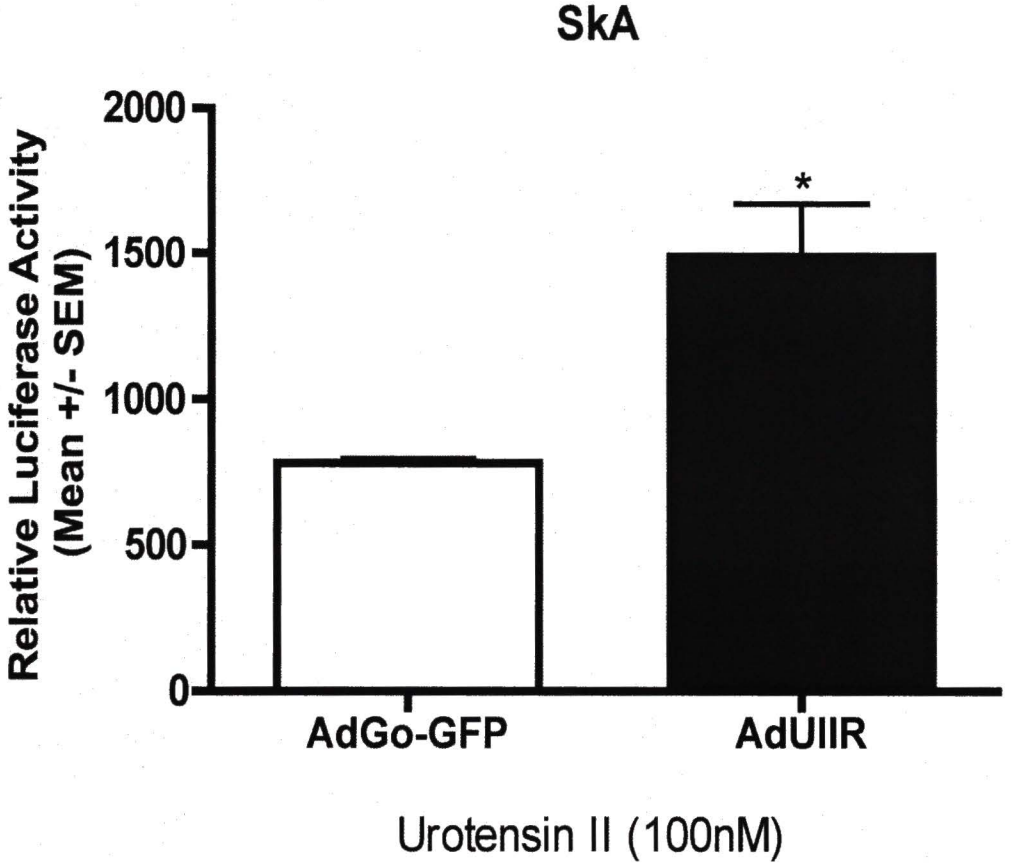

SkA
Relative Luciferase Activity
(Mean +/- SEM)
2000
1500
1000
500
0
*
AdGo-GFP
AdUIIR
Urotensin II (100nM)

Figure 13. UII stimulates MEF2 enhancer activity in a dose-dependent manner. Cardiomyocytes were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter using LipofectAMINE ™ Plus reagent. Three hours post-transfection, cells were re-fed with medium 199 containing 0.2% FBS and allowed to recover for 24 hours. Cells were then infected with AdUIIR for 24 hours, washed and re-fed with medium 199 with 0.2% FBS. After 24 hours, cells were stimulated with the indicated doses of UII for 48 hours. After stimulation, luciferase activity was assayed by luminometry. The data represent the mean ± SEM for triplicate samples.

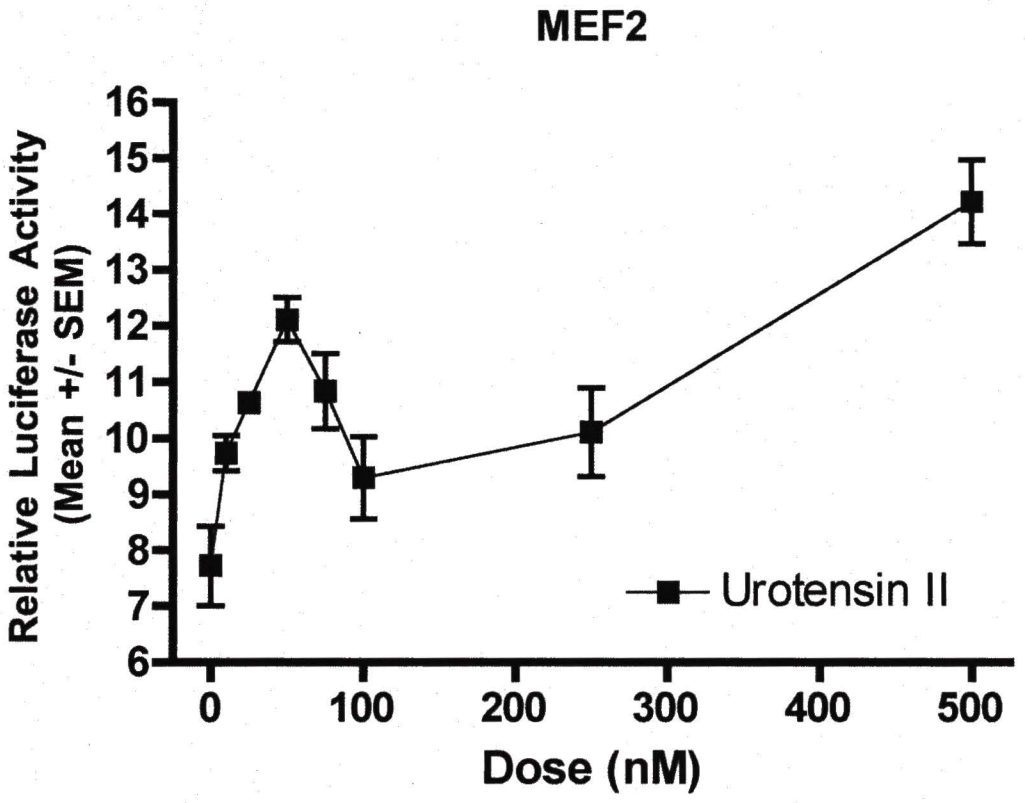

MEF2
Relative Luciferase Activity
(Mean +/- SEM)
16
15
14
13
12
11
10
9
8
7
6
0
100
200
300
400
500
Dose (nM)
Urotensin II

Figure 14. UII specifically stimulates the MEF2 enhancer-promoter in cardiomyocytes infected with an adenovirus containing UIIR. Cardiomyocytes were transiently transfected with 50ng/well of the SkA promoter-reporter for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. After recovery, cells were infected with AdUIIR or a control virus that expressed only GFP (AdGO-GFP) for 24 hours, washed and re-fed with medium 199 and 0.2% FBS, and allowed to recover for 24 hours. After recovery, cells were stimulated for 48 hours with UII (100 nM). After UII stimulation, luciferase activity was assayed by luminometry. The data represent the mean ± SE of six replicate samples per group. The data were analyzed for significance by Student's T test (* indicates $p < 0.05$ compared with AdGO-GFP infection).

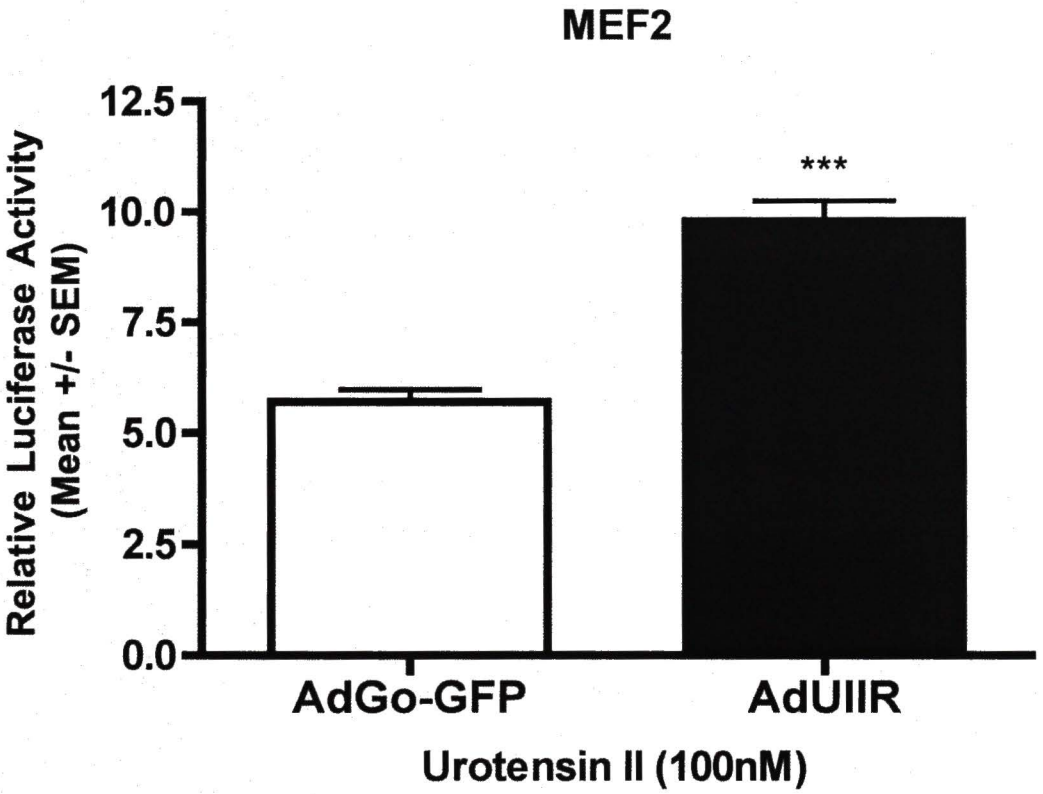

MEF2
Relative Luciferase Activity
(Mean +/- SEM)
12.5
10.0
7.5
5.0
2.5
0.0
***
AdGo-GFP
AdUIIR
Urotensin II (100nM)

Figure 15. CaMKK is required for UII stimulation of the ANF promoter. Cardiomyocytes were transiently transfected with 50 ng/well of the ANF promoter-reporter for three hours using LipofectAMINE™ Plus reagent and then re-fed with medium 199 and 0.2% FBS. After 24 hours, designated wells were infected with AdUIIR (24 hours) and then were allowed to recover for 24 hours in medium 199 with 0.2% FBS. Designated wells were pre-incubated with STO-609 (250 ng/mL) 45 minutes prior to stimulation with UII. Cells were stimulated with UII (100 nM) for 48 hours with or without STO-609 as designated. The data represent the mean ± SEM for three replicate samples (*** indicates $p < 0.001$).

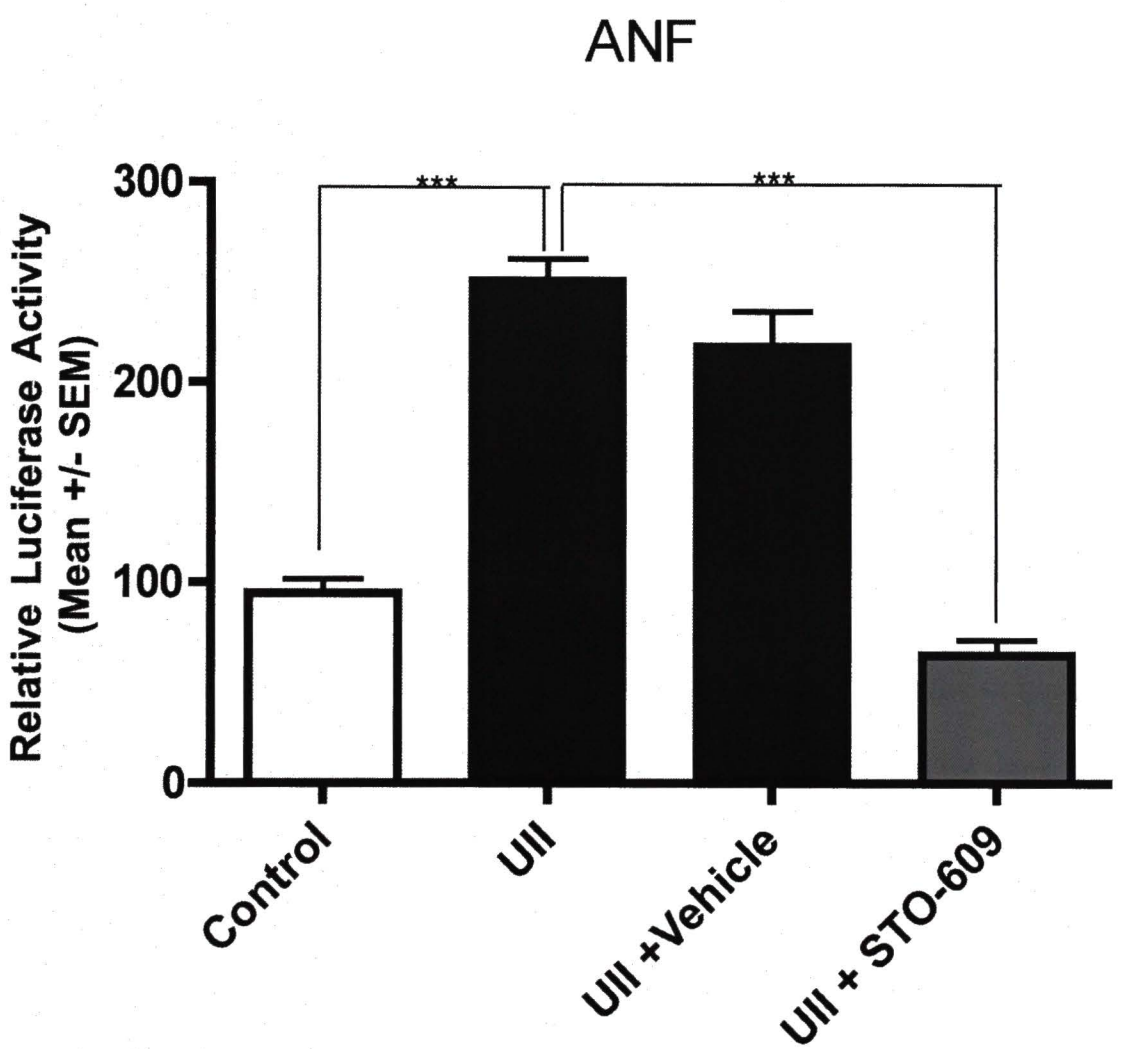

ANF
Relative Luciferase Activity
(Mean +/- SEM)
300
200
100
0
***
***
Control
UII
UII +Vehicle
UII + STO-609

Figure 16. CaMKK is required for UII stimulation of the SkA promoter.

Cardiomyocytes were transiently transfected with 50 ng/well of the SkA promoter-reporter for three hours using LipofectAMINE™ Plus reagent and then re-fed with medium 199 and 0.2% FBS. After 24 hours, designated wells were infected with AdUIIR (24 hours) and then were allowed to recover for 24 hours in medium 199 with 0.2% FBS. Designated wells were pre-incubated with STO-609 (250 ng/mL) 45 minutes prior to stimulation with UII. Cells were stimulated with UII (100 nM) for 48 hours with or without STO-609 as designated. The data represent the mean ± SEM for three replicate samples (** indicates $p < 0.01$; *** indicates $p < 0.001$).

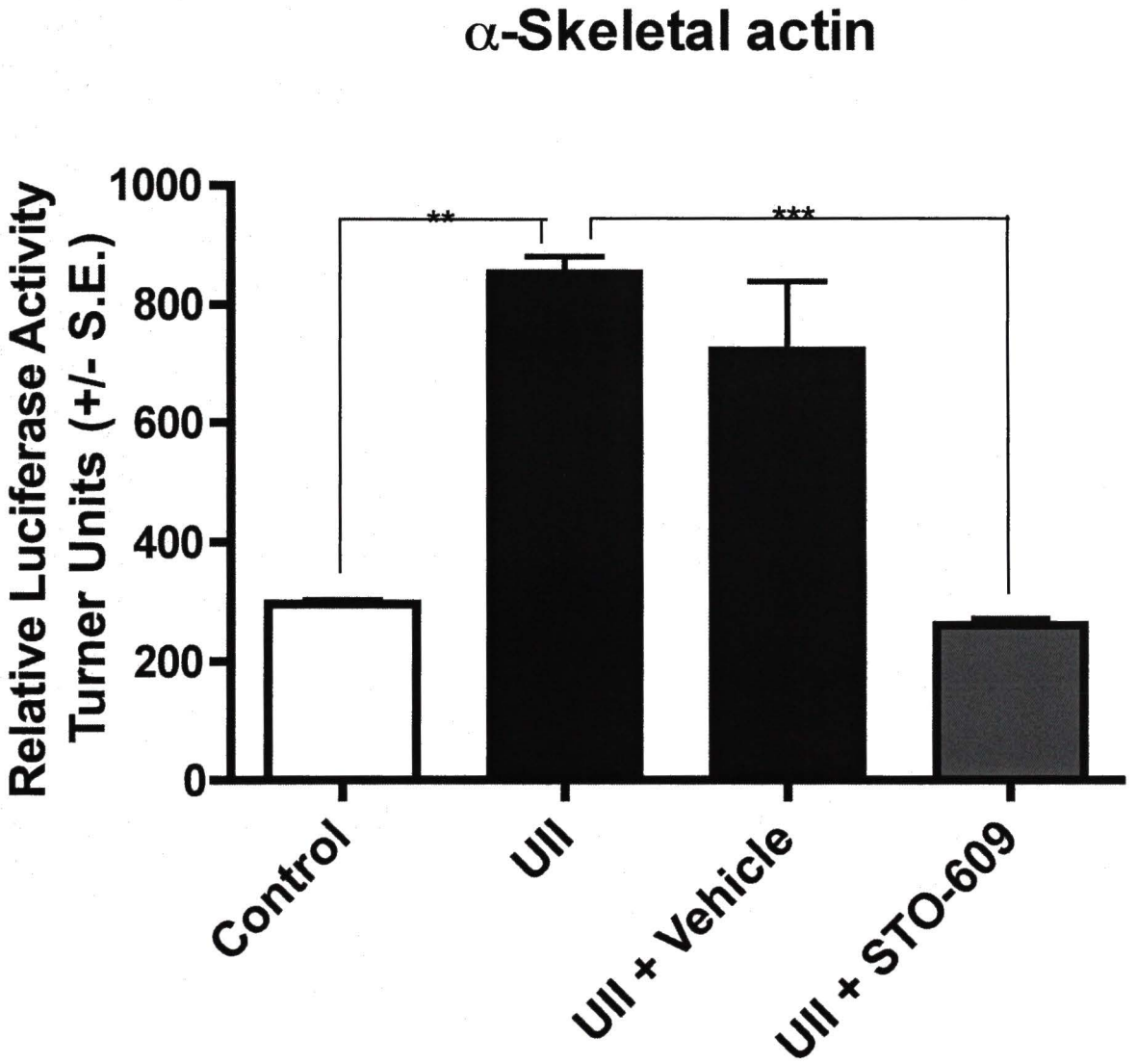

α-Skeletal actin
Relative Luciferase Activity
Turner Units (+/- S.E.)
1000
800
600
400
200
0
**
***
Control
UII
UII + Vehicle
UII + STO-609

Figure 17. CaMKK is required for UII stimulation of MEF2. Cardiomyocytes were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter for three hours using LipofectAMINE™ Plus reagent and then re-fed with medium 199 and 0.2% FBS. After 24 hours, designated wells were infected with AdUIIR (24 hours) and then were allowed to recover for 24 hours in medium 199 with 0.2% FBS. Designated wells were pre-incubated with STO-609 (250 ng/mL) 45 minutes prior to stimulation with UII. Cells were stimulated with UII (100 nM) for 48 hours with or without STO-609 as designated. The data represent the mean ± SEM for three replicate samples (*** indicates $p < 0.001$).

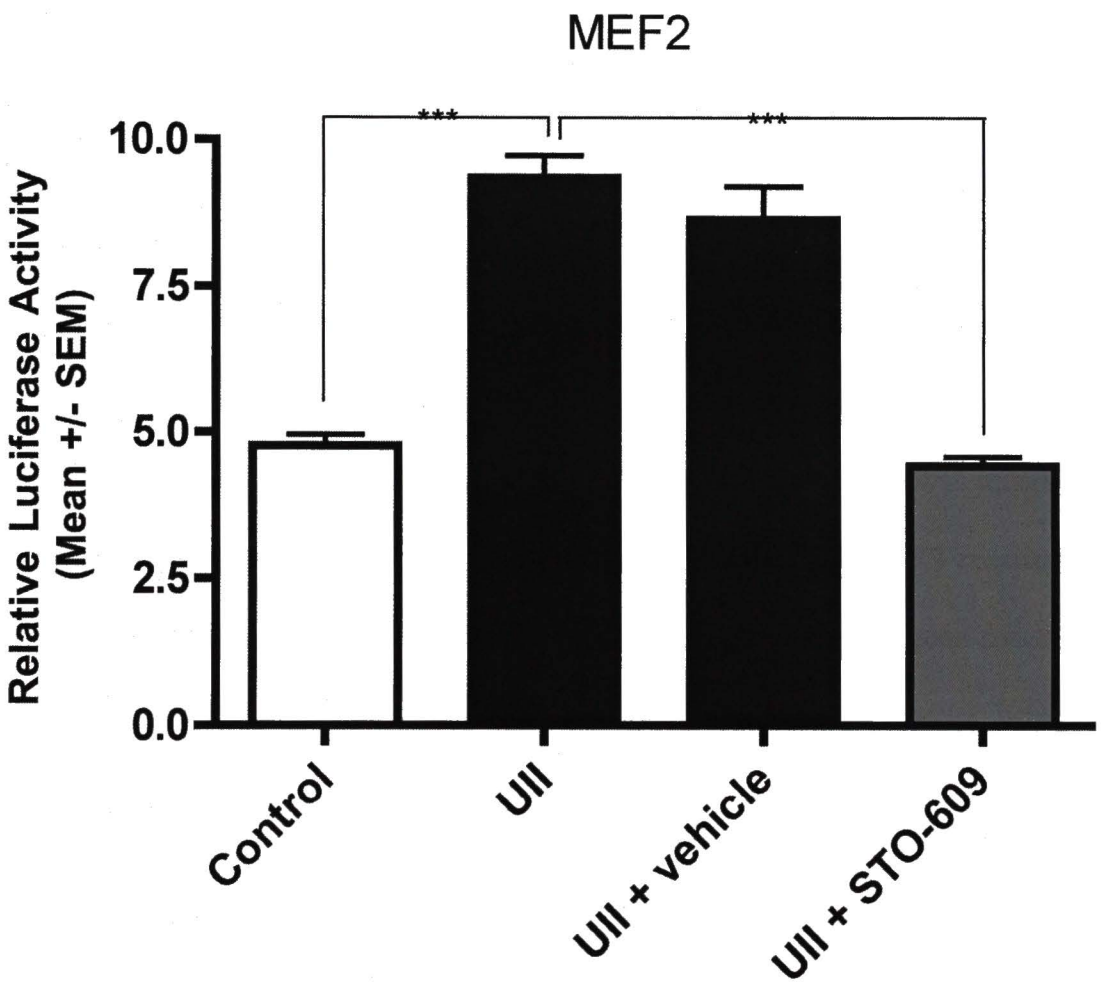

MEF2
***
***
10.0
7.5
5.0
2.5
0.0
Relative Luciferase Activity
(Mean +/- SEM)
Control
UII
UII + vehicle
UII + STO-609

Figure 18. CaMKK is required for UII-dependent increase in hypertrophy-sensitive marker gene expression. Cardiomyocyte cultures were pre-incubated with STO-609 (250 ng/mL) 45 minutes prior to UII stimulation. Cells were treated with UII (100 nM) with or without STO-609 for 48 hours as indicated. After stimulation, total RNA was collected using Trizol™ reagent. One μg of total RNA from each sample was reverse transcribed using Superscript™ III reverse transcriptase. One μl of each RT reaction was used as template for amplification of ANF, BNP, βMHC and SkA using gene-specific DNA primers. For an internal control, GAPDH was amplified in each reaction using gene-specific primers.

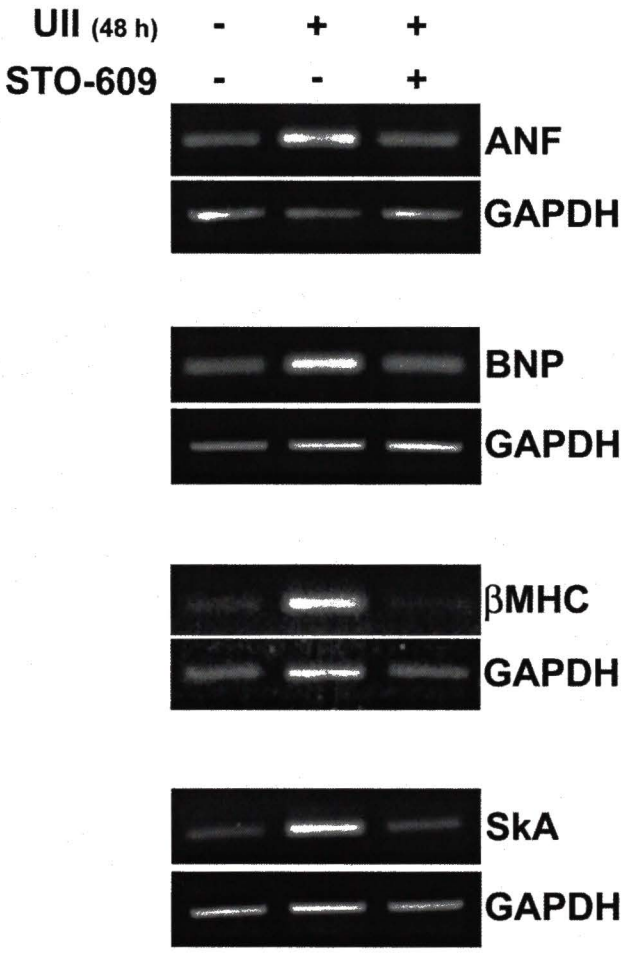

UII (48 h) - + +
STO-609 - - +
ANF
GAPDH
BNP
GAPDH
βMHC
GAPDH
SkA
GAPDH

Figure 19. UII stimulates the activation of CaMKI. Cardiomyocyte cultures were stimulated with UII (100 nM) for 0, 2, 5, 10, 30 and 60 minutes. Total protein from each treatment was isolated and total protein concentration measured by BCA assay. A total of 20 μg of total protein from each treatment was resolved by 12% PAGE and transferred to a nitrocellulose membrane. Active CaMKI was detected by Western Blot. The membrane was stripped and re-probed for GAPDH to ensure equal loading.

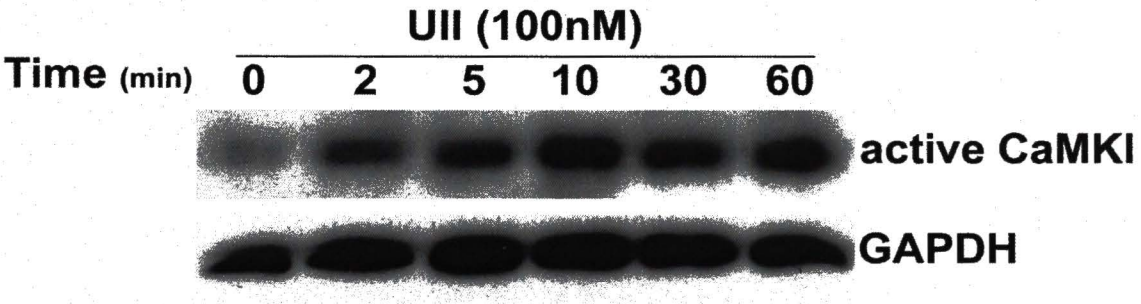

Ull (100nM)
Time (min)
0
2
5
10
30
60
active CaMKI
GAPDH

Figure 20. UII-dependent activation of CaMKI requires CaMKK. Designated cardiomyocyte cultures were pre-incubated with STO-609 (250 ng/mL) prior to UII stimulation. Cells were then stimulated with UII (100 nM) for the indicated times with or without STO-609. Total protein was isolated from each treatment and total protein measured by BCA assay. 20 μg of total protein from each treatment were resolved by 12% PAGE and transferred to a nitrocellulose membrane. Active CaMKI was detected by Western Blot. The membrane was then stripped and re-probed for GAPDH to ensure equal loading.

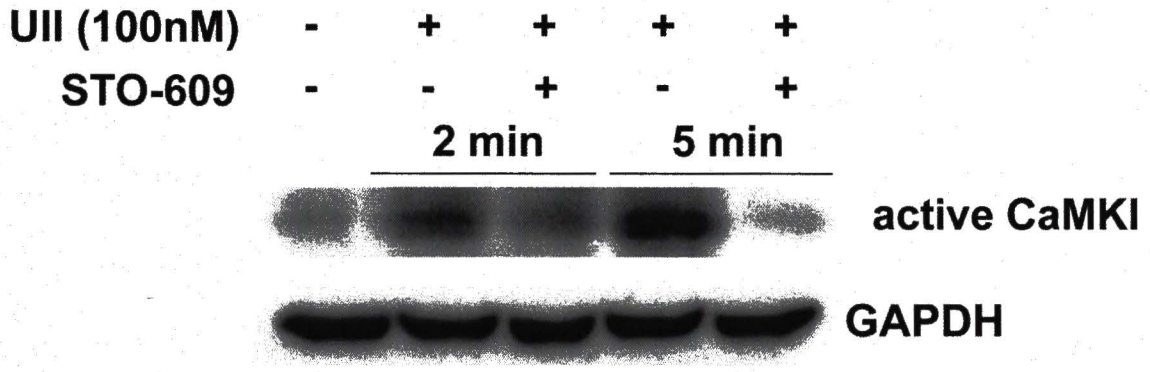

UII (100nM) - + + + +
STO-609 - - + - +
2 min
5 min
active CaMKI
GAPDH

Figure 21. Constitutively-active CaMKI rescues STO-609 inhibition of UII-dependent ANF promoter activity. Cardiomyocytes were transiently transfected with 50 ng/well of the ANF promoter-reporter plasmid and constitutively-active CaMKI (as indicated) for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. After recovery, designated cultures were infected with AdUIIR for 24 hours and then washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. Cells were treated with STO-609 (250 ng/well) as indicated prior to UII stimulation. Designated wells were then stimulated with UII (100 nM) for 48 hours after which, luciferase activity was assayed by luminometry. The data represent the mean ± SEM for triplicate cultures (** indicates $p < 0.01$ compared with control; ††† indicates $p < 0.001$ compared with UII + STO-609).

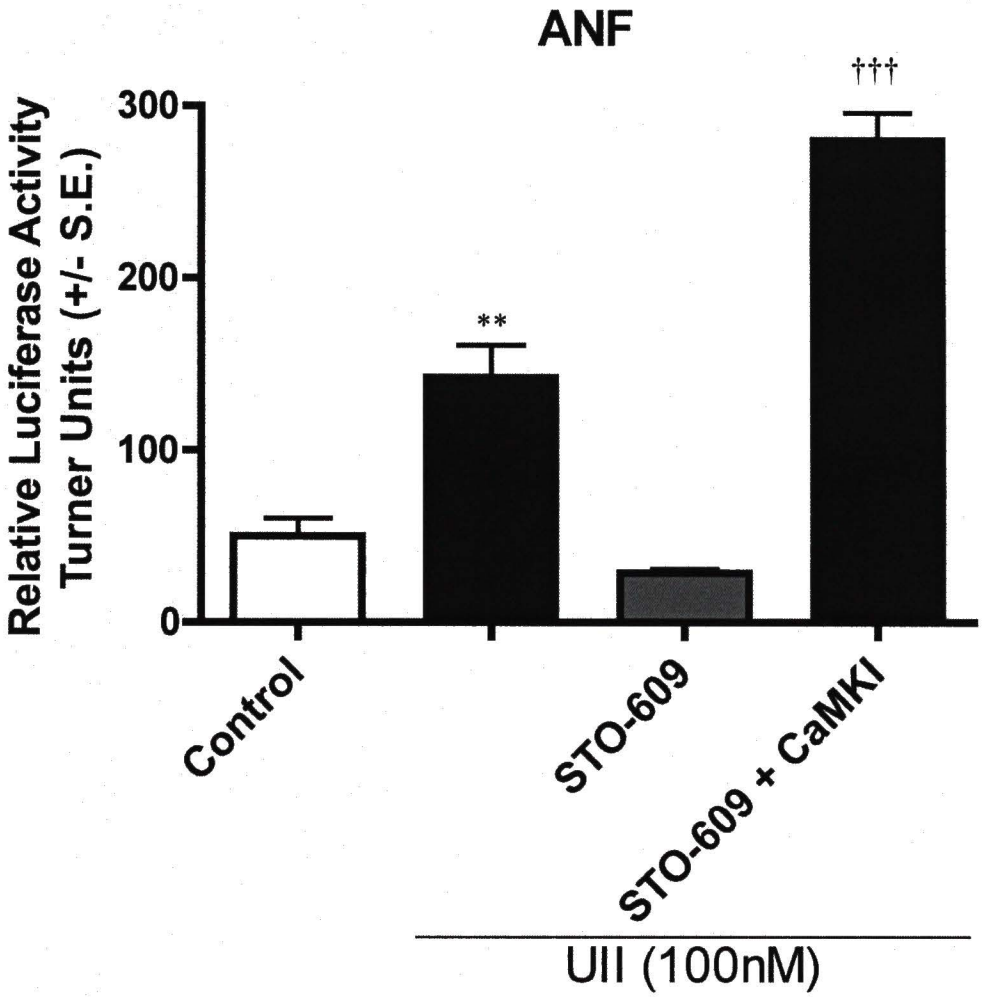

ANF
Relative Luciferase Activity
Turner Units (+/- S.E.)
300
200
100
0
**
†††
Control
STO-609
STO-609 + CaMKI
UII (100nM)

Figure 22. Constitutively-active CaMKI rescues STO-609 inhibition of UII-dependent SkA promoter activity. Cardiomyocytes were transiently transfected with 50 ng/well of the SkA promoter-reporter plasmid and constitutively-active CaMKI (as indicated) for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. After recovery, designated cultures were infected with AdUIIR for 24 hours and then washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. Cells were treated with STO-609 (250 ng/well) as indicated prior to UII stimulation. Designated wells were then stimulated with UII (100 nM) for 48 hours after which, luciferase activity was assayed by luminometry. The data represent the mean ± SEM for triplicate cultures (*** indicates $p < 0.001$ compared with control; ††† indicates $p < 0.001$ compared with UII + STO-609).

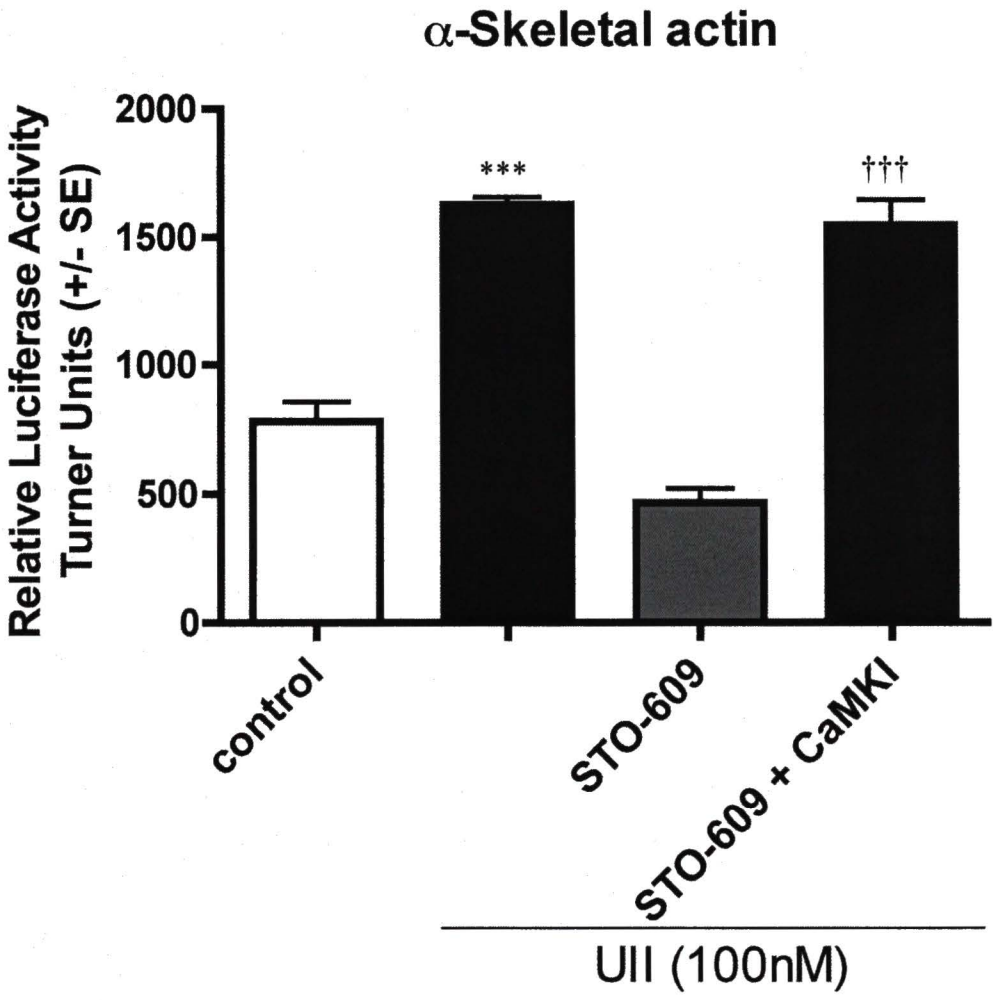

α-Skeletal actin
Relative Luciferase Activity
Turner Units (+/- SE)
2000
1500
1000
500
0
***
†††
control
STO-609
STO-609 + CaMKI
UII (100nM)

Figure 23. Constitutively-active CaMKI rescues STO-609 inhibition of UII-dependent MEF2 enhancer activity. Cardiomyocytes were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter plasmid and constitutively-active CaMKI (as indicated) for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. After recovery, designated cultures were infected with AdUIIR for 24 hours and then washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. Cells were treated with STO-609 (250 ng/well) as indicated prior to UII stimulation. Designated wells were then stimulated with UII (100 nM) for 48 hours after which, luciferase activity was assayed by luminometry. The data represent the mean ± SEM for triplicate cultures (** indicates $p < 0.01$ compared with control; ††† indicates $p < 0.001$ compared with UII + STO-609).

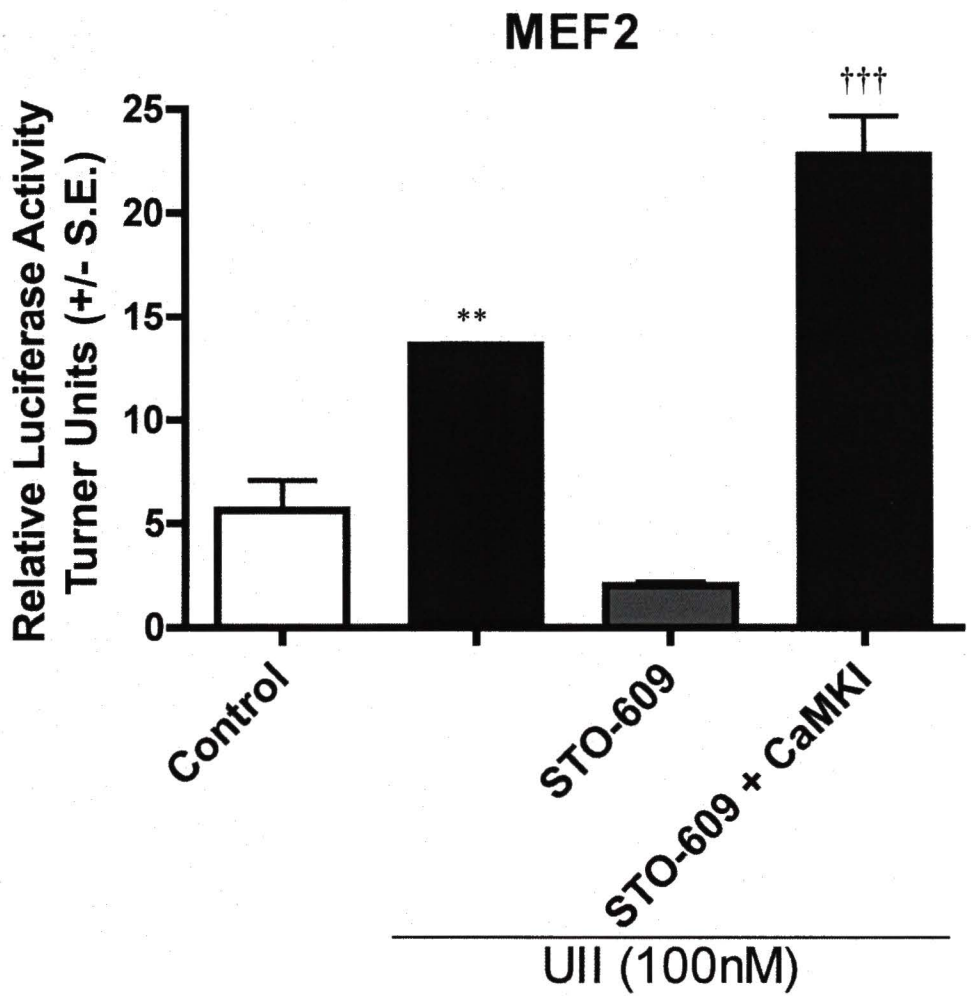

MEF2
Relative Luciferase Activity
Turner Units (+/- S.E.)
25
20
15
10
5
0
**
†††
Control
STO-609
STO-609 + CaMKI
UII (100nM)

Figure 24. Inhibition of AKT has no effect on UII stimulation of the ANF promoter. Cardiomyocyte cultures were transiently transfected with 50 ng/well of the ANF promoter-reporter plasmid for three hours using LipofectAMINE™ Plus reagent and then washed and re-fed with medium 199 and 0.2% FBS. Cultures were allowed to recover from transfection for 24 hours at which point, designated cells were infected with AdUIIR for 24 hours. After infection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. Prior to UII stimulation, cell cultures were treated with 5μM 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (AKTi) for 45 minutes. Cardiomyocytes were then stimulated with UII for 48 hours as indicated. The data represent the mean ± SEM of three samples per group (* indicates $p < 0.05$; n.s indicates not significant).

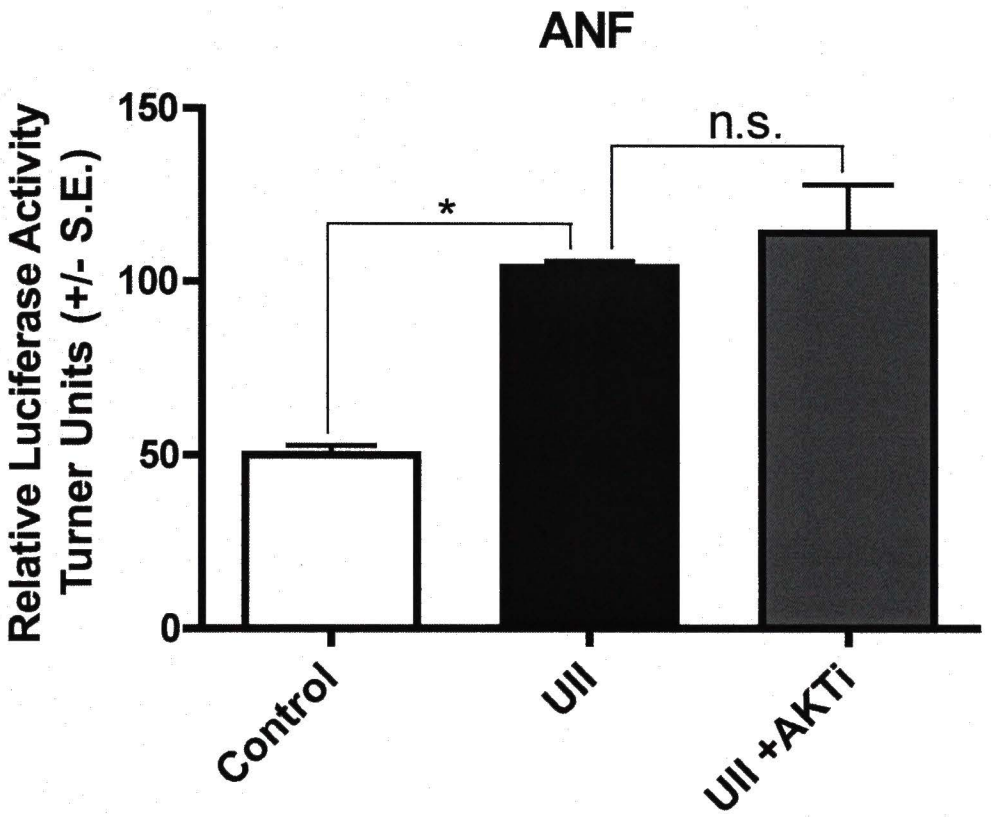

ANF
Relative Luciferase Activity
Turner Units (+/- S.E.)
150
100
50
0
*
n.s.
Control
UII
UII +AKTi

Figure 25. Inhibition of AKT has no effect on UII stimulation of the SkA promoter. Cardiomyocyte cultures were transiently transfected with 50 ng/well of the SkA promoter-reporter plasmid for three hours using LipofectAMINE™ Plus reagent and then washed and re-fed with medium 199 and 0.2% FBS. Cultures were allowed to recover from transfection for 24 hours at which point, designated cells were infected with AdUIIR for 24 hours. After infection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. Prior to UII stimulation, cell cultures were treated with 5μM 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (AKTi) for 45 minutes. Cardiomyocytes were then stimulated with UII for 48 hours as indicated. The data represent the mean ± SEM of three samples per group (* indicates $p < 0.05$; n.s indicates not significant).

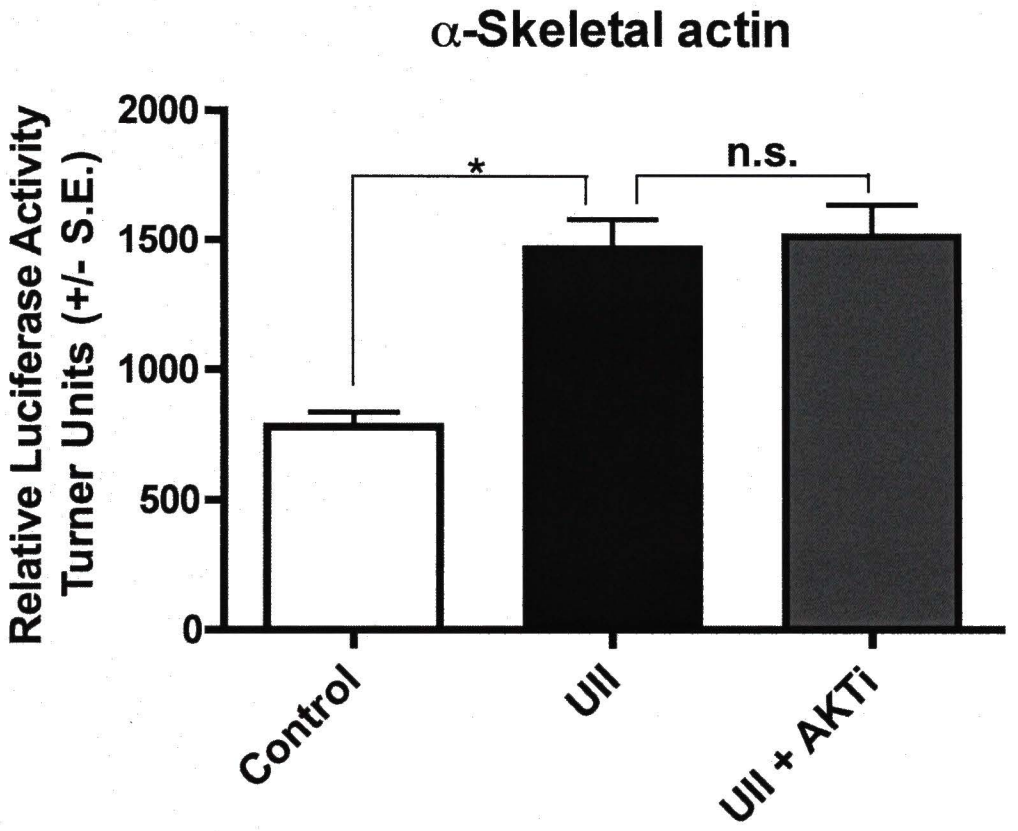

α-Skeletal actin
Relative Luciferase Activity
Turner Units (+/- S.E.)
2000
1500
1000
500
0
*
n.s.
Control
UII
UII + AKTi

Figure 26. Inhibition of AKT has no effect on UII stimulation of MEF2 activity. Cardiomyocyte cultures were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter plasmid for three hours using LipofectAMINE™ Plus reagent and then washed and re-fed with medium 199 and 0.2% FBS. Cultures were allowed to recover from transfection for 24 hours at which point, designated cells were infected with AdUIIR for 24 hours. After infection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. Prior to UII stimulation, cell cultures were treated with 5μM 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (AKTi) for 45 minutes. Cardiomyocytes were then stimulated with UII for 48 hours as indicated. The data represent the mean ± SEM of three samples per group (*** indicates $p < 0.001$; n.s indicates not significant).

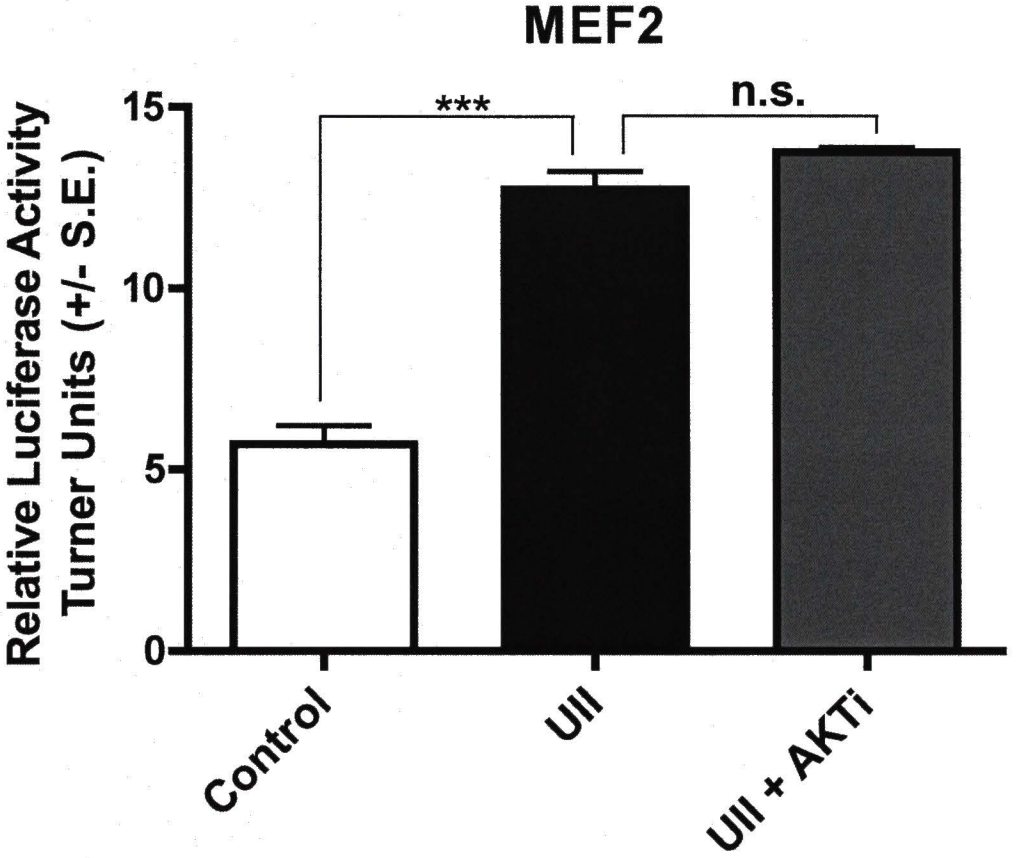

MEF2
Relative Luciferase Activity
Turner Units (+/- S.E.)
15
10
5
0
***
n.s.
Control
UII
UII + AKTi

Figure 27. UII activates p38 and ERK1/2 MAP kinases. Cardiomyocyte cultures were stimulated with UII (100 nM) for 0, 2, 5, 10, 30 and 60 minutes. Total protein from each treatment was isolated and total protein concentration measured by BCA assay. A total of 20 μg of total protein from each treatment was resolved by 12% PAGE and transferred to a nitrocellulose membrane. Active p38 and ERK1/2 were detected by Western Blot. The membrane was stripped and re-probed for GAPDH to ensure equal loading.

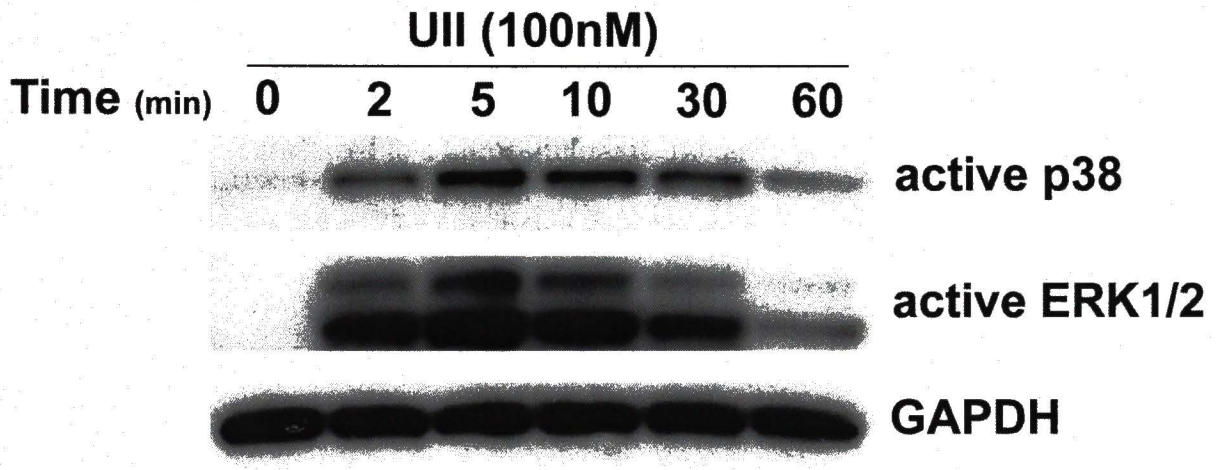

UII (100nM)
Time (min)
0
2
5
10
30
60
active p38
active ERK1/2
GAPDH

Figure 28. UII-dependent activation of p38 and ERK1/2 requires CaMKK.

Designated cardiomyocyte cultures were pre-incubated with STO-609 (250 ng/mL) prior to UII stimulation. Cells were then stimulated with UII (100 nM) for 5 minutes with or without STO-609. Total protein was isolated from each treatment and total protein measured by BCA assay. 20 μg of total protein from each treatment were resolved by 12% PAGE and transferred to a nitrocellulose membrane. Active p38 and ERK1/2 were detected by Western Blot. The membrane was then stripped and re-probed for GAPDH to ensure equal loading.

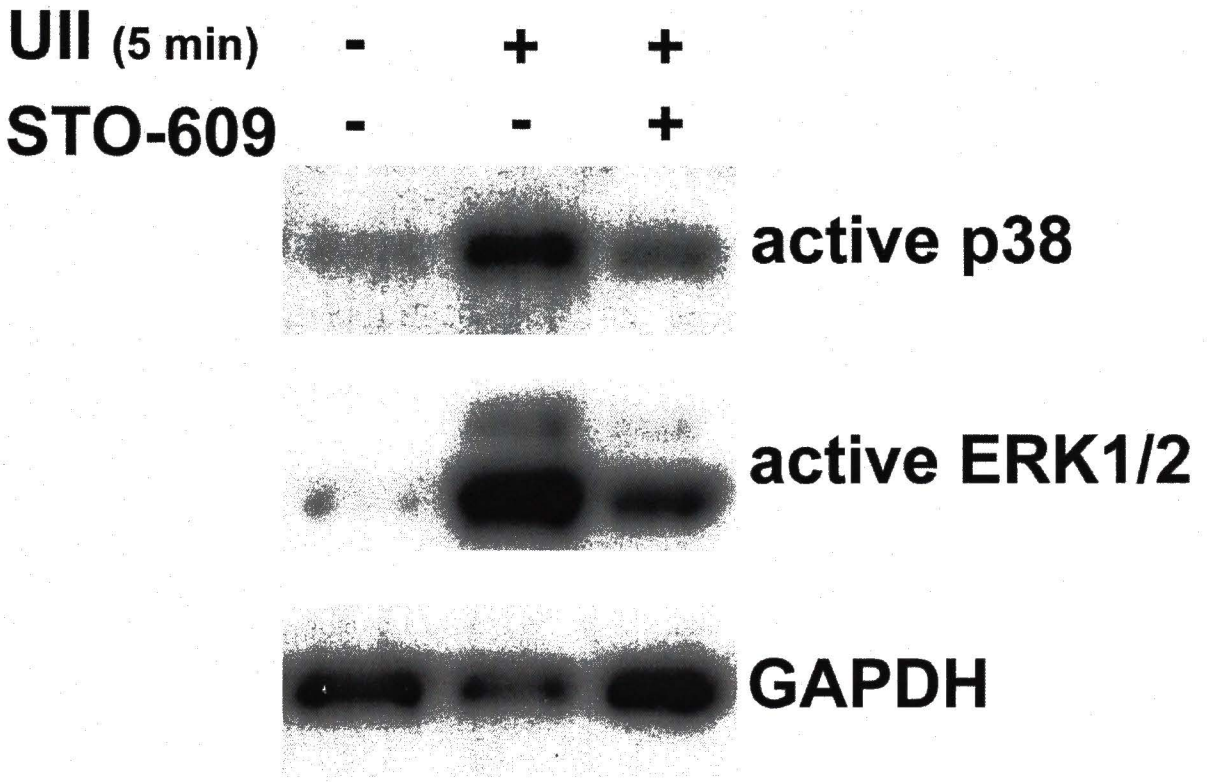

UII (5 min) - + +
STO-609 - - +
active p38
active ERK1/2
GAPDH

Figure 29. UII activation of the ANF promoter requires p38 and ERK1/2 MAP kinases. Cardiomyocytes were transiently transfected with 50 ng/well of the ANF promoter reporter for three hours using LipofectAMINE™ Plus reagent and were then washed and re-fed with medium 199 and 0.2% FBS. 24 hours later, cardiomyocyte cultures were infected with AdUIIR (24 hours) and then washed and re-fed with medium 199 and 0.2% FBS. Prior to stimulation with UII, designated wells were treated with either STO-609 (250 ng/mL), SB203580 (10 μM) or U0126 (10 μM) for 45 minutes. Designated cultures were then stimulated with UII (100 nM) for 48 hours after which, luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (*** indicates $p < 0.001$ compare with control; ††† indicates $p < 0.001$ compared with UII alone; n.s. indicates not significant).

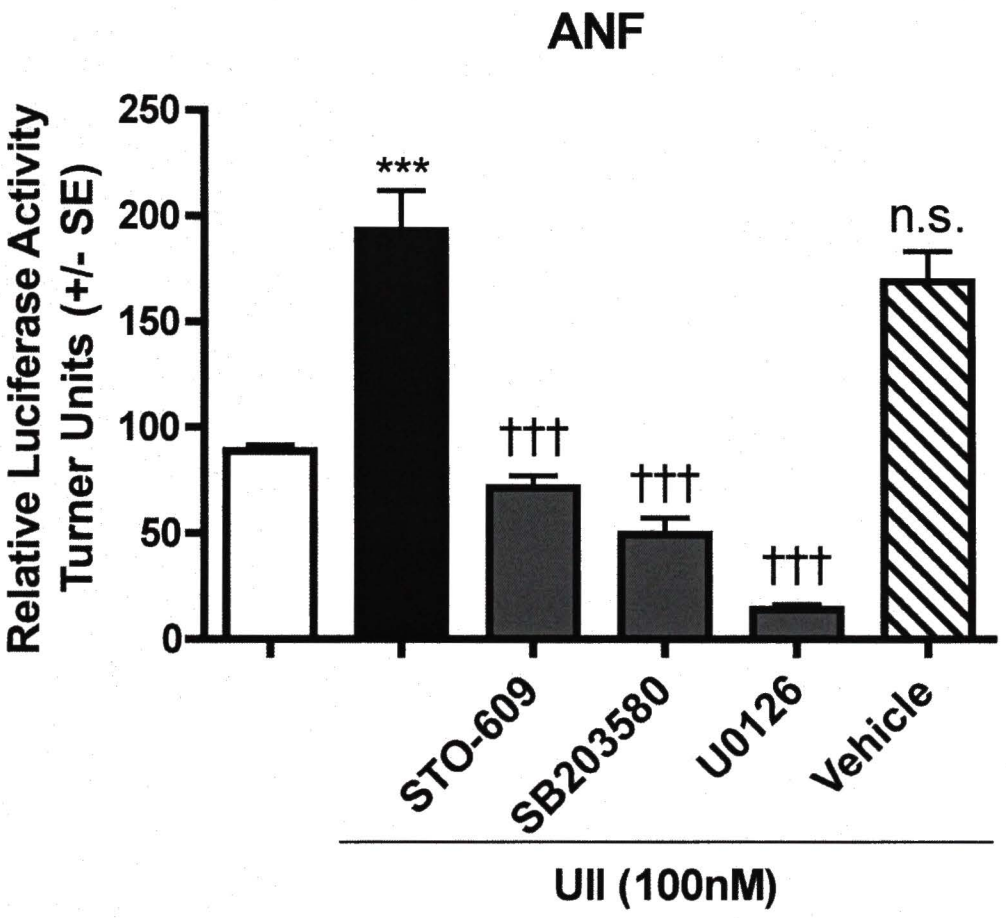

ANF
Relative Luciferase Activity
Turner Units (+/- SE)
250
200
150
100
50
0
***
n.s.
†††
†††
†††
STO-609
SB203580
U0126
Vehicle
UII (100nM)

Figure 30. UII activation of the SkA promoter requires p38 and ERK1/2 MAP kinases. Cardiomyocytes were transiently transfected with 50 ng/well of the SkA promoter reporter for three hours using LipofectAMINE™ Plus reagent and were then washed and re-fed with medium 199 and 0.2% FBS. 24 hours later, cardiomyocyte cultures were infected with AdUIIR (24 hours) and then washed and re-fed with medium 199 and 0.2% FBS. Prior to stimulation with UII, designated wells were treated with either STO-609 (250 ng/mL), SB203580 (10 μM) or U0126 (10 μM) for 45 minutes. Designated cultures were then stimulated with UII (100 nM) for 48 hours after which, luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (*** indicates $p < 0.001$ compare with control; ††† indicates $p < 0.001$ compared with UII alone; n.s. indicates not significant).

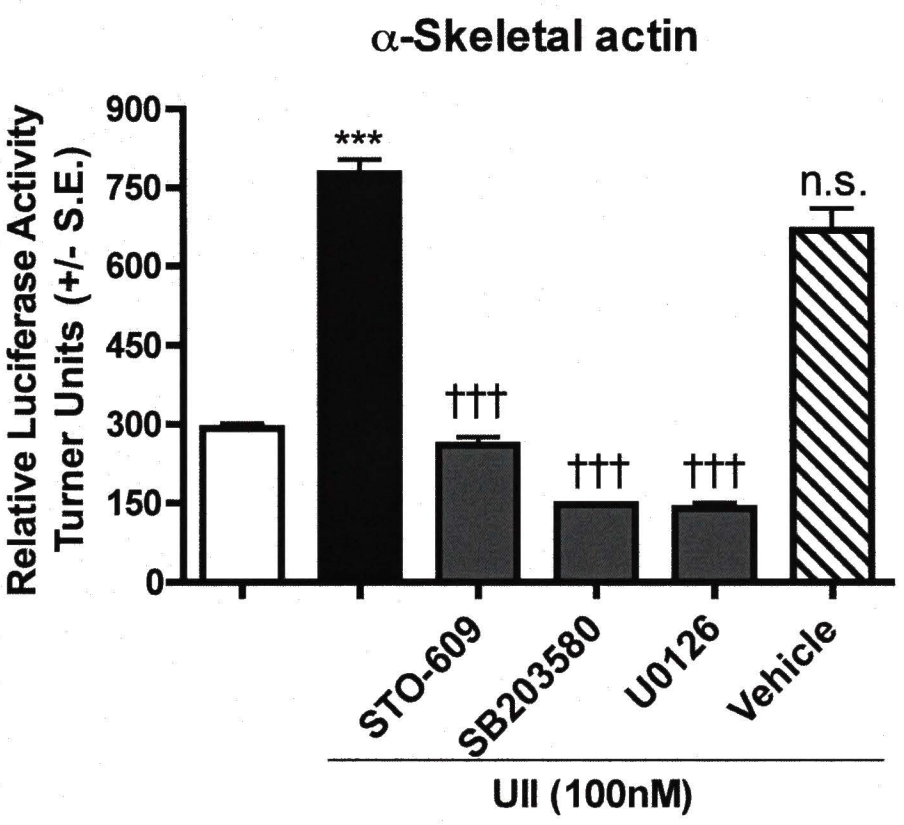

α-Skeletal actin
Relative Luciferase Activity
Turner Units (+/- S.E.)
900
750
600
450
300
150
0
***
n.s.
†††
†††
†††
STO-609
SB203580
U0126
Vehicle
UII (100nM)

Figure 31. UII activation of MEF2 requires p38 and ERK1/2 MAP kinases. Cardiomyocytes were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter for three hours using LipofectAMINE™ Plus reagent and were then washed and re-fed with medium 199 and 0.2% FBS. 24 hours later, cardiomyocyte cultures were infected with AdUIIR (24 hours) and then washed and re-fed with medium 199 and 0.2% FBS. Prior to stimulation with UII, designated wells were treated with either STO-609 (250 ng/mL), SB203580 (10 μM) or U0126 (10 μM) for 45 minutes. Designated cultures were then stimulated with UII (100 nM) for 48 hours after which, luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (*** indicates $p < 0.001$ compare with control; ††† indicates $p < 0.001$ compared with UII alone; n.s. indicates not significant).

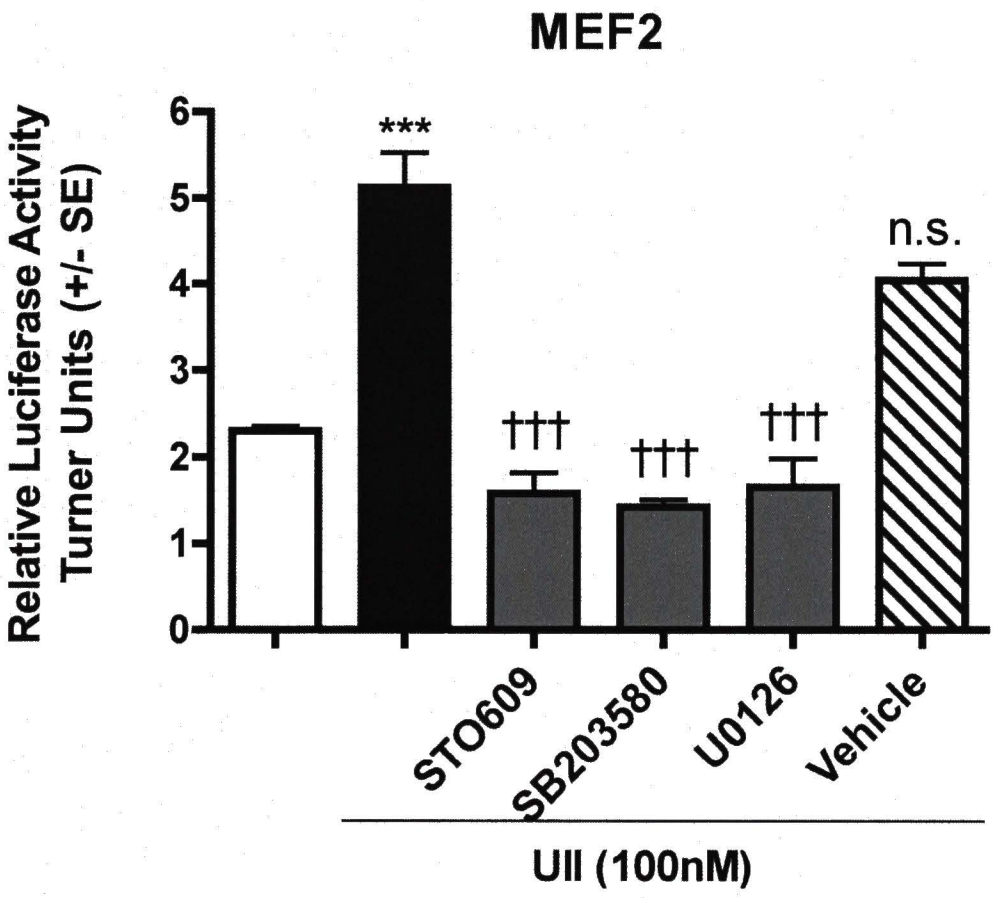

MEF2
Relative Luciferase Activity
Turner Units (+/- SE)
6
5
4
3
2
1
0
***
n.s.
†††
†††
†††
STO609
SB203580
U0126
Vehicle
UII (100nM)

Figure 32. CaMKI activation of the ANF promoter requires p38 and ERK1/2 MAP kinases. Cardiomyocytes were transiently transfected with 50 ng/well of the ANF promoter-reporter plasmid and constitutively-active CaMKI (as indicated) for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS. Designated wells were treated with SB203580 (10 μM) or U0126 (10 μM) for 48 hours, after which, luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (* indicates $p < 0.05$ compared with control; *** indicates $p < 0.001$ compare with control; †† indicates $p < 0.01$ compared with CaMKI alone).

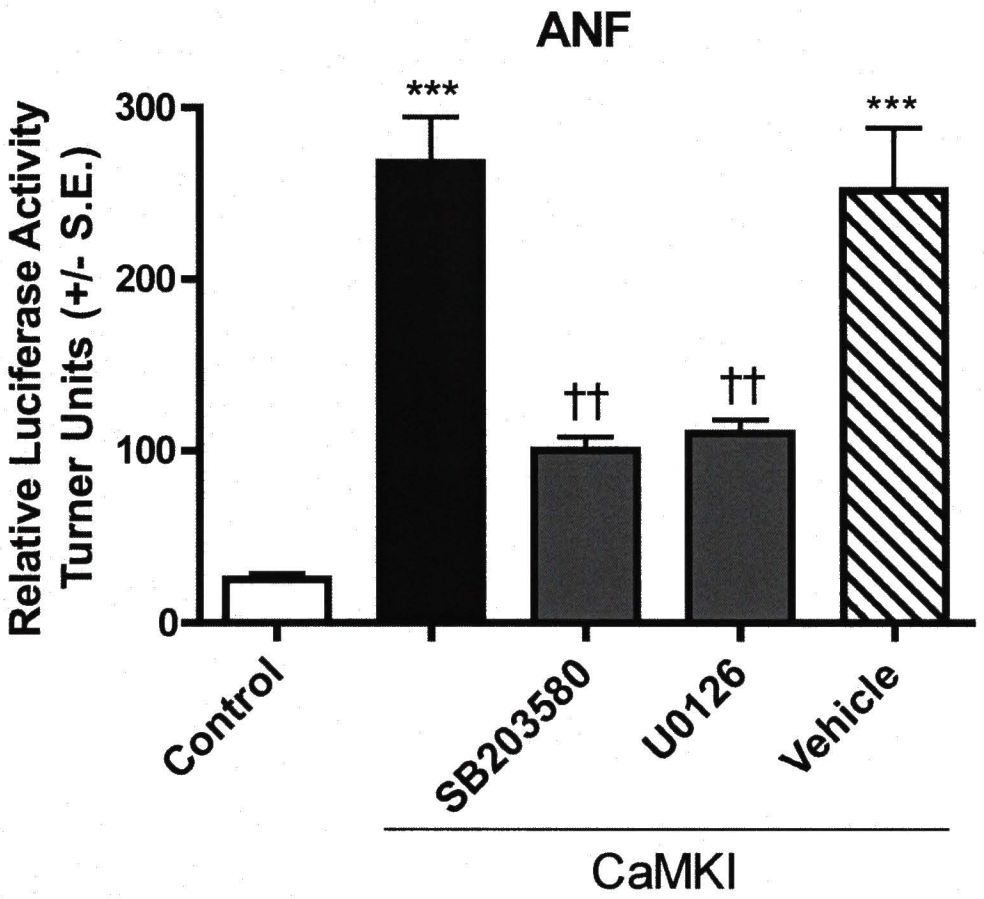

ANF
Relative Luciferase Activity
Turner Units (+/- S.E.)
300
200
100
0
***
***
††
††
Control
SB203580
U0126
Vehicle
CaMKI

Figure 33. CaMKI activation of the SkA promoter requires p38 and ERK1/2 MAP kinases. Cardiomyocytes were transiently transfected with 50 ng/well of the SkA promoter-reporter plasmid and constitutively-active CaMKI (as indicated) for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS. Designated wells were treated with SB203580 (10 μM) or U0126 (10 μM) for 48 hours, after which, luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (*** indicates $p < 0.001$ compare with control; †† indicates $p < 0.01$ compared with CaMKI alone).

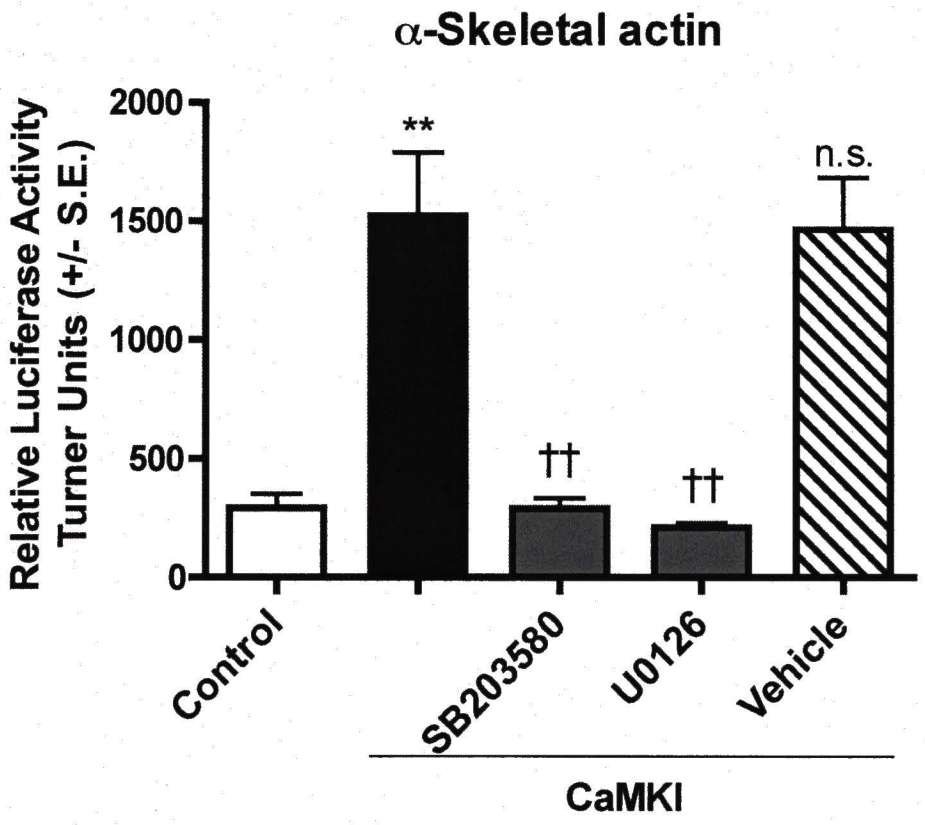

α-Skeletal actin
Relative Luciferase Activity
Turner Units (+/- S.E.)
2000
1500
1000
500
0
**
n.s.
††
††
Control
SB203580
U0126
Vehicle
CaMKI

Figure 34. CaMKI activation of MEF2 requires p38 and ERK1/2 MAP kinases. Cardiomyocytes were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter plasmid and constitutively-active CaMKI (as indicated) for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS. Designated wells were treated with SB203580 (10 μM) or U0126 (10 μM) for 48 hours, after which, luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (* indicates $p < 0.05$ compared with control; *** indicates $p < 0.001$ compare with control; †† indicates $p < 0.01$ compared with CaMKI alone).

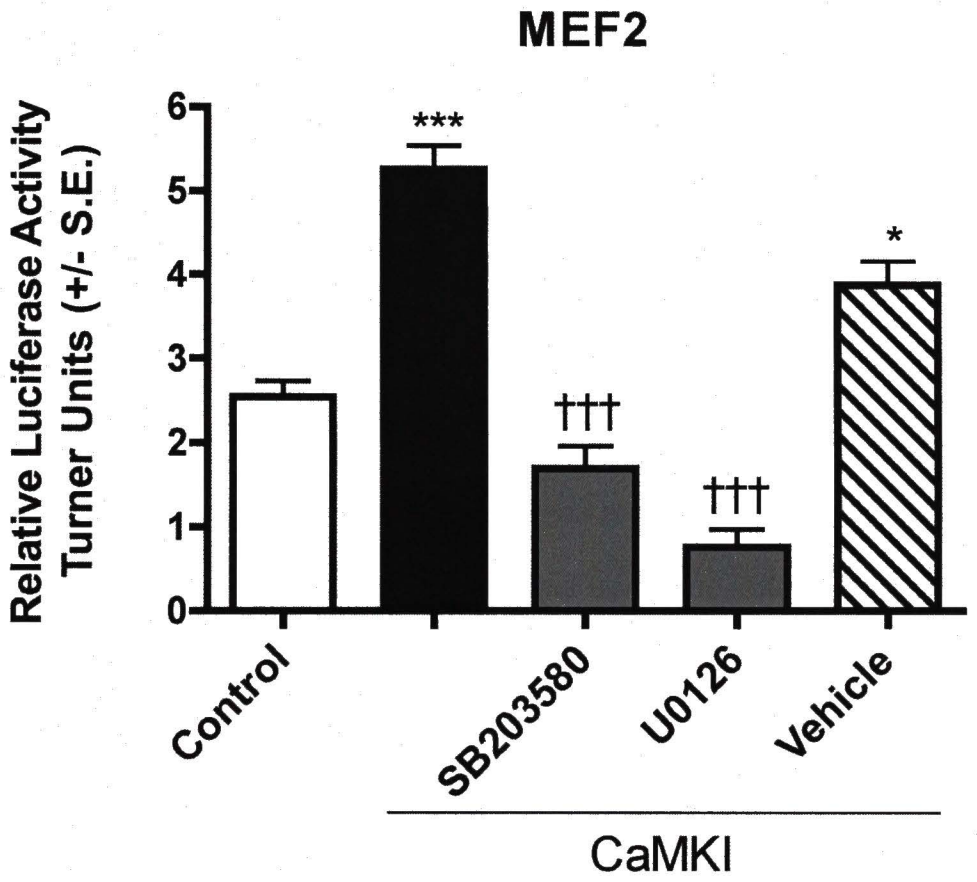

MEF2
Relative Luciferase Activity
Turner Units (+/- S.E.)
6
5
4
3
2
1
0
***
†††
†††
*
Control
SB203580
U0126
Vehicle
CaMKI

Figure 35. Dominant-negative p38α inhibits UII activation of MEF2.

Cardiomyocytes were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter plasmid and dominant-negative p38 (100 ng/well) as indicated for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS and allowed to recover for 24 hours. After recovery, designated culture wells were infected with AdUIIR for 24 hours and then washed and re-fed with medium 199 and 0.2% FBS. Designated wells were stimulated with UII (100 nM) for 48 hours, after which, luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (** indicates $p < 0.01$; *** indicates $p < 0.001$).

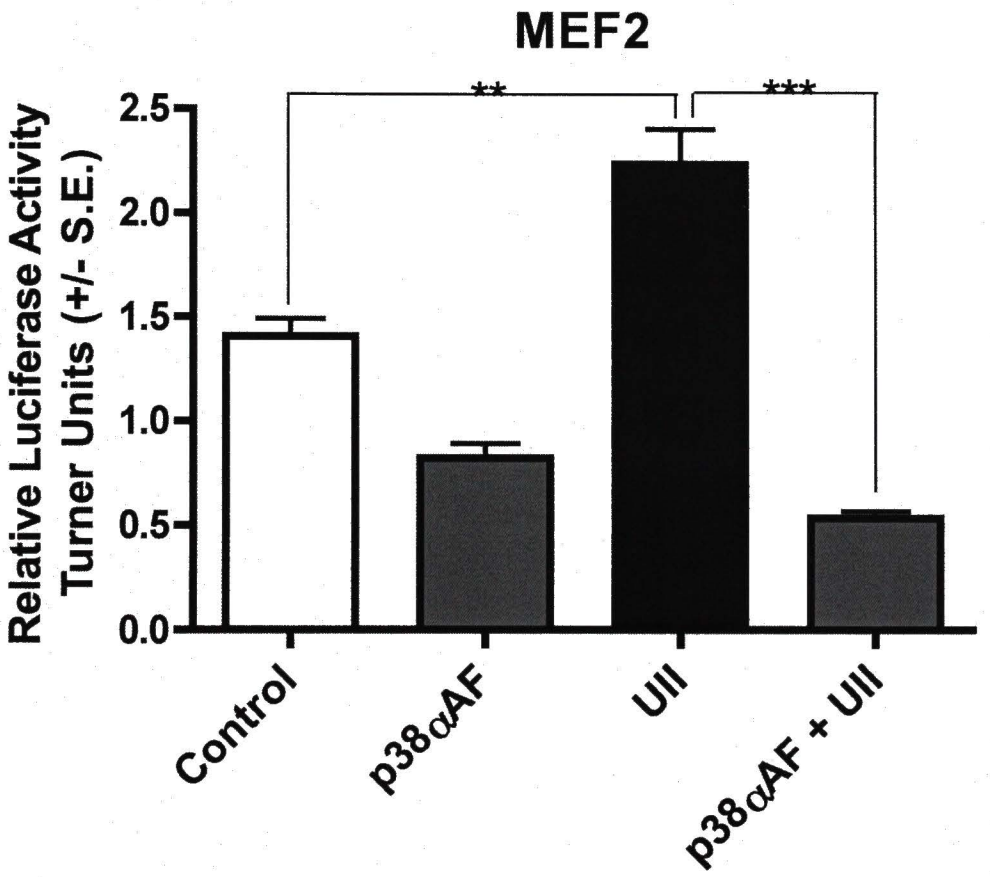

MEF2
**
***
Relative Luciferase Activity
Turner Units (+/- S.E.)
2.5
2.0
1.5
1.0
0.5
0.0
Control
p38αAF
UII
p38αAF + UII

Figure 36. Dominant-negative p38α inhibits CaMKI activation of MEF2.

Cardiomyocytes were transiently transfected with 250 ng/well of the MEF2 enhancer-reporter plasmid , constitutively-active CaMKI (10 ng/well) and dominant-negative p38 (100 ng/well) as indicated for three hours using LipofectAMINE™ Plus reagent. After transfection, cells were washed and re-fed with medium 199 and 0.2% FBS. After 48 hours luciferase activity was measured by luminometry. The data represent the mean ± SEM of three samples per treatment group (*** indicates $p < 0.001$).

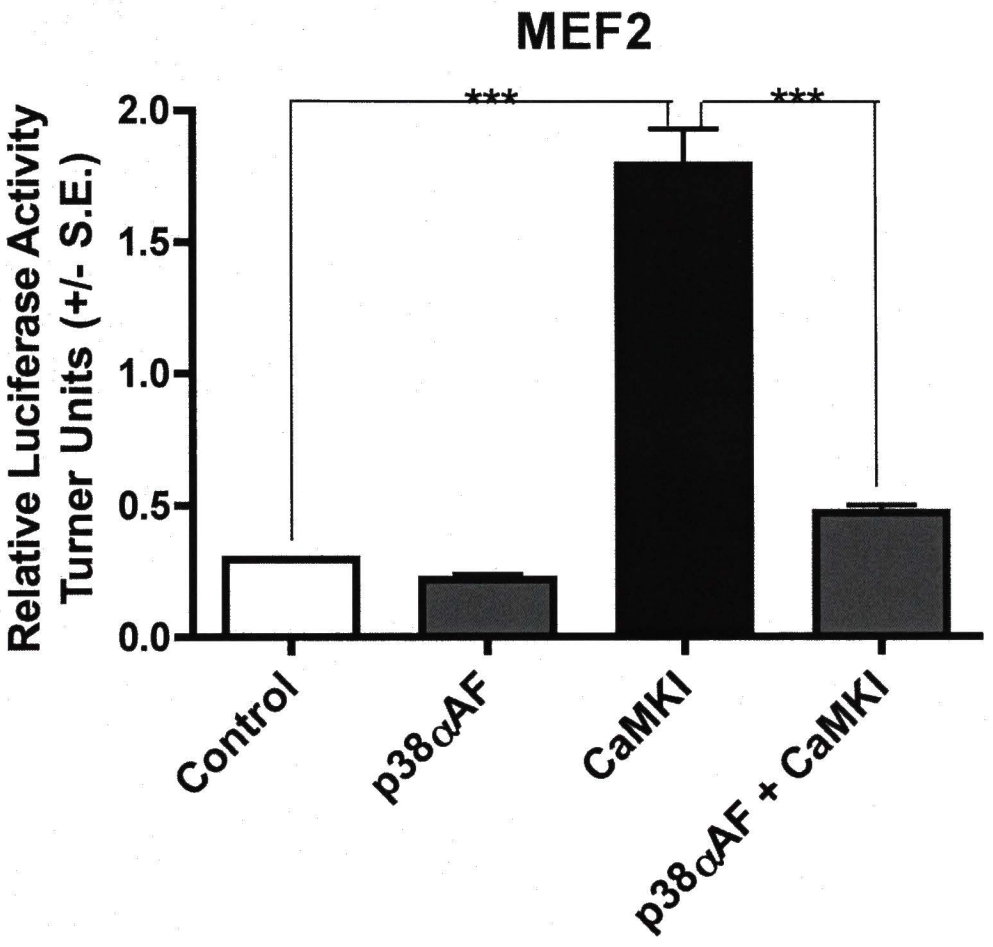

MEF2
Relative Luciferase Activity
Turner Units (+/- S.E.)
2.0
1.5
1.0
0.5
0.0
***
***
Control
p38αAF
CaMKI
p38αAF + CaMKI

Figure 37. UII stimulates the association of HDAC5 with 14-3-3β. Cardiomyocyte cultures were stimulated with UII (100 nM) for 5, 10, 30 and 60 minutes. After stimulation, total protein was isolated and 14-3-3β was immunoprecipitated. The immunoprecipitate was resolved by 12% PAGE and transferred to a nitrocellulose membrane. HDAC5 was detected by Western Blot. The membrane was stripped and re-probed for input (14-3-3β) to ensure equal loading.

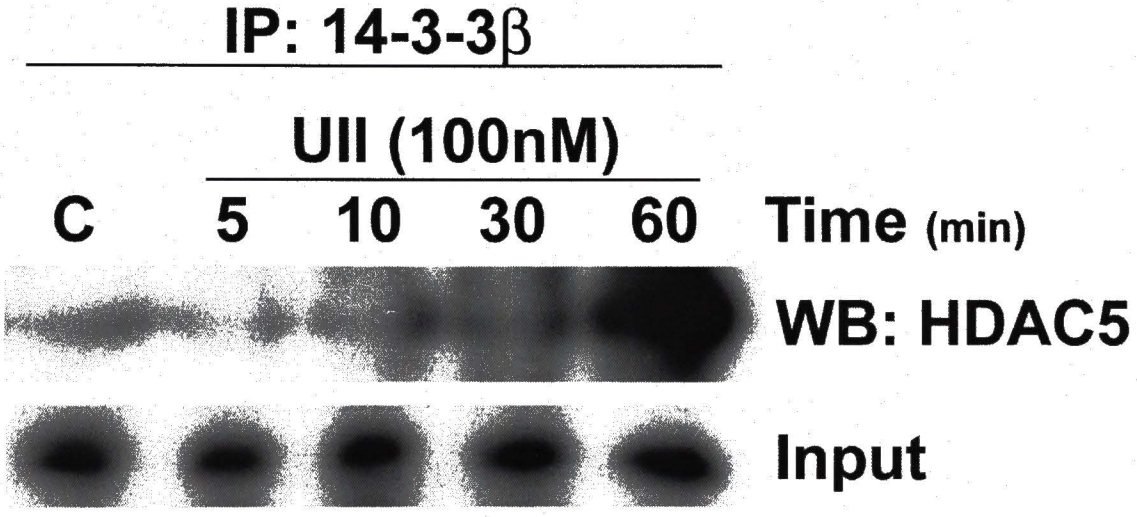

IP: 14-3-3β
UII (100nM)
C
5
10
30
60
Time (min)
WB: HDAC5
Input

Figure 38. UII-mediated association of HDAC5 with 14-3-3β is inhibited by STO-609 and U0126. Cardiomyocyte cultures were treated with STO-609 (250 ng/mL), SB203580 (10 μM) or U0126 (10 μM) 45 minutes prior to UII stimulation. Cardiomyocyte cultures were stimulated with UII (100 nM) 60 minutes. After stimulation, total protein was isolated and 14-3-3β was immunoprecipitated. The immunoprecipitate was resolved by 12% PAGE and transferred to a nitrocellulose membrane. HDAC5 was detected by Western Blot. The membrane was stripped and re-probed for input (14-3-3β) to ensure equal loading.

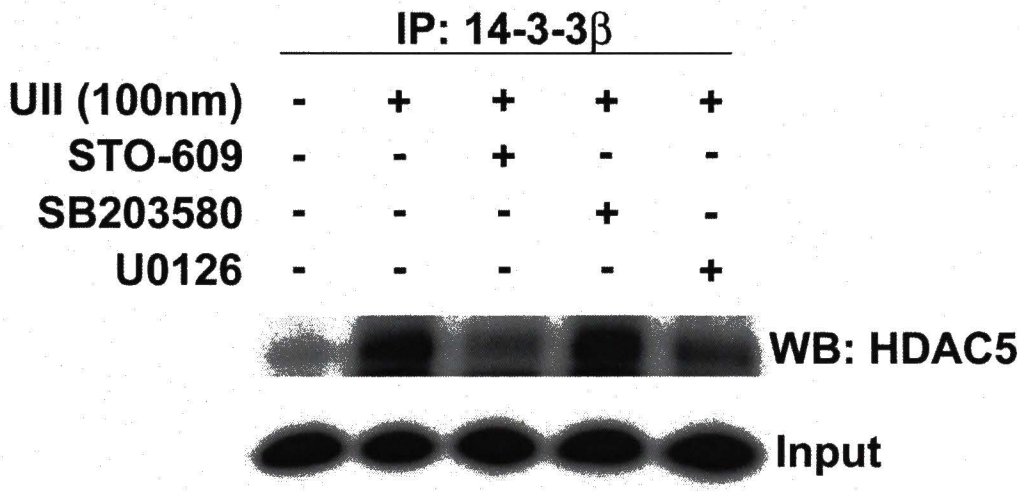

IP: 14-3-3β
UII (100nm) - + + + +
STO-609 - - + - -
SB203580 - - - + -
U0126 - - - - +
WB: HDAC5
Input

Figure 39. UII stimulates the activation of PKD. Cardiomyocyte cultures were stimulated with UII (100 nM) for 0, 2, 5, 10, 30 and 60 minutes. Total protein from each treatment was isolated and total protein concentration measured by BCA assay. A total of 20 μg of total protein from each treatment was resolved by 12% PAGE and transferred to a nitrocellulose membrane. Active PKD was detected by Western Blot. The membrane was stripped and re-probed for GAPDH to ensure equal loading.

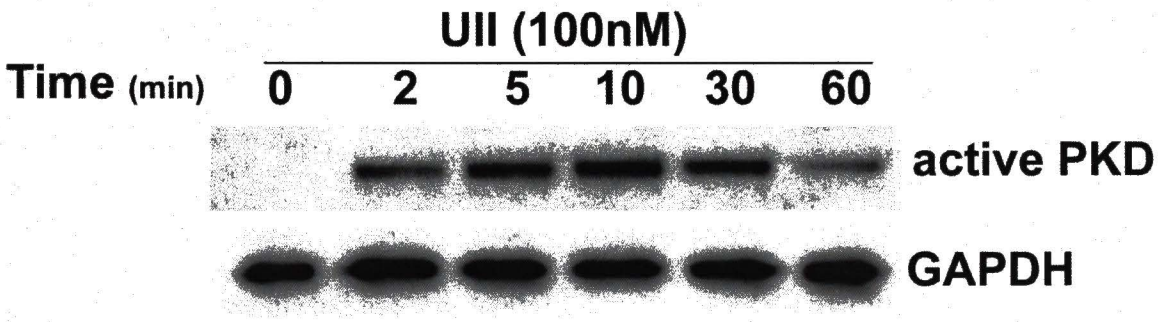
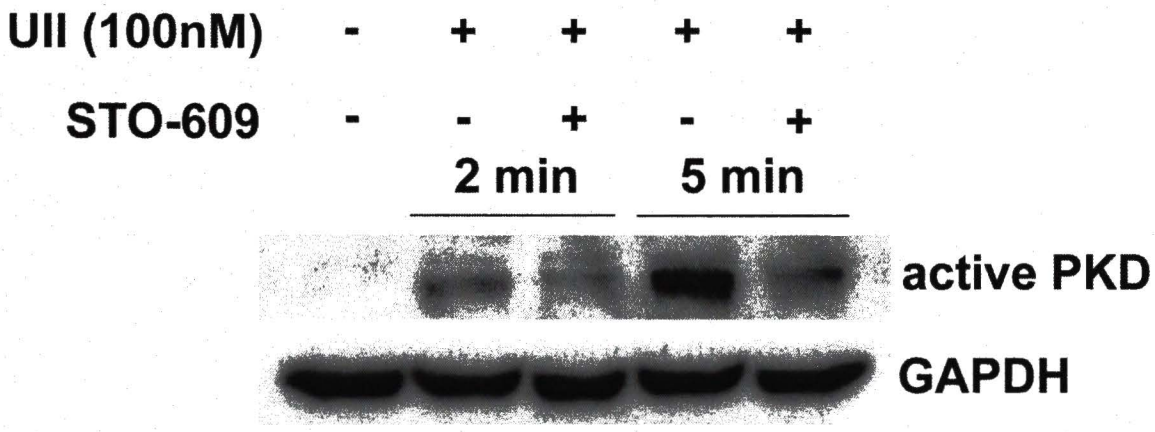

UII (100nM)
Time (min)
0
2
5
10
30
60
active PKD
GAPDH

Figure 40. UII-dependent activation of PKD requires CaMKK. Designated cardiomyocyte cultures were pre-incubated with STO-609 (250 ng/mL) prior to UII stimulation. Cells were then stimulated with UII (100 nM) for the indicated times with or without STO-609. Total protein was isolated from each treatment and total protein measured by BCA assay. 20 μg of total protein from each treatment were resolved by 12% PAGE and transferred to a nitrocellulose membrane. Active PKD was detected by Western Blot. The membrane was then stripped and re-probed for GAPDH to ensure equal loading.


UII (100nM) - + + + +
STO-609 - - + - +
2 min
5 min
active PKD
GAPDH

CHAPTER IV

DISCUSSION

Recently, UII has arisen as a probable contributor to cardiovascular physiology and pathology. More specifically, UII is capable of inducing the hypertrophic phenotype in cultured cardiomyocytes. It has been shown that UII stimulation of cardiomyocytes results in increased cell size, increased protein to DNA ratio and sarcomeric reorganization[148]. Presently, the signaling mechanisms that couple UII to hypertrophy are not completely known.

The expression of UIIR is undetectable to slight in healthy myocardium. It is only in states of myocardial disease or dysfunction that UIIR expression becomes markedly up-regulated[39, 137, 141, 149]. The etiology of heart disease and failure nearly always includes cardiac hypertrophy. UII is known to induce hypertrophy in cell culture, but only when the UIIR is sufficiently expressed. Sustained mechanical stress to the myocardium often leads to cardiac hypertrophy. There is evidence which suggests that mechanical stress itself increases the availability of humoral factors known to induce myocyte hypertrophy; for instance, AngII[11, 12, 14]. It is possible that at some point during the etiology of heart failure, available hypertrophic agonists stimulate the expression of UIIR over the necessary threshold for UII to elicit its biological activity thereby contributing to a downward spiral toward dilated cardiomyopathy and failure. Therefore, we set out to

determine whether known inducers of cardiac hypertrophy are capable of up-regulating UIIR in cardiomyocytes. We first searched the GEO database at the NCBI (URL) for microarray experiments using whole heart or cultured cardiomyocytes as a model. We found one such experiment whereby cultured cardiomyocytes were treated with PE for 24 hours. Upon further inspection of the results, we found that under these conditions, the expression of UIIR was increased. The array data itself was scored as absent meaning that at least 8 of the 11 probes for UIIR on the microarray did not achieve strong enough signal strength. Though absent, the data is still informative as the threshold for an absence call is set arbitrarily (personal communication with Patrick Cooke, Affymetrix). With this, we designed gene specific primers for rat UIIR and replicated the array experiment. Through the use of RT-PCR, we demonstrate that stimulation of cardiomyocytes with PE and AngII results in an increase of UIIR mRNA. Corresponding with the increase of UIIR mRNA, we show that PE and AngII stimulation of cardiomyocytes results in the increase of UIIR protein.

There is evidence that signaling through G_q-coupled receptors increases the expression of other G_q-coupled receptors; for example, Angiotensin II stimulates the up-regulation of Endothelin receptor B (ET_BR) in cardiomyocytes[16]. Of particular interest, it has been observed that moderate degrees of G_q signaling stimulate adaptive hypertrophy[17-19], whereas high degrees of G_q signaling result in maladaptive cardiomyocyte apoptosis[20-23]. Though more work needs to be done, our results show that hypertrophy-stimulating humoral factors such as AngII result in the up-regualtion of another Gq-coupled receptor, UIIR

Signaling through the UIIR is known to activate PLC producing IP_3 and DAG. IP_3 activates the sarcoplasmic reticulum IP_3 receptor that releases Ca^{2+} to the cytoplasm. However, Onan et al. showed that inhibition of PKC does not result in the inhibition of UII-mediated hypertrophy induction[78]. The complete signaling pathway through UIIR has not been fully delineated; however, others have shown that UII stimulation activates members of the MAP kinase pathway. CaMKI is immediately downstream of and requires CaMKK to be active. CaMKI has been shown to activate ERK 1/2[58] and UII stimulation activates both ERK1/2 and p38[78]. Therefore, we hypothesized that ERK 1/2 and p38 activation through the UIIR may be dependent on CaMKI activation by CaMKK.

Initial experiments were conducted whereby we stimulated cardiomyocytes with varying doses of UII and measured ANF promoter activity. Under the culture conditions that we used for this study, cardiomyocytes were incompetent to respond to UII (data not shown). This makes sense in light of the fact that the expression of UIIR in normal rat cardiomyocytes is slight to nonexistent. In addition, this suggests that UII is incapable of stimulating cardiomyocytes through a non-receptor mediated mechanism. In order to study the hypertrophic effects of UII in our cell culture model, we used to adenoviral delivery of UIIR.

Data from the present study clearly demonstrate that UII stimulation of cardiomyocytes expressing UIIR results in the induction of hypertrophy marker genes. This was first shown by promoter reporter assays whereby cardiomyocytes were transfected with either ANF or SkA promoter-reporters (Figures 9-12). In similar

experiments, we show for the first time that UII is capable of activating MEF2 (Figures 13 and 14).

Since UII elicits the mobilization of intracellular calcium through classical G_q coupling mechanisms, we hypothesized that UII stimulation of hypertrophy gene induction would include the involvement of CaMKK. In order to demonstrate this, we initially utilized promoter reporter assays measuring the activities of the ANF and SkA promoters. In the presence of STO-609, a potent and selective inhibitor of CaMKK, UII was incapable of stimulating neither the ANF nor the SkA promoter (Figures 15 and 16). We are very confident in the selectivity of STO-609 for CaMKK as the IC_{50} value is 120ng/mL for CaMKKα and 40ng/mL for CaMKKβ. Other kinases are inhibited by STO-609 (CaM-KII, MLCK (IC50 ~ 10μg/mL), CaM-KI, CaM-KIV, PKA, PKC, and p42 MAP kinase (IC_{50} >10μg/mL)), but only well above the dose used in the current study (250ng/mL). In addition, we assayed the ability of UII to increase the message of several hypertrophy-sensitive genes in the cardiomyocyte. UII was able to increase the expression of all hypertrophy marker genes studied. However, in the presence of STO-609, UII was unable to stimulate the expression of ANF, BNP, βMHC or SkA (Figure 18). These data implicate CaMKK as a major component in UII-induced hypertrophic gene induction.

Through the use of western blots, we determined that UII stimulation of cardiomyocytes results in a time-dependent activation of CaMKI (Figure 19). These data make sense in that CaMKI is a major downstream effector of CaMKK. Indeed, in the presence of STO-609, the UII-dependent activation of CaMKI was inhibited (Figure 20).

The activation of CaMKI by UII provides a critical insight into the mechanism by which UII is able to elicit a hypertrophic response in cardiomyocytes. Our laboratory has previously demonstrated that constitutively active CaMKI transfection of cardiomyocytes results in a robust activation of ANF and SkA reporters, MEF2 activity as well as the 14-3-3 dependent translocation of class II HDACs to the cytoplasm[38, 126]. The constitutively active mutant of CaMKI is active independent of Ca^{2+}/CaM and CaMKK. In addition, we show herein that constitutively active CaMKI completely rescues UII stimulation of ANF, SkA and MEF2 when CaMKK is inhibited (Figures 21-23). These results demonstrate that under UII stimulation, CaMKI is active and is a major component of the downstream signaling from the UII receptor. The ability of CaMKI to rescues UII stimulation under conditions where CaMKK was inhibited further implicates the CaM kinase pathway as necessary for UII-dependent hypertrophy gene induction. If an additional signaling component downstream of CaMKK was necessary for full UII stimulation, we would not expect that CaMKI alone could fully recapitulate UII stimulation of ANF, SkA and MEF2. To date, only three kinases are known downstream targets of CaMKK: CaMKI, CaMKIV and AKT. Of these, only CaMKI and AKT are expressed in the heart. Although confident of the role of CaMKI, we could not rule out the involvement of AKT. We therefore designed experiments to test whether inhibition of AKT could affect UII stimulation of hypertrophy-sensitive promoters. We found no reduction of UII stimulation of ANF, SkA and MEF2 promoter activities when AKT was inhibited (Figures 24-26). Additionally, AKT inhibition had no effect on the ability of

UII to stimulate the activity of MEF2. Our AKT data are in agreement with others who have shown that inhibition of PI3K had no effect on UII stimulation of hypertrophy[78].

It is known that UII stimulation of cardiomyocytes results in a time-dependent activation of p38 and ERK1/2 MAP kinases[78]. In fact, several studies demonstrate the overall importance of p38[101, 150-152] and ERK1/2[69, 76, 151, 153] in the progression of cardiomyocyte hypertrophy both *in vivo* and *in vitro*. Onan, et al. demonstrated that UII stimulation of p38 and ERK1/2 activities might be dependent on the ability of the UIIR to transactivate the EGFR. Additionally, UII induced hypertrophy was completely prevented only if the EGFR and ERK1/2 were inhibited. These data led the authors to suggest that there may be an EGFR independent pathway leading to ERK1/2 activation and myocyte hypertrophy. In other cell types, Ca^{2+} is capable of activating both MAPKs[58]. More important, Ca^{2+} activation of MAPKs is dependent on the CaMK cascade. This was demonstrated using neuronal cells lines—excitable cells like cardiomyocytes[58]. In line with this, we hypothesized that UII stimulation of cardiomyocytes resulting in the activation of p38 and ERK1/2 is dependent on CaMKK. We have already shown that UII stimulation of cardiomyocytes resulted in a time-dependent activation of CaMKI. Using antibodies that detect only the active forms of p38 and ERK1/2, we determined by western blot analysis that both p38 and ERK1/2 were indeed activated in a time-dependent manner by UII stimulation of our cardiomyocyte cell model (Figure 27). These data confirmed the findings by Onan, et al. When we inhibited CaMKK, UII stimulation of active p38 and ERK1/2 was almost completely repressed (Figure 28). The portion of activated p38 and ERK1/2 that remained after

CaMKK inhibition may be due to EGFR transactivation by UII as seen by Onan, et al; however, recall that CaMKK inhibition completely blocked UII activation of ANF, SkA and MEF2 in earlier experiments. It is probable that MAP kinase activation must meet a threshold of activity prior to stimulating hypertrophy marker genes and the UII-induced EGFR component is not capable of reaching this threshold. An interesting note must be made regarding the EGF receptor. It has been shown that CaM is capable of binding EGFR and thus inhibits PKC-dependent activation[154]. Under our experimental conditions, we demonstrate the dependence of CaMKK—which requires CaM—as well as the activation of CaMKI. Onan et al. found that UII stimulation of hypertrophy did not require PKC activity. More important, the authors show that p38 and ERK1/2 activation by UII did not depend on PKC. Here we provide a mechanism that accounts for the fact that UII activation of MAP kinases and hypertrophy does not require PKC. Namely, we show that UII stimulates hypertrophy marker gene expression and MAP kinase activation through CaMKK and consequently, through CaMKI.

In our hands, either inhibition of p38 or ERK1/2 resulted in a complete loss of the ability of UII to stimulate both ANF and SkA promoter activities or MEF2 enhancer activity (Figure 29-31). More important, we demonstrated that CaMKI stimulation of ANF, SkA and MEF2 was also prevented when p38 and ERK1/2 were inhibited (Figures 32-34). Taken together, these data demonstrate a novel crosstalk mechanism between the CaM kinase cascade and MAP kinases not previously described in cardiomyocytes. This crosstalk mechanism not only helps to explain UII-dependent stimulation of cardiomyocyte hypertrophy, but also may be relevant for other G_q coupled receptors such

as for AngII. Indeed, in additional experiments we found that the AngII- or ET-1-dependent activation of ERK1/2 also required CaMKK (Figure 41A).

As p38 MAPKs require dual phosphorylation of both a Thr and a Tyr residue to be active, it is improbable that CaMKI directly activates p38. The CaMKI consensus phosphorylation motif is [M/V/L/I/F]-X-X-R-X-X-[S/T]-X-X-X-[M/V/L/I/F][155]. MKK3 and MKK6 are the canonical immediate upstream activators of p38. MKK3 and MKK6 are phosphorylated at Ser_{189} and Ser_{207}, respectively. These phosphorylations result in kinase activity. When we looked at the regions immediately surrounding Ser189 (MKK3) and Ser207 (MKK6), we found that the sequence bears a striking similarity to that of the CaMKI consensus sequence. It is therefore reasonable to suspect CaMKI phosphorylation of MKK3 and MKK6 leading to the activation of p38.

MEF2 is a transcritption factor that is a major component of the signaling involved with cardiomyocyte hypertrophy. MEF2 is critical for the development of the heart. In the adult heart, MEF2 is held inactive by class II HDACs. Under conditions that result in cardiomyocyte hypertrophy, HDACs are phosphorylated and transported to the cytoplasm via 14-3-3 proteins, thereby relieving repression of MEF2. We have previously demonstrated that CaMKI is capable of stimulating the phosphorylation and nucleocytoplasmic shuttling of HDAC5 resulting in MEF2 transcriptional activity[126]. In addition to the removal of HDACs, the activation of MEF2 is dependent on phosphorylation by p38[104].

Using a dominant negative p38, we demonstrated that the UII-dependent activation of MEF2 required functional p38 (Figure 35). In addition, we showed that

CaMKI-dependent activation of MEF2 also required p38 (Figure 36). These data corroborate our earlier findings using a pharmacological inhibitor of p38 whereby either UII or CaMKI stimulation of MEF2 activity was abolished by p38 inhibition. More important, we found that the stimulation of cardiomyocytes with UII resulted in the time-dependent association of HDAC5 with 14-3-3β (Figure 37). In addition, inhibition of CaMKK with STO-609 resulted in a decreased association of HDAC5 with 14-3-3β in cardiomyocytes stimulated with UII (Figure 38). Inhibition of p38 had no effect on HDAC5 association with 14-3-3β under UII stimulation. This was to be expected as p38 activates MEF2 directly through phosphorylation whereas p38 phosphorylation of HDACs has never been described. Therefore, we show two independent mechanisms of MEF2 activation by UII: i) relief of HDAC repression and ii) activation of MEF2 by p38. When we inhibited ERK1/2 with U0126, there was a reduced UII-dependent association with 14-3-3β.

These results suggest that ERK1/2 may be able to regulate HDAC5. In fact, the association of ERK1/2 with HDAC4 has been reported; however, it appears that ERK1/2 kinase activity induces nuclear localization of HDAC4[156]. It is interesting to note that U0126 also inhibits ERK5. ERK5 was shown to interact with MEF2[157]. Whether ERK5 is capable of phosphorylating HDACs remains to be seen and should be explored.

In cardiomyocytes, two parallel pathways have been proposed which result in the export of class II HDACs from the nucleus. The first pathway is through CaMKI as we have shown, and the second is through PKD[127]. PKD was shown to directly phosphorylate HDAC5 resulting in its nuclear export. Since UII was able to induce the

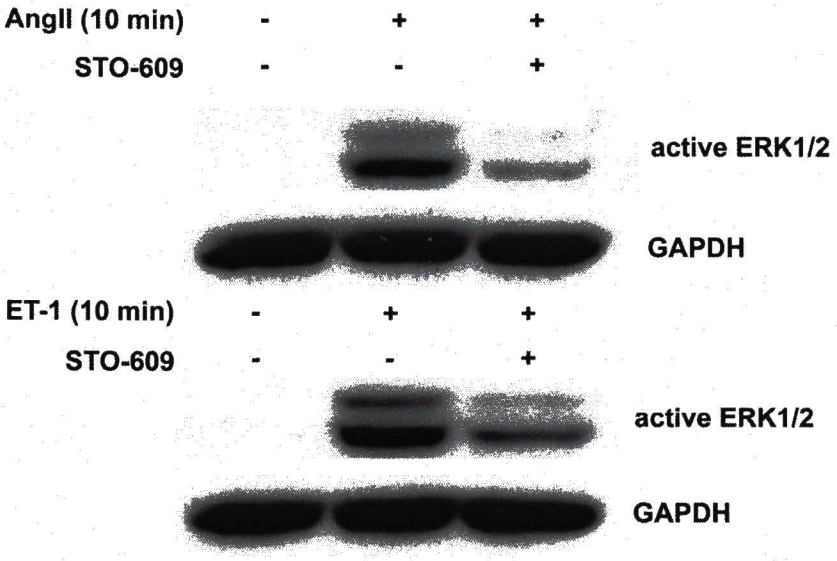
association of HDAC5 with 14-3-3β, we could not rule out the possibility that PKD was involved. Using an antibody specific for active PKD phosphorylated at Ser_{744} and Ser_{748}, we demonstrated that UII stimulation of cardiomyocytes resulted in the time-dependent activation of PKD (Figure 39). More interesting, we demonstrated that CaMKK inhibition blocked the UII-dependent phosphorylation of PKD. These data argue that PKD may be downstream of CaMKK or CaMKI. To date, no kinase activity has been proposed downstream of CaMKI that results in the export of HDAC from the nucleus. CaMKI is localized to the cytoplasm and has not been reported in the nucleus. Though further work must be done, we cannot rule out the possibility that the CaM kinase cascade activates PKD resulting in HDAC nuclear export. Indeed, preliminary data that we have gathered suggests that activation of PKD by AngII and ET-1 requires CaMKK (Figure 41B).

The results presented in this dissertation clearly demonstrate an important role for CaMKK in UII-mediated cardiomyocyte hypertrophy. UII was able to stimulate the promoter activity of ANF and SkA and the transcriptional activity of MEF2 in a CaMKK-dependent manner. UII stimulation of ANF, BNP, βMHC and SkA gene expression was dependent on CaMKK. UII stimulation caused the CaMKK-dependent activation of CaMKI. Constitutively-active CaMKI completely rescued UII stimulation of ANF and SkA promoter activities as well as MEF2 activity with CaMKK pharmacologically inhibited. The inhibition of AKT had no effect on the ability of UII to stimulate hypertrophy-sensitive promoters or MEF2 activity. We demonstrated that the UII-induced activation of p38 and ERK1/2 MAP kinases was dependent on CaMKK

suggesting a novel cross-talk mechanism not previously described in cardiomyocytes. Both UII- and CaMKI-mediated induction of ANF, SkA and MEF2 reporter activities was dependent on p38 and ERK1/2. The data presented in this dissertation allow us to construct a more complete pathway whereby UII stimulation results in hypertrophic gene induction. Moreover, this new pathway involves a crosstalk mechanism between the CaMKs and the MAPKs not previously reported in cardiomyocytes (Figure 42).

Figure 41. AngII and ET-1 dependent activation of ERK1/2 and PKD requires CaMKK. We have begun to explore the importance of CaMKK-mediated activation of ERK1/2 MAPKs as well as PKD by other Gq-coupled receptor agonists. Both AngII and ET-1 activation of ERK1/2 and PKD require CaMKK. These data begin to demonstrate a common theme in Gq-mediated cardiac hypertrophy events.

A.


AngII (10 min) - + +
STO-609 - - +
active ERK1/2
GAPDH
ET-1 (10 min) - + +
STO-609 - - +
active ERK1/2
GAPDH

B.

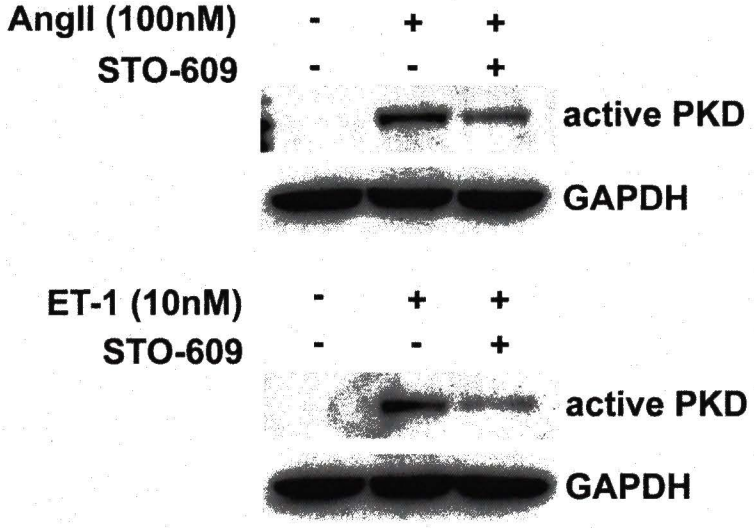
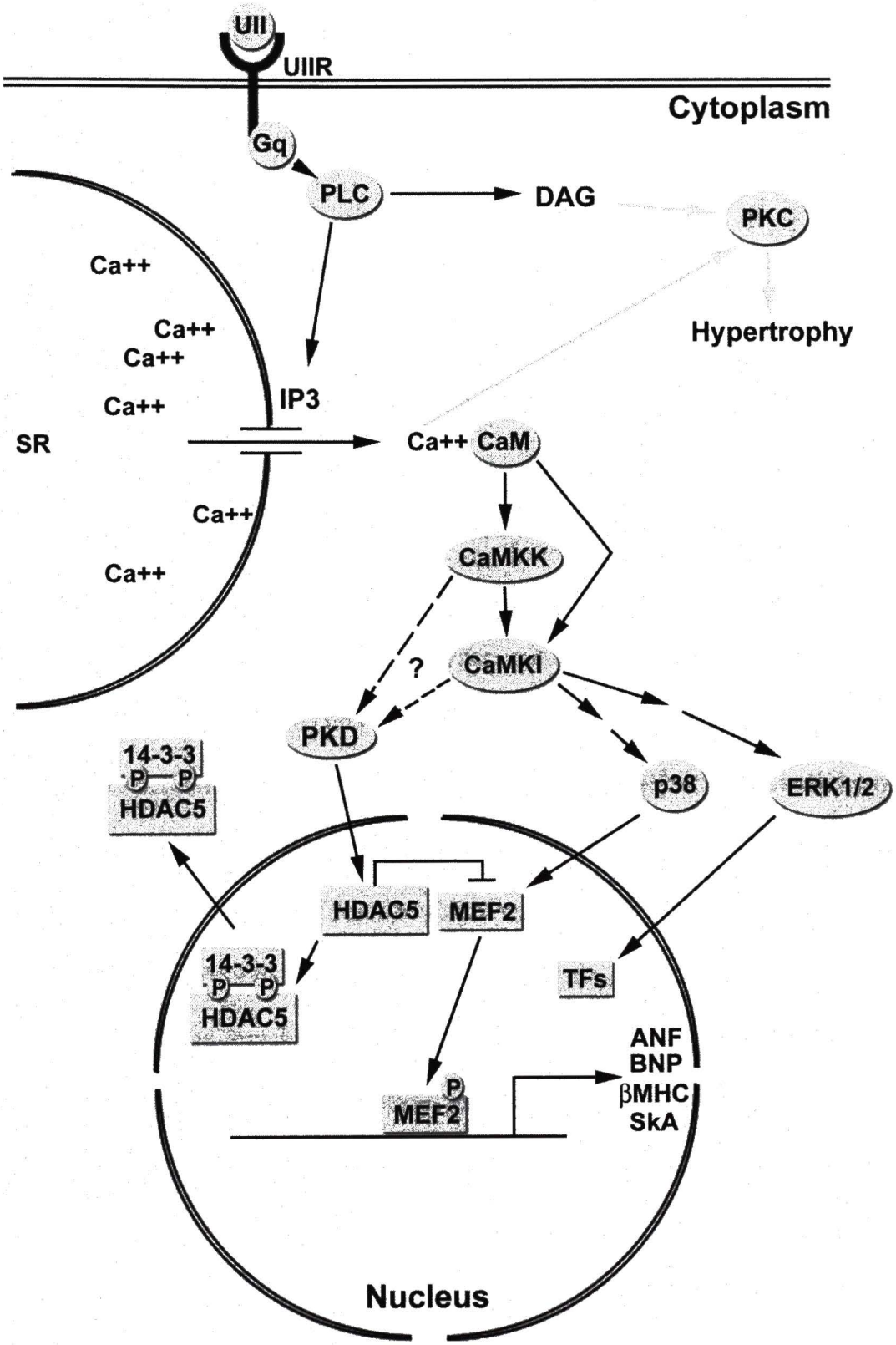

AngII (100nM) - + +
STO-609 - - +
active PKD
GAPDH
ET-1 (10nM) - + +
STO-609 - - +
active PKD
GAPDH

Figure 42. Pathway describing novel signaling mechanisms for UII-induced hypertrophic gene induction. A requirement for CaMKK in the UII-induced expression of hypertrophy sensitive genes and activation of MAPKs has been demonstrated. Though more work needs to be done, these data begin to describe an important role for CaMKK in Gq-mediated cardiac hypertrophy.


UII
UIIR
Cytoplasm
Gq
PLC
DAG
PKC
Hypertrophy
Ca++
Ca++
Ca++
Ca++
IP3
SR
Ca++ CaM
Ca++
Ca++
CaMKK
?
CaMKI
PKD
14-3-3
P P
HDAC5
p38
ERK1/2
HDAC5
MEF2
14-3-3
P P
HDAC5
TFs
ANF
BNP
βMHC
SkA
P
MEF2
Nucleus

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Since UIIR is a newly discovered G_q coupled receptor, many questions still remain concerning UIIR expression during cardiac disease states as well as its contribution to downstream signaling mechanisms that contribute to the hypertrophic phenotype in cardiomyocytes. The experiments carried out in this dissertation were designed to test the overall hypothesis that hypertrophic up-regulation of UIIR and the resultant increase in G_q signaling and intracellular calcium activates CaMKK resulting in the downstream activation of CaMKI. In addition, we hypothesized that CaMKI cross-talks with p38 and ERK1/2 MAP kinases. We will now describe specific conclusions drawn from the current work and describe future work needed to further explain the role of UII in cardiac hypertrophy and failure.

Microarray data found at the GEO database (NCBI) demonstrated the likelihood that UIIR is up-regulated in cardiomyocytes as a result of stimulation by other G_q-coupled receptor agonists. UIIR is normally expressed at very low levels in the heart; however, UII and its receptor are co-expressed in cardiomyocytes and up-regulated in cardiac disease states [39, 137, 141, 149]. Therefore, we first set out to determine the effects of other G_q-stimulatory hypertrophic agonists on the expression of UIIR. Data described in

this dissertation clearly demonstrated that PE and AngII stimulation of cardiomyocytes results in the up-regulation of UIIR gene expression and, more importantly, the up-regulation of the UIIR protein.

Mechanical stresses to the left ventricle, such as increased afterload (pressure overload) or increased preload (volume overload), lead to the stretching of cardiomyocytes. This stretch mechanism results in the local release of AngII and ET-1 from cardiomyocytes, which can act via an autocrine mechanism to stimulate a compensatory hypertrophic phenotype. *In vivo* models of pressure-overload should be used to measure the expression of UIIR. In addition, ACE inhibition of pressure-overload hypertrophy would be useful to determine whether AngII up-regulates UIIR in the heart *in vivo*.

There are presently no data regarding the precise spatio-temporal control of the expression of UIIR. Therefore studies should be designed to uncover the specific mechanisms of transcriptional control of the UIIR gene. Careful inspection of the 5' flanking region of the UIIR gene will yield key insights into the combination of transcription factors required for both proper as well as aberrant expression of the receptor. Initially, some of this work could be accomplished *in silico* utilizing the rVISTA[158] multiple alignment algorithm, which would allow for the inspection of evolutionary conserved promoter regions across divergent species. The data derived from the *in silico* promoter analysis would be validated by cloning the 5' region of UIIR and generating mutations of key transcription factor binding sites within conserved promoter regions to study loss/gain of expression of UIIR.

When coupled with expression of other Gq-coupled receptors, such as UIIR, the overall G_q signaling component in cardiomyocytes is increased under sustained myocardial stress. It is this increase in Gq signaling that has been reported to lead from compensatory hypertrophy toward myocyte cell death thereby contributing to decompensated failure. In fact, as mentioned above, UII has been reported to contribute to three major causes of decompensation: i) wall thickening (hypertrophy), ii) fibrosis and iii) cardiomyocyte apoptosis[78, 141]. Of particular note, the UII peptide is up-regulated in injured myocardium after experimental myocardial infarction and is increased in human congestive heart failure. It is therefore not improbable that UII signaling in the diseased heart also functions as an autocrine mechanism. In order to determine this, more work needs to be done to assess the *in vivo* contribution of UII to hypertrophy and failure. This could be accomplished with genetic knock-out models of UII and/or UIIR in the heart. Already, data derived from experimental models of ischemia have demonstrated the importance of UII using UIIR antagonists[159]. Others have described an animal model of the progression from hypertrophy to failure in rats using the Dahl Salt-Sensitive line[160]. The data generated in this dissertation would benefit from utilizing the salt-sensitive rat model to assess i) the contribution of UII signaling from hypertrophy to failure and ii) UIIR blockade to assess the contribution of UII from hypertrophy to failure. In addition, others have developed a hypertensive transgenic mouse model of salt-sensitivity[161]. The role of UIIR signaling from the progression of hypertrophy to failure should be studied by crossing heart-targeted UIIR transgenic knockout mice with the hypertensive transgenic mouse model of salt-sensitivity.

As the expression of UIIR is non-existent to slight in normal non-hypertrophied cardiomyocytes, it was necessary for us to exogenously express UIIR via an adenoviral vector. We first considered up-regulating UIIR in cardiomyocytes with PE or AngII, but we reasoned that since these signal through Gq-coupled receptors as well, downstream results would be confounded. As a result of initial experimentation, we were able to conclude that exogenous expression of UIIR in cardiomyocytes resulted in the dose-dependent activation of the ANF and SkA promoters as well as MEF2 activity. In addition, we were able to conclude that the stimulatory effect of UII was specific to cardiomyocyte cultures infected with the adenoviral vector for UIIR and not the control vector (AdGO-GFP). It would be of particular interest to study the effects of transgenic over-expression of UIIR in the heart. This would best be accomplished using an inducible expression system such as the TET-inducible system[162].

A major effector of Gq signaling in the heart is PKC. However, UII-stimulation of cardiomyocyte hypertrophy does not require PKC. Therefore, a key aim of this work was to determine whether UII requires the CaMK cascade for hypertrophy stimulation. Through the use of promoter-reporter assays and a CaMKK specific pharmacological inhibitor, we were able to conclude that CaMKK is required for the UII-dependent activation of the ANF and SkA promoters. In addition, we clearly demonstrated that the stimulation of MEF2 by UII was dependent on CaMKK. In support of reporter data, RT-PCR assays clearly showed that UII stimulation of cardiomyocytes resulted in increased gene expression of key hypertrophy indicators (ANF, BNP, βMHC and SkA)—all of which were decreased under CaMKK blockade. Taken together, these data allow us to

conclude that CaMKK is required for UII stimulation of key hypertrophy-sensitive marker genes as well as MEF2 activity.

Of the three known substrates of CaMKK (CaMKI, CaMKIV and AKT), only CaMKI and AKT are expressed in heart. Through the use of western blots, we were able to conclude that UII stimulates the activation of CaMKI in a time-dependent manner. In addition, we conclude that the UII activation of CaMKI requires CaMKK. Importantly, we demonstrated that the loss of UII stimulation of ANF, SkA and MEF2 by CaMKK inhibition was completely rescued by co-transfection with constitutively-active CaMKI. We excluded the possibility that AKT was involved with UII-dependent activation of ANF, SkA and MEF2 as we clearly demonstrated that inhibition of AKT had no effect on UII stimulation of these hypertrophy indicators. These data clearly demonstrate that CaMKI is the major downstream effector of UII-stimulated CaMKK activity. Experiments in the future should employ the use of dominant-negative mutants of CaMKI to further demonstrate its role in UII stimulated hypertrophy. To date, there are currently no transgenic models of CaMKI over-expression or knockout. It would be of interest to determine the *in vivo* effects of CaMKI. Generation of CaMKI transgenic knockout models could be used to assess its role in pressure overload hypertrophy.

The MAP kinases p38 and ERK1/2 have been implicated in hypertrophic signaling from G_q-coupled receptors. UII stimulation of cardiomyocytes resulted in the PKC-independent activation of both p38 and ERK1/2. The data presented here show that treatment of cardiomyocytes with UII result in the activation of the MAP kinases p38 and ERK1/2 under our cell culture and experimental conditions; however, we go on to

conclude that UII-dependent activation of p38 and ERK1/2 requires CaMKK. Our conclusions were further supported by data showing that both UII stimulation and constitutively-active CaMKI stimulation of ANF, SkA and MEF2 were abolished by pharmacological inhibition of either p38 or ERK1/2. In addition, dominant-negative p38 completely inhibited the activation of MEF2 by UII and CaMKI. CaMKI is a known activator of MAP kinases in cell types other than cardiomyocytes. These results clearly demonstrate that at some point within their respective pathways, CaMKI interacts with and leads to the activation of p38 and ERK1/2 in cardiomyocytes. Since the MAP kinases require dual phosphorylation at threonine and tyrosine residues from upstream kinases, it is unlikely that CaMKI is able to directly phosphorylate and activate either p38 or ERK1/2. Therefore, work needs to be done in order to determine the point at which CaMKI interacts with the upstream components of the p38 and ERK1/2 MAP kinase pathways.

It must also be noted that the ERK1/2 inhibitor, U0126, also inhibits the activation of ERK5/Big MAP kinase. Interestingly, ERK5 is also activated by G_q-coupled receptors[163]. Furthermore, ERK5 activation by G_q-coupled receptors involves a pathway separate from those regulated by Ras and Rho GTPases. ERK5 has also been shown to bind to and enhance the activation of MEF2[157]. Therefore, conclusions drawn from the present study cannot be used to rule out the possibility of the involvement of ERK5 in UII stimulation of hypertrophy. For these reasons, it is important that future work addresses the contribution of ERK5 to UII stimulated cardiac hypertrophy through MEF2. This should be accomplished when a specific MEK5/ERK5 inhibitor becomes

available. In the mean time, work should be done to produce dominant-negative mutants of ERK5 or transgenic knockout models whereby the effects of UII stimulation of hypertrophy could be studied in the absence of ERK5 activity.

MEF2 is a major transcription factor in cardiomyocytes that leads to the expression of hypertrophy-sensitive genes. In order for MEF2 to achieve full activity, two events must occur: i) phosphorylation of partnered class II HDACs (relief of repression) and ii) phosphorylation of MEF2. As mentioned above, the former is accomplished as a result of CaMKI signaling as well as 14-3-3-dependent nucleocytoplasmic shuttling and the latter by p38. Results in this dissertation clearly demonstrate that UII stimulates the CaMKK-dependent association of HDAC5 with 14-3-3β. When combined with the fact that dominant-negative p38 completely inhibited UII and CaMKI-induced MEF2 activation, these results allow us to conclude that a major mechanism for UII-induced hypertrophy is through MEF2. Since CaMKI is a cytoplasmic kinase and HDAC5 is normally partnered with MEF2 in the nucleus, we can reasonably conclude that there is some intervening kinase activity bridging CaMKI to HDAC5. It has been demonstrated that CaMKI and PKD are members of parallel pathways that both lead to HDAC phosphorylation[127]. PKD is a cytoplasmic kinase with access to the nucleus. To date, no data has been presented demonstrating a kinase downstream of CaMKI with nuclear access. Through the use of western blots, we conclude that UII stimulation of cardiomyocytes leads to the activation of PKD. Furthermore, we found that the UII-dependent activation of PKD was also dependent on CaMKK. These are the first data to suggest that the CaMK cascade, presumably through

CaMKI, activates PKD. In order to confirm this novel cross-talk mechanism, further work needs to be done in order to i) confirm protein-protein interaction, ii) confirm that PKD is a direct substrate for CaMKI both *in vitro* and *in vivo*, and iii) determine whether CaMKI is also active under other conditions where PKD is active.

In conclusion, data described in this dissertation clearly demonstrate an important role for UII in the regulation of genes involved with the hypertrophic phenotype in cardiomyocytes. This was demonstrated using promoter-reporter assays and RT-PCR. In addition we conclude that the CaM kinase cascade is required for hypertrophic gene expression by UII. This work is the first to demonstrate crosstalk between the CaM kinase cascade and the MAP kinases in cardiomyocytes—a mechanism that may shed light on other G_q-coupled signaling pathways that lead to hypertrophy. We were able to conclude that UII stimulation of cardiomyocytes results in the CaMKK-dependent association of HDAC5 with 14-3-3β, leading to the activation of MEF2. Moreover, we conclude that rather than being parallel pathways leading to 14-3-3-mediated HDAC5 nuclear shuttling, PKD may in fact be downstream of CaMKI. Taken together, these data contribute to our understanding of the regulation and signaling mechanism whereby UII stimulates cardiac hypertrophy.

APPENDIX

LIST OF ABBREVIATIONS USED

$[Ca2+]_i$	Intracellular calcium concentration
AKTi	1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate
αMCH	alpha Myosin heavy chain
ANF	Atrial natriuretic factor
AngII	Angiotensin II
AngIIR	Angiotensin II receptor
AraC	Cytosine _-D-arabinofuranoside
ATF-1	Activating transcription factor-1
βMHC	beta Myosin heavy chain
BNP	Brain natriuretic factor
CaM	Calmodulin
CaMK	Calcium/calmodulin-dependent kinase
CaMKI	Calcium/calmodulin-dependent protein kinase I
CaMKIV	Calcium/calmodulin-dependent protein kinase IV
CaMKK	Calcium/calmodulin-dependent protein kinase kinase
CREB	Calcium response element binding protein
DMSO	Dimethylsulfoxide
Erk	Extracellular signal related kinase
ET-1	Endothelin-1
ET-1R	Endothelin-1 receptor
GEO	Gene expression omnibus
GPCR	G protein coupled receptor
HAT	Histone acetyltransferase
HBSS	Hanks buffered salt solution
HDAC	Histone deacetylase
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor 2
MEK	MAP/ERK kinases
NCBI	National Center for Biotechnology Information
PAMH	Pyrimidine activator of myocyte hypertrophy
PD98059	2'-amino-3'-methoxyflavone
PE	Phenylephrine

PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
RTK	Receptor tyrosine kinase
SB203580	4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1H-imidazole
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SkA	alpha skeletal actin
Ser	Serine
SR	Sarcoplasmic reticulum
STO-609	7H-Benz[de]benzimidazo[2,1-a]isoquinoline-7-one-3-carboxylic Acid
TBS	Tris-buffered saline
TBST	Tris -buffered saline 0.1% Tween-20
Thr	Threonine
Tyr	Tyrosine
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
UBF	Upstream binding factor
UII	Urotensin II
UIIR	Urotensin II receptor

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