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THE CELLULAR AND MOLECULAR MECHANISMS OF OXIDANT-INDUCED CARDIOMYOCYTE HYPERTROPHY

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by CHUNYI TU

entitled THE MOLECULAR AND CELLULAR MECHANISMS OF OXIDANT-INDUCED CARDIOMYOCYTE HYPERTROPHY

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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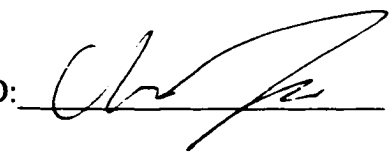
SIGNED: 

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ABBREVIATIONS

ANF: atrial natriuretic factor
AngII: angiotensin II
BNP: B-type natriuretic prptide
DAG: 1,2-diacylglycerol
DOX: doxorubicin
ERK: extracellular signal-regulated kinase
ET-1: endothelin-1
FBS: fetal bovine serum
IP3: inositol 1,4,5-trisphosphate
JNK: c-Jun N-terminal kinase
L-NAME: N-nitro-L-arginine methyl ester
MAPK: mitogen activated protein kinase
MHC: myosin heavy chain
MLC-2v: ventricular myosin light chain-2
PE: phenylephrine
PI3K: phosphoinositide 3-kinase or phosphatidylinositol 3-kinase
PKB: Akt kinase or protein kinase B
PKC: protein kinase C
PLC: phospholipase C
PtdIns: phosphatidylinositol
ROS: reactive oxygen species
RT-PCR: reverse transcription-polymerase chain reaction
S6K: S6 kinase
SERCA2: sarco(endo)plamic reticulum Ca^{2+}-ATPase 2α
SkA: skeletal α-actin
SR: sarcoplasmic reticulum
TEF-1: transcription enhancer factor-1

ABSTRACT

Cardiac hypertrophy is a common consequence of many cardiovascular diseases. It is often a transition to heart failure. Although oxidants have been indicated to mediate heart failure, whether they induce hypertrophy and how they are able to induce hypertrophy are still unknown. The objective of this dissertation is to investigate the underlying molecular and cellular mechanisms of oxidant-induced heart hypertrophy. My hypothesis is that H_2O_2 induces cardiomyocyte hypertrophy through activating specific signaling pathways.

First, signaling pathways that contribute to H_2O_2-induced cell size enlargement were investigated. *In vitro* kinase assay and Western Blot analysis were conducted to examine activation of PI3K and p70S6K1. We found that H_2O_2 is able to activate PI3K and p70S6K1 in a time- and dose- dependent manner. Inhibitor studies indicate that the activation of PI3K is upstream of p70S6K1. Both PI3K and p70S6K1 inhibitors are able to abolish H_2O_2 induced cell size enlargement and protein content increase, suggesting that this pathway is necessary for H_2O_2 induced hypertrophy. We also tested the activation of three branches of MAPKs: ERK, p38 and JNK. Experiments examining the possible relationship between MAPKs and p70S6K1 revealed that p38 MAPK is able to regulate p70S6K1 in part.

Second, signaling pathways related to gene expression alteration associated with hypertrophy induced by H_2O_2 were investigated. This study focused on two important hypertrophy transcription factors, AP-1 and NFAT3. Using both gel shift assay and promoter reporter activation analysis, we found that H_2O_2 is a potent inducer of AP-1. This activation is downstream of ERK. NFAT3 was recently implicated to play a role in cardiac hypertrophy. Whether and how it is activated by H_2O_2 is unknown. When cardiomyocytes transfected with pNFAT-luc, a NFAT luciferase plasmid with a luciferase gene under control of repetitive NFAT/AP-1 composite sites derived from the IL-2 promoter, were treated by H_2O_2, luciferase activity increased in a time and dose dependent manner. Experiments using pharmacological inhibitors demonstrated that this NFAT3 activation detected by luciferase assay is ERK and AP-1 dependent but calcineurin independent. This discovery reveals a novel mechanism of NFAT3 regulation in cardiomyocytes that can play an important role in hypertrophic gene regulation.

Chapter 1 INTRODUCTION

The focus of this dissertation is to study the underlying molecular and cellular mechanism of oxidant-induced cardiomyocyte hypertrophy. This chapter introduces the basic terminologies, methodologies and previous research findings that are important for my dissertation work. It is organized as following: First, we define cardiovascular disease in general and heart failure in particular. We also introduce cardiac hypertrophy, its relationship with heart failure, and the significance of cardiac hypertrophy. We then point to commonly used *in vitro* systems and pathophysiological inducers of hypertrophy. The literature on hypertrophy-related signal transduction pathways is reviewed, followed by an introduction to the relationship between oxidative stress and heart hypertrophy. We conclude this chapter by presenting the research proposal for this dissertation.

Cardiovascular Disease, Heart Failure, and Cardiac Hypertrophy

As a vital organ in the human body, the heart pumps blood to flow through all human tissues, supplying these tissues with oxygen and nutrients. Since cardiovascular diseases greatly interfere with human blood supply, they are often severe, if not lethal. The statistics recently published by the American Heart Association (AHA) shows that at least one out of every 2.5 deaths in United States is from cardiovascular disease, making the cardiovascular disease the No. 1 killer in the United States. Cardiovascular disease

alone causes more deaths than the next 7 causes of death combined. It is not an overstatement that cardiovascular disease is currently the greatest threat to human lives among all diseases in the developed countries.

The leading forms of cardiovascular disease include ischemia, hypertension, coronary heart disease and heart failure. Among them, ischemia and heart failure are major causes of disability and mortality. Heart failure occurs when the heart is unable to pump efficiently to meet the workload demand of the body. It is the end point of many forms of cardiac disease. Each year, about 300,000 Americans die from acute heart failure, and about 600,000 Americans develop and suffer from chronic heart failure. The estimated annual cost for the care of heart failure patients is more than 17.5 billion dollars. So far, the effective clinical treatment of heart failure has not been achieved. Therefore, defining the triggers and biomedical mechanisms of heart failure is important for developing preventive and therapeutic strategies for this deadly disease.

Important features of heart failure are loss of cardiomyocytes and development of hypertrophy of the surviving cardiomyocytes (Colucci and Braunwald 1997). Cardiomyocyte hypertrophy, observed histologically as the enlargement of cardiacmyocytes, initially takes place to compensate for the functional loss of the heart that occurs in cardiac pathological conditions, such as hypertension, atherosclerosis, myocardial infarction and valvular disease. However, sustained hypertrophy is decompensatory and often transforms into heart failure How hypertrophy develops in

response to cardiovascular disease and when the transition to heart failure occurs are believed to be the key issues for understanding of the heart failure process. In this dissertation, the focus is on the mechanism of oxidant-induced cardiac hypertrophy. We believe that this research will lay a foundation for understanding how oxidants might contribute to heart failure at the molecular level.

Physiological hypertrophy vs. pathological hypertrophy

Cardiac hypertrophy is defined as a phenomenon where the heart increases its volume and/or the thickness of ventricular wall. This often occurs as a compensatory reponse to an increased demand of workload under certain physiological or pathological conditions. These conditions divide hypertrophy into physiological versus pathological hypertrophy.

With physiological hypertrophy, enlargement of cardiomyocytes is the major mechanism of the growth of the postnatal heart. Since cardiomyocytes stop proliferating soon after birth in most mammal species, the growth of the heart is achieved through the increase of cardiomyocyte size. Therefore, all human beings experience physiological cardiac hypertrophy throughout their juvenile ages. Physiological cardiac hypertrophy can also occur in adults under the circumstance of prolonged physiological training. Nevertheless, physiological hypertrophy is characterized by coordinated increases in the

muscle thickness versus chamber size of the heart and proportional increases in the length versus diameter of cardiomyocytes. These coordinated changes result in an increase in the volume of hearts. When stimuli are withdrawn, hearts with physiologic hypertrophy are able to return to its normal status (Hunter and Chien 1999).

Cardiac hypertrophy can also develop with certain cardiovascular diseases. This type of disease-associated cardiac hypertrophy is referred to as pathologic hypertrophy. For example, hypertrophy is observed following myocardial infarction, where the surviving cardiomyocytes have to work harder to compensate for the loss of cardiomyocytes due to infarction. Pathologic hypertrophy can also result from increased demand of workload placed on the heart. For example, in patients with systemic hypertension, pathologic hypertrophy is an adaptive response of the heart to pressure overload. Although pathologic hypertrophy can be initially beneficial, prolonged hypertrophy that occurs at disease states lacks an increased cardiac output and is viewed by clinicians as a transition to heart failure. Severe hypertrophy is often observed in a failing heart (Colucci and Braunwald 1997).

Unlike physiologic hypertrophy, cardiac hypertrophy as a result of diseases is usually asymmetric or uncoordinated. Left ventricular hypertrophy is a prominent form found in the clinic. Uncoordinated hypertrophy can be either concentrical or eccentrical. Concentrical hypertrophy represents an overall increase in heart mass in the absence of increased heart volume. In another word, the overall diameter of the heart remains the

same, but the thickness of the heart walls increases, which is often caused by mutations of contractile proteins and familiar hypertrophic cardiomyopathy. Hypertrophy observed with dilated cardiomyopathy shows an increased heart volume, however, a coordinated increase in muscle mass is not observed and the chamber walls are actually thinner than normal. This form of hypertrophy is referred to as eccentrical hypertrophy.

Molecular characteristics of cardiomyocyte hypertrophy

At the cellular and molecular level, cardiomyocyte hypertrophy has three major features: enlarged cell size, increased protein content per cell and expression of hypertrophy marker genes. Increased protein content per cell and its corresponding cell size enlargement have been linked to changes in signaling transduction pathways. In addition to changes in cell size and protein content per cell, hypertrophic cardiomyocytes also alter their gene expression profile. A group of constitutively expressed contractile genes, including ventricular myosin light chain-2 (MLC-2v) and troponin C, increase their expression levels. In addition, reactivation of the fetal gene program often leads to an increased expression of atrial natriuretic factor (ANF), β myosin heavy chain (βMHC) and skeletal α-actin (SkA) (Zak 1995; Copper 1997; Zhu 1997). In contrast, α myosin heavy chain (αMHC) and the Ca^{2+} pump proteins of sarcoplasmic reticulum are often downregulated in cardiac hypertrophy (Schaub et al. 1997).

In Vitro Models for Studying Cardiac Hypertrophy

Because changes at the cellular level are the main contributor to cardiac hypertrophy, *in vitro* models have been developed to study these changes. Although there are always concerns about the relevance of hypertrophic growth of cardiomyocyte *in vitro* to adult cardiac hypertrophy *in vivo*, *in vitro* systems provide the best available systems for examining individual stimuli and cellular signaling pathways. In this section, we briefly introduce two *in vitro* cell culture systems used in cardiac hypertrophy studies and describe their pros and cons. We also introduce several cardiac hypertrophy inducers commonly used in these systems.

Rat cardiomyocytes in culture: neonatal versus adult

Since heart failure and cardiac hypertrophy often happen to adults, it would be natural to use adult rat cardiomyocytes to study the mechanism of cardiac hypertrophy. The isolation method for adult rat cardiomyocytes has been established. Briefly, isolated adult rat hearts are perfused by collegenase solution until heart tissues become soft. Then, the hearts are dissected to small pieces and subjected to further digestion by collegenase. Individual cardiomyocytes are collected from the supernatant of digested heart tissues (Welder et al. 1991; Mitcheson et al. 1998). Despite the relevance of this system to study changes occurred during heart failure, some obvious problems prevent it from being widely used in research. First, cardiomyocytes in adult hearts are fully differentiated and they are tightly connected, which make it extremely difficult to separate cardiomyocytes from each other. As a result, the yield of individual adult

cardiomyocytes is usually low. Although this yield is acceptable for electrophysiologic studies, it hampers signaling and molecular studies in which more cells are needed. Secondly, the viability of adult cardiomyocytes in culture is low. In serum-free culture, which is needed for hypertrophy studies, the viability of adult cardiomocytes is generally less than 1 week) (Liu et al. 1998). With these considerations, although the adult cardiomyocytes have been used in cardiac researches including hypertrophy, an alternative *in vitro* system was developed to better serve the requirements of investigating the molecular mechanisms of cardiac hypertrophy.

Since Simpson *et al.* (Simpson et al. 1982; Simpson 1983) introduced neonatal rat cardiomyocyte cultures as an *in vitro* model of cardiac hypertrophy, this system has become widely used for investigating the cellular and molecular mechanisms of cardiac hypertrophy. The majority of the important findings about signaling pathways and gene regulation relating to cardiac hypertrophy were generated using this system. The specific neonatal rat cardiomyocyte isolation protocol may vary slightly from one laboratory to another. In this research, neonatal rat cardiomyocytes are usually isolated from 1-2 days old rats. Individual cardiomyocytes are released from heart tissues by repetitive digestion in a solution containing collegenase or trypsin. Cardiomyocytes are then separated from non-muscle cells (mainly fibroblasts) by differential plating. This process normally generates 90-95% cardiomyocytes in culture. Contracting cardiomyocytes are usually observed at 24-48 hours after plating. Since serum encourages the growth of

fibroblasts, after being kept in serum-containing medium for 1 or 2 days, cardiomyoctes are normally placed in serum-free medium during endocrine factor treatment.

Neonatal rat cardiomyocyte culture is a popular system in the field of molecular cardiology, because neonatal cardiomyocytes are relatively easy to isolate, to grow in culture, and to manipulate. However, a possible disadvantage of this system is that the characteristics of neonatal cardiomyocytes, compared with adult cardiomyoctes, are less close to *in vivo* situations in terms of cellular responses to various treatments. These differences may warn cautions on extrapolating data obtained from neonatal culture to animal studies.

Inducers of cardiomyocyte hypertrophy

Angiotensin II (AngII), endotheiin 1 (ET-1) and catecholamines, have been shown to induce cardiac hypertrophy. An elevated blood level of these factors has been observed in association with a number of disease conditions. AngII is an 8-amino-acid peptide derived from angiotensin I by cleavage with angiotensin converting enzyme. Angiotensin I is produced by the renin-angiotensin system in the kidney. Activation of renin-angiotensin system occurs when the kidney responds to the demand of increasing blood pressure or retaining salt and water in the body. Hypertension can cause the renin-angiotensin system to become overactive. An increased amount of AngII is therefore produced, inducing cardiac hypertrophy (Varagic et al. 2001). ET-1 is another

hypertrophy inducer. ET-1 is a 21-amino-acid peptide mainly produced by endothelial cells. Pressure overload and ischemia stimulate endothelial cells and cardiomyocytes to secrete ET-1 (Tonnessen et al. 1995; Battistini and Kingma 2000; Shichiri et al. 1990). The third type of hypertrophy inducers, catecholamines, is a group of tyrosine-derived neurotransmitters or neuroendocrine factors such as epinephrine and norepinephrine. They are synthesized in the sympathetic adrenal glands and nerve terminals. Stress and exercise enhance catecholamine synthesis, which is thought to contribute to exercise-induced hypertrophy (Scheuer 1999). An increased level of catecholamines is also observed with pressure overload, ischemia, ischemia-reperfusion and heart failure (Schomig 1990; Rapacciuolo et al. 2001).

In addition to AngII, ET-1 and chatecholamines, physical stress, such as mechanical stretching in the case of hypertension, is also able to induce cardiac hypertrophy. Hypertension-induced cardiac hypertrophy can be studied in animal models with aortic banding. Both endocrine factors and mechanical stretching have been shown to induce cardiomyocyte hypertrophy in neonatal cardiomyocytes in culture. In recent years, more hypertrophy inducers have been identified, for example cardiotropin-1, prostaglandin F2α and inhibitors of fatty acid oxidation (Higgins et al. 1985; Pennica et al. 1995; Lai et al. 1996). Reactive oxygen species (ROS) have also been implicated in inducing cardiomyocyte hypertrophy. One mechanism of ROS inducing hypertrophy involves activation of the apoptosis signal-regulating kinase (ASK) and the nuclear factor-κB (NF-κB) signaling pathway (Force et al. 2002).

Signaling Pathways of Cardiac Hypertrophy

Because of increased interest in the pharmaceutical industry and the emerging possibility of drug design, a huge effort is underway to characterize the molecular mechanisms, especially signaling cascades, of heart failure and hypertrophy. In this section, the major signaling pathways contributing to cardiac hypertrophy are summarized.

Unlike cancer cells and many types of immune cells, cardiomyocytes in adult hearts lack the ability to replicate. However, the signaling pathways contributing to cardiomyocyte hypertrophy share much of the similarity with cancer cell proliferation. Hypertrophy-inducing endocrine factors bind to their specific receptors on the plasma membrane of cardiomyocytes. Multiple signaling pathways are turned on and eventually affect the gene expression profile of cardiomyocytes. The orchestrated and balanced performance of this signaling network appears important for the development of hypertrophy. Experimental data have suggested that in some cases, blocking one particular signaling pathway is able to attenuate the whole hypertrophic response induced by endocrine factors (Molkentin et al. 1998).

In general, the signaling transduction pathways of hypertrophy in cardiomyocytes are initiated by binding of endocrine factors to their receptors on the cell membrane. This binding activates G proteins that are associated with the receptors. Several distinct but

related signaling pathways are therefore activated. First, phospholipase C (PLC) is activated. Protein kinase C and Ca^{2+} dependent calcineurin pathway are activated downstream of PLC. Secondly, through small GTP binding proteins, the ligand binding also induces the activation of phosphotidylinositide 3-kinase (PI3K) and its downstream kinase, p70 S6 kinase. Thirdly, in parallel with the PI3K and p70 S6 kinase pathway, all the three members of mitogen activated protein kinase (MAPK) are activated through small GTP binding proteins. These signaling cascades from receptors to gene expression in the nucleus will now be discussed in detail.

G proteins

GTP binding proteins (G proteins) transduce either stimulatory or inhibitory signals from agonist-occupied receptors to intracellular molecular events. G proteins are composed of three subunits, Gα and Gβγ. Upon binding to GTP, the α subunit dissociates from the βγ subunits. Gβγ activates signaling pathways in the cell. In the heart, it is known that free Gβγ subunits regulate the activity of Ras protein, MAPKs and PI3K (Simmon et al. 1991; Crespo et al. 1994; Pumiglia et al. 1995). Based on different signaling pathways downstream, three major subtypes of G protein, Gαs, Gαi, and Gαq, are defined. Cardiomyocytes have receptors on the plasma membrane associated with all three subtypes of G proteins. β-adrenergic receptors (βAR), which couple primarily with Gαs, mediate acute enhancement of heart contraction rate in response to epinephrine or norepinephrine stimulation. Gαi mainly associates with cholinergic receptors. Receptors

coupled primarily to Gαq are a group of receptors important for hypertrophic response, including AngII, ET-1, and α-adenergic receptors (αAR).

Receptor mediated activation of the Gαi subunit results in the attenuation of adenylyl cyclase (AC) in the heart. Inhibited AC activity leads to suppression of the protein kinase A signaling pathway. Increased Gαi protein levels and depressed basal AC activity were observed in heart failure patients and animal models (Neumann et al. 1988; Kawamoto et al. 1994). These changes were also found in experimental and genetic cardiac hypertrophy associated with hypertension (Bohm et al. 1992b; Bohm et al. 1992a). Taken together, enhanced Gαi activity may present a signaling transduction pathway that contributes significantly to cardiac hypertrophy. However, the function of this pathway in cardiac hypertrophy is not clear.

The role of Gαs in cardiac hypertrophy has not been well studied. Gαs activation by agonist binding augments the activity of AC and induces the protein kinase A signaling pathway. Interestingly, Gaudin *et al.* reported that transgenic mice overexpressing Gαs did not show increased AC activity but had an enhanced cardiac contractility when stimulated by isoproterenol, a βAR agonist (Gaudin et al. 1995). Overall, the phenotype of transgenic mice Gαs overexpressing resembles the alterations

in cardiac function and pathology associated with exogenously administrated catecholamines (Imperato-McGinley et al. 1987).

Unlike Gαs or Gαi, Gαq subunit participates in the regulation of the phospholipase C (PLC) signaling pathway. The critical role of the Gαq subunit in cardiac hypertrophy has been supported by a large amount of evidence from *in vitro* and *in vivo* experimental models and from clinical studies. In the early 1980's, Simpson and colleagues first showed that the αAR agonist PE, but not the βAR agonist isoproterenol, increased the cell size of cultured neonatal rat cardiomyocytes (Simpson et al. 1982). Later on, hypertrophic effects of additional αAR agonists, phenylephrine (PE), AngII, ET-1 and prostaglandin F2α, have also been established (Adams et al. 1996; Knowlton et al. 1993; Sadoshima et al. 1993; Shubeita et al. 1990). Without exception, all of these αAR agonists activate PLC via Gαq, which implies that Gαq and PLC could be important mediators of hypertrophy. The significant role of Gαq was further demonstrated from experiments in which Gαq was constitutively expressed in neonatal cardiomyocytes. These transfected cardiomyocytes developed the phenotype of hypertrophy (LaMorte et al. 1994).

Further evidence for the role of Gαq in hypertrophy includes transgenic mouse studies. Gαq -overexpressing transgenic mice exhibited a hypertrophic phenotype

similar to pressure overload hypertrophy with regard to the extent of cell size enlargement and the pattern of fetal gene expression (D'Angelo et al. 1997; Sakata et al. 1998). However, Gαq over-expressing animals showed additional features different from compensatory pressure overload hypertrophy, such as eccentric ventricular remodeling, resting sinus bradycardia, and left ventricular contractile depression (Sakata et al. 1998). When Gαq-overexpressing mice were subjected to pressure overload induced by aortic banding, their hearts developed eccentric hypertrophy with progressively declining in ventricular function, instead of centric and compensatory hypertrophy observed in their littermates with normal genetic background in response to the same challenge (Sakata et al. 1998). The Gαq signaling in pathologic forms of hypertrophy was also demonstrated recently. Akhter *et al.* reported that transgenic mice overexpressing a dominant-negative Gαq gene in the heart are resistant to pressure overload induced hypertrophy (Akhter et al. 1998). These data argue that the signaling pathways activated by Gαq are sufficient for maladaptive cardiac hypertrophy. Along with these findings, the activity of Gαq and PLC have been shown to be up-regulated in the myocardium of experimentally infarcted rats (Ju et al. 1998). Figure 1.1 summerizes signaling pathways associated with different G proteins.

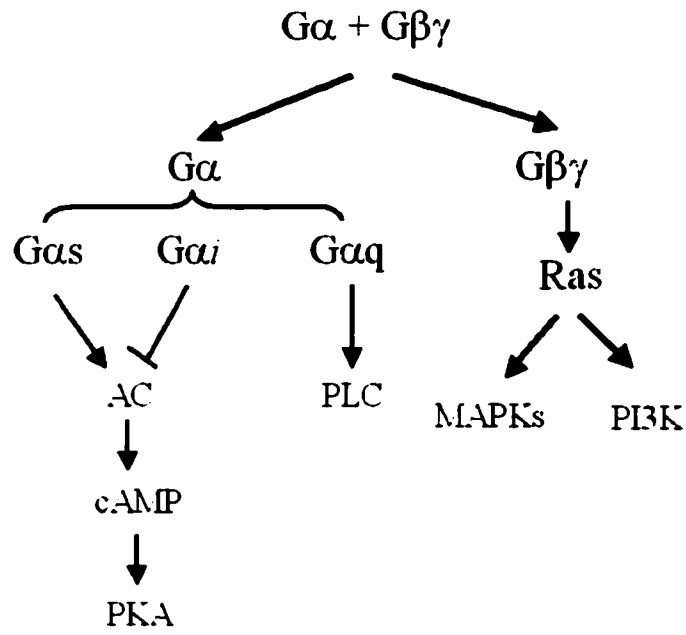


Figure 1.1 Signaling Pathways Associated with Different G Proteins

PI3K and p70 S6 kinase

An important event downstream of G-proteins is the activation of PI3K. As a lipid kinase, PI3K adds a phosphate group to the 3-position of the inositol ring of phosphatidylinositol (PtdIns) and phosphoinositides. Its substrates include PtIns-4-P, PtdIns-5-P, PtIns-4, 5-P_2. The resulting products participate in multiple signaling events (Rameh and Cantley 1999). For example, PtIns-3, 4-P_2 regulates the activity of Akt kinase (also called protein kinase B or PKB) and PKC. Akt can activate p70 S6 kinase, which regulates protein translation and contributes to an increase of the protein content in hypertrophic cells (Pullen and Thomas 1997; Dufner and Thomas 1999). Another PI3K

product, PtIns-3, 4, 5-P_3, activates PLCγ, which releases IP_3 from the lipids and triggers an increase in cytosolic calcium. Normally, lipids that contain inositol phosphorylated at the 3-position are low in abundance. Less than 0.25% of the inositol-containing lipids or 0.025% of total lipids in a cell are phosphorylated at the 3-position (Rameh and Cantley 1999). Therefore, activation of PI3K is a rate-limiting step for the signaling events involving 3-position phosphorylated inositol or inositides. Nevertheless, Shioi *et al.* (Shioi et al. 2000) used transgenic approaches to demonstrate that cardiac-specific expression of constitutively active PI3K resulted in mice with larger hearts. In contrast, expression of dominant-negative PI3K resulted in mice with smaller hearts (Rameh and Cantley 1999). Heart enlargement correlates with increases in the size of cardiomyocytes (Rameh and Cantley 1999). In parallel with these transgenic studies, activation of PI3K has been reported with AngII, ET-1 and αAR agonists in various cell types including cardiomyocytes (Saad et al. 1995; Hu et al. 1996; Foschi et al. 1997; Rabkin et al. 1997; Schluter et al. 1998; Su et al. 1999). These data support the hypothesis that PI3K plays an essential role in determining the size of cardiomyocytes.

An important downstream event of PI3K and PKC is the activation of S6 kinases. The S6 kinase family contains 2 genes: S6 kinase 1 and 2 (Dufner and Thomas 1999). S6 kinase 1 gene splices into two variants and encodes for a p70 (cytosolic, p70S6K1) and a p85 (nuclear, p85S6K1) protein. AngII, ET-1 and αAR agonists can activate both p70S6K1 and p85S6K1 (Sadoshima and Izumo 1995; Boluyt et al. 1997; Laser et al. 1998; Simm et al. 1998). p70S6K1 regulates the phosphorylation of the 40S ribosomal

S6 protein. Although the function of p85S6K1 is not fully understood, it is expected to phosphorylate nuclear ribosomes (Pullen and Thomas 1997; Dufner and Thomas 1999). As a result of p70S6K1 activation, the translation machinery selectively translates a family of mRNAs with an oligopyrimidine tract at the 5'-transcriptional start site (Dufner and Thomas 1999). About 20-30% of total translated mRNA species contain such a tract. These mRNA species encode components of the translational apparatus (Dufner and Thomas 1999). Due to an ultimate increase in translational activity, activated S6 kinases contribute to increased protein content in hypertrophic cells.

MAPKs

Endocrine hypertrophy inducers activate three subtypes of MAPKs: extracellular signal-regulated kinases (ERKs), p38 and c-Jun N-terminal kinases (JNKs, also called stress activated protein kinases or SAPKs) as a result of G protein activation (Hunter and Chien 1999).

ERK has two major isoforms, ERK1 and ERK2, which are encoded by two genes and have similar functions but distinct molecular weights (Boulton et al. 1991). Both of them are coordinately phosphorylated and activated by a wide array of stimuli. Its major upstream regulator, MAPK kinase 1 and 2 (MEK1 and MEK2), phosphorylate ERKs at the site of Thr-Glu-Tyr. Activation of ERK1/2 by artificially expressing a constitutively active form of the upstream regulator MEK1 is sufficient to induce enlargement of cell size and expression of hypertrophy marker genes *in vitro* (Gillespie-Brown et al. 1995).

Clerk *et al.* defined a role for ERK1/2 in ET-1 and PE-induced increases in the sarcomeric content in cultured cardiomyocytes (Clerk et al. 1998). However, whether ERK activation is necessary for the development of hypertrophy is questioned by several recent studies. Thorburn *et al.* reported that although ERK activation results in enhanced c-fos and ANF promoter activity in cardiomyocytes, inhibition of ERK activity does not attenuate hypertrophic morphology or cytoskeletal organization in response to PE (Thorburn et al. 1995). Furthermore, dominant-negative ERK1/2 and a MEK1 inhibitor PD98059 failed to inhibit ANF expression induced by phenylephrine (PE). Overexpressing a constitutively activated form of ERK could induce c-Fos, but had no effect on ANF or myosin light-chain-2V promoter activity (Thorburn et al. 1995). There is also a lack of *in vivo* data supporting the functional significance of ERK activation in cardiac hypertrophy. Collectively, although ERK1/2 activation is a downstream event of receptor activation by hypertrophy inducers, the role of ERK1/2 in the development of hypertrophy remains unclear.

A second subfamily of MAPKs, p38 proteins, has four isoforms, α, β, γ and δ, present in various mammalian cell types (Widmann et al. 1999). The heart mainly expresses the α- and β- isoforms of p38 (Sugden and Clerk 1998). p38 can phosphorylate the transcription factor ATF-2 and activate the AP-1 transcription factor containing ATF-2. Hypertrophy inducers and a variety of physical or chemical stresses activate p38 MAPK *in vitro* and *in vivo*. For example, PE and ET-1 can strongly activate p38 (Clerk and Sugden 1998). An increased p38 activity was also observed with pressure

overload induced hypertrophy (Wang et al. 1998a). Overexpressing the p38β transgene or a constitutively active upstream kinase of p38 can produce hypertrophic responses *in vitro* (Wang et al. 1998a). Using pharmacological inhibitors for p38 or dominant negative forms of p38 gene, Zechner *et al.* was able to abolish PE induced hypertrophy *in vitro* (Zechner et al. 1997). However, further experiments are in need to test if p38 is sufficient for inducing hypertrophy *in vivo.*

Like p38, JNKs are activated by various hypertrophy inducers and stress conditions. JNKs are phosphorylated and activated by upstream kinases MKK4 and MKK7 at the site, Thr-Pro-Thr. The JNK family is encoded by three distinct genes: JNK1, JNK2 and JNK3. JNK1 and JNK2 are the two major isoforms expressed in the heart. JNK activation has been associated with load-induced cardiac hypertrophy in both rat and human heart failure (Choukroun et al. 1999; Cook et al. 1999). JNKs can activate the AP-1 transcription factor by phosphorylating ATF-2 and c-Jun. Overexpressing dominant negative forms of JNK or SEK-1, an upstream kinase of JNK, is able to abolish ET-1 induced cardiomyocyte hypertrophy *in vitro* (Wang et al. 1998b; Choukroun et al. 1998). Futhermore, in an *in vivo* study, dominant negative forms of SEK-1 are able to prevent pressure overload from inducing hypertrophy(Choukroun et al. 1999). These data strongly support the important role of JNKs in inducing cardiomyocyte hypertrophy.

From the above discussion, it is clear that activation of G-proteins, calcium signaling, certain isoforms of PKC, JNKs or PI3K alone is sufficient to induce

hypertrophic response *in vitro* and *in vivo*. *In vitro* data support the importance of ERKs or p38 in hypertrophic responses. Regardless, studies on the relationships between these signaling molecules suggest the existence of crosstalk and overlap between signaling pathways. For instance, the nature of PI3K and its products suggest that PI3K activation can switch on PKC, PLC and calcium signaling pathways. A recent publication indicated that PI3K also regulates Calcineurin-Nuclear Factor of Activated T cell (NFAT) activity through glycogen synthase kinase 3 (GSK3) in hypertrophy (Rommel et al. 2001). PKCs are also kinases with multiple functions. They have been shown as upstream regulators for MAPKs in hypertrophic responses (Yamazaki et al. 1995; Zou et al. 1996). PKCs have also been shown to increase cytosolic calcium concentration despite the fact that cytosolic calcium normally increases in response to IP_3 elevation (Huang et al. 2001). In addition, the groups of Molkentin and Force recently found that calcium-dependent activation of calcineurin regulates JNK activation (De Windt et al. 2000). Murat *et al.* reported that inhibiting calcineurin blocks activation of p38 and JNKs by pressure overload (Murat et al. 2000). The existence of crosstalk and overlapping mechanisms suggest that activation of one or a few key signaling molecules by any stimulus can be sufficient for inducing hypertrophy.

Transcription Factors in Cardiac Hypertrophy

Transcription factors are proteins that are downstream of signaling pathways and regulated by these pathways through mainly phosphorylation or dephosphorylation. Upon activation, transcription factors are able to bind to specific regions in the promoters

of genes to regulate these genes' transcription. As we discussed above, altered gene expression profile is a key feature of cardiac hypertrophy. Several transcription factors have been found involving the gene expression regulation in hypertrophy (Molkentin and Dorn, 2001). This dissertation focuses on two important transcription factors in hypertrophy, NFAT3 and AP-1.

Calcineurin-NFAT Transcription Factor

Calcineurin is a serine-threonine phosphatase that is activated by calcium-calmodulin. It has been known for a long time that in immune cells calcineurin dephosphorylates the NFAT transcription factor, causing its translocation to the nucleus. A number of immune response genes are under the control of NFATs (Masuda et al. 1998). The immunosuppressive drugs cyclosporine A and FK506 are inhibitors of calcineurin and preventing NFAT nuclear translocation (Klee et al. 1998).

NFAT signaling pathway has drawn a great deal of attention in recent years since Molkentin et al. first reported a critical role of this pathway in cardiac hypertrophy in 1998 (Molkentin et al. 1998; Olson and Williams 2000; Sugden 2001). Overexpression of a constitutively active form of calcineurin in the hearts of transgenic mice resulted in severe cardiac hypertrophy that underwent a transition to dilated heart failure within 2 months (Molkentin et al. 1998). Transgenic mice overexpressing a constitutively nuclear mutant of NFAT3 also showed cardiac hypertrophy (Molkentin et al. 1998). Cyclosporine A was able to inhibit cardiac hypertrophy in calcineurin-trangenic mice, but

not in NFAT3-transgenic mice. However, recent conflicting data on sufficiency and necessity of this signaling pathway has made its importance uncertain. First, the effect of calcineurin inhibitor on pressure overload hypertrophy is not clear with conflicting reports(Meguro et al. 1999; Shimoyama et al. 1999; Lim et al. 2000; Ding et al. 1999; Luo et al. 1998; Zhang et al. 1999). Secondly, whether calcineurin activity increases in response to hypertrophic stimuli is not clear. It was found that the amount of calcineurin and enzymatic activity in the heart are increased by hypertrophic stimuli (Hill et al. 2000; Lim et al. 2000). However, other studies reported an unchanged or decreased calcineurin activity after pressure overload (Meguro et al. 1999; Zhang et al. 1999; Ding et al. 1999). Thirdly, the reported calcineurin activity in failing hearts also differs from one study to another (Lim and Molkentin 1999; Tsao et al. 2000). Different experimental systems, surgical procedures and drug dosing may all contribute to the above discrepancies. Further investigation on this pathway is needed.

NFAT family consists of five members. Northern blot analysis indicated that at least four different NFAT genes are expressed in the heart (Molkentin and Dorn 2001). Transgenic mice expressing a constitutively nuclear mutant of NFAT3 demonstrated cardiac hypertrophy (Molkentin et al. 1998). However, the necessity of NFAT in cardiac hypertrophy has not been established.

AP-1 Transcription Factor in Cardiac Hypertrophy

Although the transcription factor Activating Protein-1 (AP-1) was first discovered 15 years ago (Angel et al. 1987; Lee et al. 1987a; Lee et al. 1987b), the biological relevance and physiological function of AP-1 and its components are still under investigation. AP-1 is involved in multiple cell functions including proliferation, survival, death and hypertrophy in response to extracellular stimuli (Wisdom 1999; Shaulian and Karin 2001). The AP-1 proteins are homodimers or heterodimers composed of basic leucine zipper (bZIP) proteins, including mainly Fos (c-Fos, Fos-B), Jun family members (c-Jun, Jun-B and Jun-D) and ATF-2. The AP-1 transcription factor recognizes a palindromic sequence 5' TGAGTCA 3'. Jun family proteins can form homodimeric complexes, as well as Jun-Fos heterodimers, while Fos family proteins are only capable of heterodimer formation with Jun family members. Jun proteins can also form heterodimers with ATF-2 protein, which recognizes a related, yet distinct AP-1 binding site with a sequence of 5' TGAGCTCA 3' (Angel and Karin 1991; Wisdom 1999).

AP-1 can be activated by a large number of stimuli. Under different stimulating conditions, AP-1 regulates cellular responses by regulating distinct sets of genes. How AP-1 is able to precisely adjustify to the diversity of cell responses is yet to be fully studied. This question can be partially answered by the fact that AP-1 activation is well regulated at different levels in a cell. First, the component of AP-1 can often be activated by phosphorylation at sites in their activation domains. The activation of c-Jun by

phosphorylation is largely regulated by the JNK family of MAPKs. The c-Fos activation domain can also be phosphorylated, but the upstream kinases remain to be identified (Shaulian and Karin 2001). Secondly, the expression of both c-fos and c-jun genes is sensitive to stimulations. The mRNA level of c-fos was transiently but strongly increased by stress stimulators. This induction is usually downstream of ERKs that phosphorylate the ternary complex factor (TCF/Elk-1), a transcription factor regulating c-fos expression (Hill et al. 1994). Increased c-Fos proteins form heterodimers with c-Jun proteins. This complex can bind to the AP-1 sites in the c-Jun gene promoter and enhance c-Jun expression, leading to amplification of AP-1 response(Angel et al. 1988). In addition, both ERK and p38 phosphorylate MEF2, which binds to the promoter of c-Jun and induces its expression (Han and Prywes 1995, Han et al. 1997). Thirdly, the fact that AP-1 proteins are able to interact with other transcription factors to form quaternary complexes binding to DNA sequences further complicates the AP-1 controlled gene regulation. A well-known example is the AP-1/NFAT cooperation in IL-2 gene regulation. A single molecule of NFAT along with a Fos-Jun dimer interacts with an extended sequence of 15 bp of DNA in IL-2 promoter. In the presence of NFAT, the affinity of AP-1 for the site is about 3 fold greater than AP-1 alone (Macian et al. 2001).

AP-1 protein plays an important role in hypertrophic gene expression. It can be activated by a number of hypertrophic stimuli (Sadoshima et al.1993; Takemoto et al. 1999; Herzig et al. 1997). Hypertrophy marker genes ANF and αSkA have AP-1 sites in their promoters, suggesting a role of AP-1 for their activation (Bishopric et al. 1992;

Rosenzweig et al. 1991). In angiotensin II induced cardiac hypertrophy, AP-1 is also responsible for an increased expression of Transforming Growth Factor β (TGF-β) (Wenzel et al. 2001). In a recently published paper, AP-1 was implicated in posttranscriptional control of BNP gene expression (Suo et al. 2002)

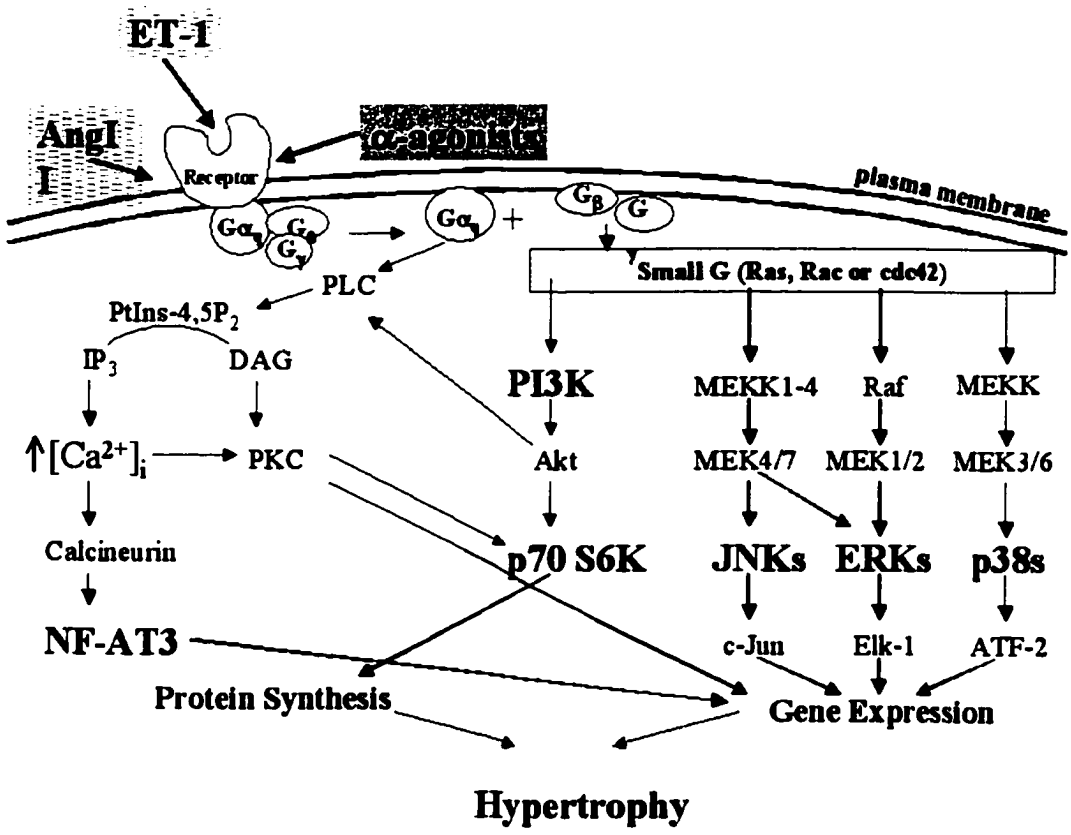


Figure 1.2 Signaling Pathways of Cardiomyocyte Hypertrophy

Oxidative Stress and Cardiovascular Disease

The life on this planet first evolved in a reducing atmosphere. Since photosynthetic algae started to release oxygen into the atmosphere millions of years ago, the shift from a reducing environment to an oxidizing one gradually took place. Today, 20% of our air is composed of oxygen. Oxygen is vital for human life. By inhaling oxygen, humans convert food, mainly carbonaceous compounds, into heat and energy. Indeed, the balance of reduction-oxidation is the core of our metabolic machinery.

Despite the dependence of oxygen in our life, the trade-off is the toxicity associated with reactive oxygen species. This results from the intrinsic chemical characteristics of oxygen. The oxygen molecule contains two electrons that are not spin-paired, each residing in an orbital of its own. Oxygen is a di-radical since a free radical is a molecule containing a single, unpaired electron. This electronic structure of the oxygen molecule determines that one oxygen molecule tends to take on additional electrons and four hydrogen molecules to produce two water molecules. In fact, the reaction of converting oxygen to water is so crucial for our life that the majority of the energy we have is produced by this reaction in mitochondria. When oxygen molecules pass through the electron transport chain on the mitochondria membrane, cytochrome oxidase transfers four electrons to oxygen to produce two molecules of water. However, when this reaction is carried out with less than four electrons, *reactive oxygen species* (ROS) are produced. Superoxide is formed when oxygen receives one more electron. About 1% to

2% of oxygen used by mitochondria leaks out as reactive oxygen species, which are the major sources of oxidative stress in our body.

ROS include all products of the incomplete reduction of oxygen such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Unlike the other two compounds, hydrogen peroxide itself is not a radical because it has no unpaired electron. However, it has a high tendency to take on two more electrons. Ferrous iron and cuprous copper are capable of transferring a third electron to hydrogen peroxide, causing cleavage of the O-O bond and formation of hydroxyl free radicals, the most reactive ROS.

The toxicity of ROS results from their ability to react with biomacromolecules in our body. Hydroxyl radical can attack any organic molecules, causing lipid peroxidation, DNA strand breaks and protein oxidization. Antioxidant enzymes naturally exist to reduce oxidative stress in an organism. The family of antioxidant enzymes includes superoxide dismutases (SODs) for elimination of superoxide radical, and catalases and glutathione peroxidases (GPXs) for elimination of hydrogen peroxide and organic peroxide. Four classes of SOD have been identified, containing either a dinuclear Cu, Zn or mononuclear Fe, Mn or Ni cofactors. According to their locations in the cell, there are three forms of SOD in humans: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular-SOD (EC-SOD) (Majima et al. 1998). All forms of SOD reduce superoxide radical to hydrogen peroxide and oxygen. Catalase and glutathione peroxidase prevent

cells from hydrogen peroxide toxicity by converting it to water and oxygen. Catalase contains four identical tetrahedrally arranged subunits of 60kDa. It is one of the most efficient enzymes known. Catalase is important for cells to deal with oxidative stress caused by external insults (Hunt et al. 1998). GPXs have a boarder substrate spectrum than catalase. They can detoxify not only H_2O_2 but also other hydroperoxides using glutathione as the reductant. There are at least five GPX isoenzymes found in mammals. They are distributed in the cytosol or membrane fraction. The glutathione redox cycle using GPXs is the major source of protection against low-level oxidative stress in cells (Mates and Sanchez-Jimenez 1999).

The significance of these antioxidant enzymes in protecting our body's normal function has been demonstrated by studies using knockout mice. The homozygous manganese SOD knockouts are born small and alive, but they die within days of birth with dilated cardiomyopathy (Li et al. 1995). Both cytosolic copper-zinc and extracellular SOD knockouts, although they survive, respond badly to stress, such as hypoxia (Carlsson et al. 1995). Hydrogen peroxide, as mentioned above, is capable of converting to hydroxyl radical in our body and therefore producing oxidative stress. Because of its chemical stability, hydrogen peroxide is widely used as an oxidant in oxidative stress research. Catalase is the major enzyme in animals to detoxify hydrogen peroxide. Transgenic mice overexpressing catalase were observed with stronger cardiac protection against the oxidative damage from ischemia/reperfusion (Kirshenbaum and Singal 1993). Recent studies using knockout mice technology also demonstrated the

importance of GPXs in defending against oxidative damage. GPX-1 knockout mice were shown to have increased infarct size and exacerbated apoptosis in the brain after ischemic reperfusion challenge, compared to wild type mice. (Crack et al. 2001; Lei 2001).

The redox status is a major regulator of signal transduction pathways and gene expression in the cell. In response to oxidative stress, cells activate a variety of kinases, such as protein kinase C (Klann et al. 1998), MAPKs and Src kinase family (Thannickal and Fanburg 2000). The activation of these kinases leads to an altered gene expression profile in the cell. Several transcription factors including AP-1 and NF-kB are activated by oxidative stress (Allen and Tresini 2000). Recent research has established the important role of NF-κB in oxidant induced cardiomyocyte hypertrophy (Force et al. 2002).

Oxidative stress has been implicated in the development of cardiac diseases. An increased level of ROS is observed in various models of heart failure (Sawyer and Colucci 2000; Singh et al. 1995). Ischemia and ischemic reperfusion are major causes of increased ROS. It is known that ischemia impairs mitochondrial function and decreases the activity of SOD and GPX, resulting in an increased amount of H_2O_2 detectable in the ischemic region of the heart (Shlafer et al. 1987; Vandeplassche et al. 1989). Unlike ischemia, ischemic reperfusion enhances oxidant generation through abnormal mitochondrial function as well as activated xanthine oxidase, which produces H_2O_2 when

converting hypoxanthine to xanthine. The level of H_2O_2 can reach 11 μM at infarcted sites during reperfusion (Goldhaber 1997). In addition, cell injury in the ischemic region recruits neutrophils to the site. The ROS producing activities of neutrophiles and other inflammatory cells result in a marked increase of oxidant at the injured site. Thus, oxidative stress is related with a number of cardiac pathological conditions.

Although intensive research has investigated the mechanism of cardiac hypertrophy, the potential role of oxidative stress in the development of hypertrophy has not been defined. The relationship between oxidative stress and heart disease, as reported by the above literature, strongly indicates that oxidants play an important role in cardiac hypertrophy. This dissertation studied the mechanisms of H_2O_2-induced cardiomyocyte hypertrophy. Early work from our lab found that H_2O_2 is able to induce cardiomyocyte hypertrophy. In this dissertation, we identified specific signaling pathways and gene transcription factors activated by H_2O_2 in this process.

Preliminary Study

The relationship between cardiac hypertrophy and apoptosis

Apoptotic cell death and hypertrophy of cardiomyocytes are two seemingly independent yet intrinsically related processes in the heart. As hypertrophic growth of the myocardium is a normal process throughout development and in response to increased workload, by the same token, apoptotic death in the myocardium is most likely a normal process during development of the heart when thinning of the right ventricular

wall occurs (Smoak, 2002). Although necessary during development, apoptosis and hypertrophy are ironically the two major responses of the heart to external insults and inherently detrimental to cardiac function. Apoptosis and hypertrophy of surviving cardiomyocytes both occur under several pathological conditions. Loss of cardiomyocytes is one of the key features of the failing heart. The surviving cardiomyocytes are usually severely enlarged. During the aging process, it is estimated that the heart loses millions of cardiomyocytes each year in healthy individuals presumably due to apoptosis. On the other hand, cardiac hypertrophy is prevalent in the aging population (Lakatta et al. 1997). The presence of apoptosis counteracts age-related hypertrophy and therefore a clear increase in the muscle thickness is often not detected. The co-occurrence of apoptosis and hypertrophy in the heart was also demonstrated in dilated cardiomyopathy. The thinning of the ventricular walls is caused by cell death, whereas the remaining cardiomyocytes are enlarged (Colucci and Braunwald 1997). The co-occurrence of apoptosis and hypertrophy in the heart suggests there are common triggers for these two processes. Defining these triggers and the mechanisms of their induced apoptosis and hypertrophy are necessary for a better understanding the mechanisms of cardiotoxicity.

Preliminary data

The preliminary studies from our laboratory indicated that H_2O_2 was able to induce both apoptosis and hypertrophy. The cells that survived H_2O_2 treatment developed

a hypertrophic morphology over 5 days or longer (Fig. 1.3). In addition to a cell surface increase, these cells showed dose-dependent increases in cell volume, protein content per cell or ratio of protein to nucleic acid (Fig. 1.4). These data demonstrate for the first time that H_2O_2 is indeed an inducer of cardiomyocyte hypertrophy. They are the foundation as well as the starting point of my dissertation research.

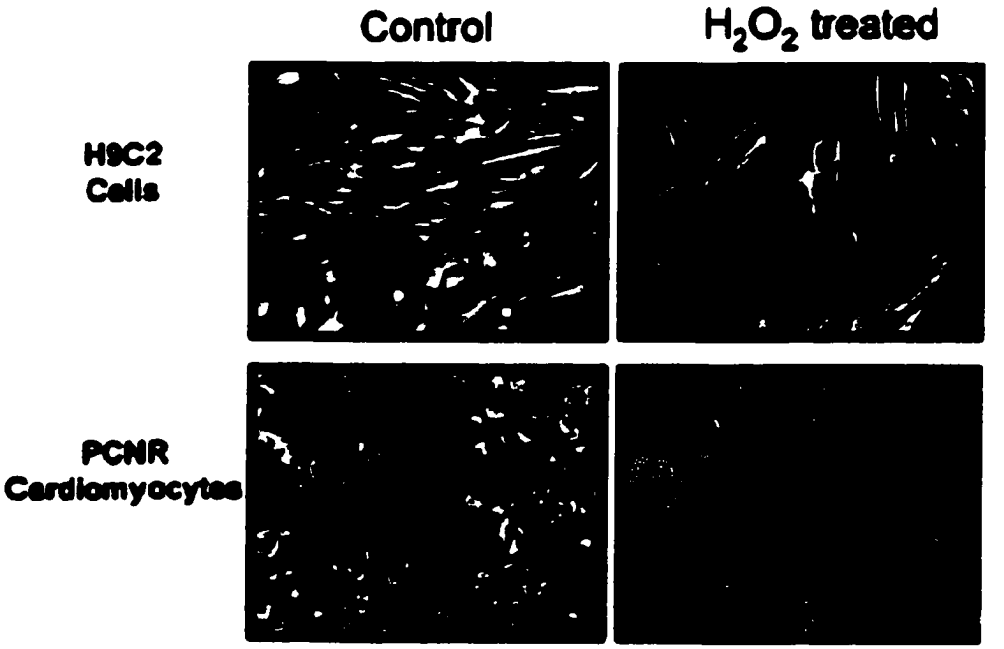


Figure 1.3 Morphological Change of Attached Cells Indicating an Increase in Cell Surface Area after H_2O_2 Treatment.

H9C2 cells ($15.3 \times 10^3/cm^2$) or primary neonatal rat cardiomyocytes ($25 \times 10^3/cm^2$) in six-well plates were treated with 200 μM H_2O_2 for 2 hr, placed in fresh medium afterwards, and cultured for 5 days. The images were acquired by a digital camera attached to a phase contrast microscope with 10 × lens.

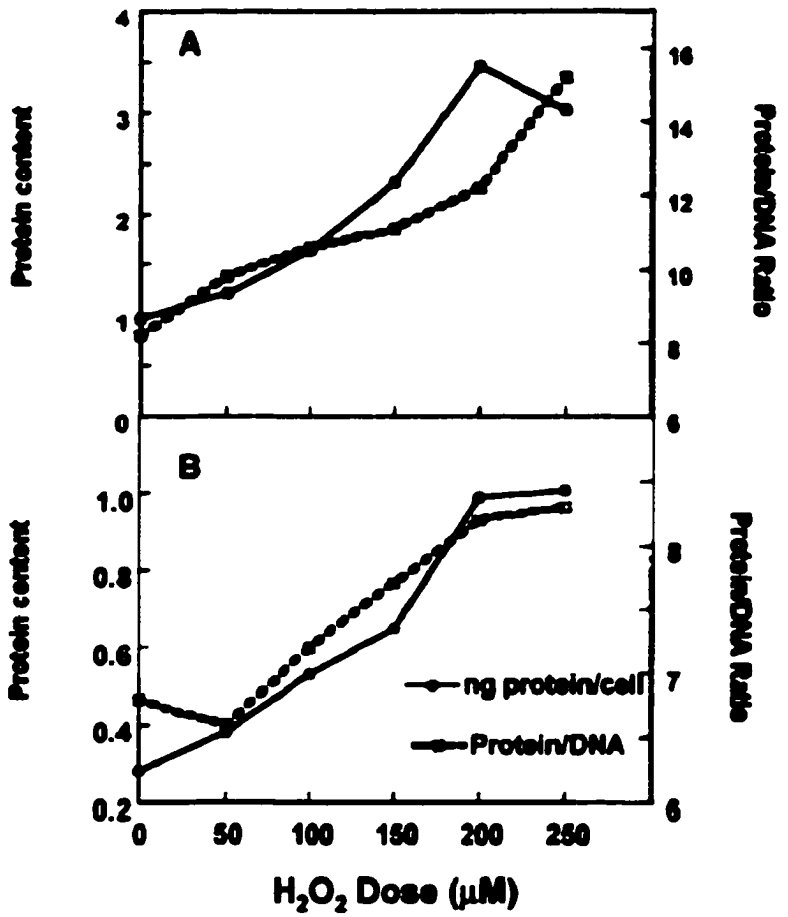


Figure 1.4 H_2O_2 Causes Increases in Protein Content per Cell and Protein to DNA Ratio in H9C2 (A) or Primary Neonatal Rat Cardiomyocytes (B).

Cells in six-well plates were harvested at 5 days after a 2-hr H2O2 treatment for measurement of protein, DNA, and cell number. The data are from one representative experiment.

My dissertation research has focused on deciphering the mechanisms of oxidant induced cardiomyocyte hypertrophy. A board spectrum of research demonstrates that oxidants are inducers of cardiomyocyte hypertrophy and it induces the hypertrophy through specific signaling transduction pathways and gene regulation machinery.

Chapter 2 ACTIVATION OF p70 S6 KINASE-1 AND PHOSPHOINOSITIDE 3-KINASE IN H_2O_2 INDUCED CARDIOMYOCYTE HYPERTROPHY

Introduction

Several lines of evidence suggest a role of oxidative stress in cardiac disease. The level of oxidants increases in the myocardium as a result of ischemia and ischemic reperfusion (Flitter 1993; Goldhaber 1997). As estimated with experimental animals, the concentration of H_2O_2 reaches 6 μM with ischemia and rises to 11 μM during reperfusion in the myocardium (Goldhaber 1997). The myocardium incurs oxidative damage over the course of heart failure (Singh et al. 1995; Hill and Singal 1996; Keith et al. 1998). Elevated levels of oxidative damage markers are observed in patients with congestive heart failure and in animal models of chronic heart failure (Dhalla et al. 1996; Hill and Singal 1996; Keith et al. 1998). Oxidative stress is thought to be a major contributor to the cardiac toxicity of the antineoplastic drug doxorubicin, which can cause acute cardiac toxicity when administrated at high doses or cause chronic heart failure at mild doses or accumulated low doses above a certain threshold (Singal and Iliskovic 1998; Singal et al. 2000). Epidemiological studies show that antioxidant vitamins reduce the risk of coronary heart disease and the mortality rate associated with this disease (Rimm et al. 1993; Kushi et al. 1996). Animal experiments reveal a beneficial effect of vitamin E therapy in reducing the rate of heart failure induced by pressure overload or doxorubicin (Dhalla et al. 1996; Singal et al. 2000). However, regardless of these positive outcomes

of antioxidants in preventing heart failure, it is not clear how oxidants cause or accelerate heart disease at the cellular and molecular levels.

Cardiac hypertrophy is a common endpoint of many cardiovascular diseases. An increase in the size of cardiomyocytes is the key feature of hypertrophic hearts. In end-stage heart failure, enlarged cardiomyocytes are often observed (Colucci and Braunwald 1997). Enlargement of cardiomyocytes has been reported to result from an increase in protein content and activation of p70S6K1 (Sadoshima and Izumo 1995; Boluyt et al. 1997). p70S6K1 plays a major role in regulating the phosphorylation of 40S ribosomal S6 protein and selective translation of a family of mRNAs that contain an oligopyrimidine tract at the 5'-transcriptional start site (Dufner and Thomas 1999). These mRNA species make up 20-30% of total translated mRNAs and encode components of the translational apparatus important for cell growth (Dufner and Thomas 1999). Activation of p70S6K1 results from phosphorylation of specific threonine (Thr) and Serine (Ser) residues such as Thr389 and Thr421/Ser424 (Dufner and Thomas 1999). Phosphorylation of Thr389 and activation of p70S6K1 can be inhibited by rapamycin, which inhibits an upstream regulator the Ser/Thr kinase mTOR (Chou and Blenis 1995; Proud 1996; Pullen and Thomas 1997; Dufner and Thomas 1999).

One of a few important upstream regulators of p70S6K1 is PI3K. PI3K can be activated by a number of receptor tyrosine kinases as well as G-protein-coupled receptors. PI3K is a heterodimer composed of a 110-kDa catalytic subunit (p110) and an

85-kDa regulatory subunit (p85). PI3K catalyzes addition of a phosphate group to the 3' position of the sugar ring in a phosphoinositide. Its products act on multiple downstream effectors that interact with Src homology-2 and pleckstrin homology domains of serine/throenine and tyrosine kinases. Some of these kinases contribute to phosphorylation and activation of p70S6K1.

Earlier, we found that the majority of cardiomyocytes in culture survive a pulse treatment with low or mild doses of H_2O_2 but become enlarged over a course of 4 to 7 days (Chen et al. 2000b). The cells respond to H_2O_2 treatment by increasing cell volume and protein content. To understand the mechanism behind this phenomenon, we determined early changes in signal transduction pathways of oxidative stress using primary cultured neonatal rat cardiomyocytes. The dose of 200 μM was chosen for most experiments since it appeared to induce maximal increases in cell size without killing the majority of the cells (Chen et al. 2000b). We demonstrate here that H_2O_2 activates PI3K and p70S6K1 in cardiomyocytes. Inhibitor approaches indicate a critical role of these two kinases in oxidant-induced enlargement of cell size. To evaluate the immediate pharmacological significance of these findings, we also tested whether the anthracycline quinones, represented by doxorubicin and daunorubicin, induce cardiomyocyte hypertrophy via an oxidative stress mechanism.

Materials and Methods

Chemicals and reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Stabilized H_2O_2 (Sigma, H-1009) was used and the concentration of the stock was verified by absorbency at 240 nm. Wortmannin, rapamycin, doxorubicin and daunorubicin were obtained from Calbiochem (La Jolla, CA).

Cell culture and treatment

Cardiomyocytes were prepared from 1 to 2-days old neonatal Sprague-Dawley rats (Harlan, Indianapolis, IN). Briefly, the heart tissue was cut into 1-2 mm pieces and washed with a cocktail containing 0.02% pancreatin (Life Technologies, Grand Island, NY) and 0.045% collagenase to dissociate the myocytes and remove red blood cells. The heart tissue was digested in fresh cocktail at 37°C for 15 min. The digestion was repeated 6-10 times. Dissociated cells were collected by centrifugation and were resuspended in Ham's F-12 medium (Life Technologies, Grand Island, NY) with 1.0% (w/v) bovine serum albumin (Life Technologies, Grand Island, NY), 0.025% (w/v) Fetuin, 0.1 mM ascorbate, 100 units/ml penicillin G, and 100 units/ml streptomycin. The dissociated cells were placed in uncoated 100 mm plastic petri dishes and incubated at 37°C in a 5% CO_2 incubator for 45 to 60 min. This procedure allows fibroblasts to attach to the dishes while most myocytes remain unattached (ref). The population of cells enriched in myocytes by this differential plating procedure was collected and counted.

The cells were seeded at a density of $2x10^6$ cells per 100 mm dish in DMEM with 1 mM pyruvate, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin. One to two days after plating, the media was replaced with fresh DMEM containing 0.5% FBS for 48 h and the cells were treated with H_2O_2, doxorubicin or daunorubicin in the medium. For groups treated with N-acetylcysteine (NAC) or buthionine sulfoximine (BSO), NAC (5 mM) or BSO (100 μM) was added to cells during the 48 h low serum (0.5%) incubation time. NAC or BSO was removed by changing medium before H_2O_2 or doxorubicin treatment. At the time of H_2O_2, doxorubicin or daunorubicin treatment, over 90% of the cells were capable of expressing sarcomeric myosin as determined by immunocytochemistry with an antibody against sarcomeric myosin heavy chain.

p70S6K1 activity assay

The protocol of Oh, *et al.* (Oh et al. 1998) was adopted for measuring p70S6K1 activity. Following H_2O_2 exposure, myocytes were lysed on ice in 0.5 ml of p70S6K1 lysis buffer (10 mM potassium phosphates, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 0.5% Triton X-100, 1 mM Na_3VO_3, 2 mM DTT, 1 mM PMSF and 10 μg/ml aprotinin). After a 20-min incubation on ice, the lysates were centrifuged for 5 min at 13,000 rpm. Supernatants were collected and measured for protein concentration by the Bradford method (Bio-Rad, Richmond, CA) before being used for immunoprecipitation. An equal amount (500 μg) of proteins from each sample

was used for a 2-h incubation on ice with 5 μl p70S6K1 antibody (Santa Cruz Biotechnology, CA) and for an additional 1 hr incubation after the protein A-Sepharose (30 μl per reaction tube) was added. The protein A-Sepharose immunocomplexes were washed twice with the kinase lysis buffer and twice with a kinase reaction buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM Na_3VO_3 and 1 mM DTT) before being resuspended in 30 μl kinase reaction buffer containing 20 μM S6 kinase substrate peptide (RRRLSSLRA, Santa Cruz Biotechnology, CA). The kinase reaction was initiated by addition of 10 μCi [γ- ^{32}P] ATP plus unlabeled ATP to a final concentration of 40 μM and $MgCl_2$ to a final concentration of 10 mM. After a 10 min incubation at 30°C, 20 μl of reaction mixtures were spotted onto p-81 phosphocellulose discs (Whatman, Clifton, NJ), which were then washed twice in 0.75% phosphoric acid and twice in acetone. After being dried in air, the discs were placed in 10 ml scintillation fluid and phosphorylated products were quantified by liquid radioactive chromatography.

Assay for p70S6K1 phosphorylation

After H_2O_2 exposure, myocytes were harvested in a lysis buffer (1% Triton X-100, 10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 50 mM NaCl, 50 mM NaF, 10 μg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 2 mM Na_3VO_4). Protein concentration was measured by the Bradford method according to the manufacturer's instruction (Bio-Rad, Richmond, CA). An equal amount of protein was loaded in each lane and separated by 8% SDS-polyacrylamide gel electrophoresis for Western blot as

previously described (Chen et al. 2000a). After transferring the proteins to a PVDF membrane, the membrane was incubated for 2 h with an antibody that recognizes phosphorylated Thr 389 or phosphorylated Thr421/Ser424 of p70S6K1 (1:1000 dilution, New England Biolab, Beverly, MA). For determining the basal level of the protein, a duplicated membrane from the same experiment was incubated with an antibody that recognizes both phosphorylated and unphosphorylated forms of p70S6K1 (1:1000 dilution; New England Biolab, Beverly, MA). Bound antibodies were detected by enhanced chemiluminescence (ECL) reaction following the incubation with a secondary antibody conjugated with the horse radish peroxidase (Chen et al. 2000a).

PI3K activity assay

Myocytes were harvested by scraping on ice using PI3K lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM Na_3VO_4, 2 μM leupeptin, 10 unit/ml aprotinin and 1 mM phenylmethanesulfonyl fluoride). Supernatants were collected for protein concentration determination as described above. Samples containing 500 μg of protein were incubated with 5 μl anti-p85 antibody (Upstate Biotechnology, Lake Placid, NY) for 2 h on ice and for additional 1 h following addition of 40 μl protein A-Sepharose. The kinase reaction was carried out at 30°C for 10 min in a 50 μl reaction mixture containing the immunoprecipitates, 0.2 mg/ml phosphatidylinositol, 10 μCi [γ-^{32}P]ATP and the kinase reaction buffer (20 mM Tris-HCL, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA,

120 μM adenosine and 50 μM ATP). The reaction was stopped by the addition of 100 μl of 1 M HCl. Phospholipids were extracted immediately using 200 μl of a chloroform and methanol mixture (1:1 volume ratio). An equal volume of organic phase from each sample was spotted onto a Silica Gel 60 plate (EM Separations Technology, Germany) for thin layer chromatography using a solvent containing chloroform, methanol, 25% ammonia hydroxide and water (43:38:5:7 volume ratio). Kinase activity was determined by autoradiography. An Instant Imager (Packard Instrument, Meriden, CT) was used to quantify the radioactivities in the product phosphatidylinositol 3-phosphate. The inhibitory effect of wortmannin was verified by incubating the immunoprecipitates with wortmannin for 30 min at 30°C before the kinase reaction.

Measurement of phosphorylation of p85 subunit of PI3K

Cells were harvested as described above using PI3K lysis buffer. For each sample, 500 μg of proteins were incubated with either anti-phosphotyrosine antibody or anti-p85 antibody (Upstate Biotechnology, Lake Placid, NY) for immunoprecipitation. Proteins were dissociated from the Sepharose by boiling and were separated by 8% SDS-polyacrylamide gel electrophoresis for Western blot as described (Chen et al. 2000a). The PVDF membrane was incubated with an anti-p85 antibody and the bound antibody was detected by ECL reaction after incubation with a secondary antibody conjugated with horseradish peroxidase as described (Chen et al. 2000a).

Measurements of cell enlargement

Cell volume and protein content per cell were determined as previously described (Chen et al. 2000b). Briefly, the cells were allowed to recover for 5 days after H_2O_2, doxorubicin or daunorubicin treatment. For the NAC treated group, cells were incubated with 2.5 mM NAC during the 5-day recovery period. The adherent cells in 6-well culture plates were detached by trypsin treatment and rounded cells were loaded onto a microslide field finder (Fisher Scientific, Pittsburgh, PA) for the measurement of cell diameters, which were used to calculate cell volume using the equation: $V=(4/3)\pi r^3$. Protein concentrations were measured by bicinchoninic acid (BCA) method according to the manufacturer's instruction (Pierce, Rockford, IL). Protein content per cell was determined by dividing the total amount of protein in each well with the cell number, which was determined by a Coulter Counter or hemocytometer after trypsin treatment.

Statistics

One-way analysis of variance (ANOVA) was used to compare groups of means followed by the Student-Newman-Keuls' method for multiple comparisons. Groups of means that are not significantly different from each other are indicated in the figure by a common letter symbol. Any mean with a letter designation different from others is significantly different from the others. When labeled with letters "*ab*", the mean is not significantly different from those labeled with *a* or *b*, although the mean with the letter designation *a* is significantly different from that with the letter *b*.

Results

Activation of p70S6K1 in cardiomyocytes by H_2O_2 treatment

Activation of p70S6K1 is an important event leading to cardiomyocyte hypertrophy induced by a variety of different inducers (Sadoshima and Izumo 1995; Boluyt et al. 1997; Laser et al. 1998; Simm et al. 1998). We determined whether H_2O_2 activated p70S6K1 by measuring its activity using cell lysates harvested after H_2O_2 treatment. Time course studies indicated a significant increase in p70S6K1 activity after 30 min of 200 μM H_2O_2 treatment (Fig. 2.1A). The activity reached a plateau of 2 fold after 60 min and the elevation level remained at 120 min (Fig. 2.1A). The activity of p70S6K1 returned to the basal level 24 h after H_2O_2 treatment (data not shown). When cells were treated with various concentrations of H_2O_2 for 60 min, the activation of p70S6K1 was detectable with 50 μM H_2O_2 and reached the highest level of 2.2-fold with 150 μM H_2O_2 (Fig. 2.1B). H_2O_2 at 100 μM or 200 μM concentration induced similar levels of activation as H_2O_2 at 150 μM (Fig. 2.1B).

Activation of p70S6K1 has been reported to result from phosphorylation of Thr389 and Thr421/Ser424 residues (Pullen and Thomas 1997; Dufner and Thomas 1999). Phospho-specific antibodies were employed to detect phosphorylation of these residues in H_2O_2 treated cells using Western blots. The results showed that 200 μM H_2O_2 caused an increase in Thr389 phosphorylation after 10 min and an increase in Thr421/Ser424 phosphorylation after 30 min (Fig. 2.2A). These residues remained

phosphorylated for 120 min (Fig. 2.2A). Dose-response studies showed that phosphorylation of Thr389 or Thr421/Ser424 was detectable with 100-250 μM H_2O_2 (Fig. 2.2B). To eliminate the possibility that the observed increases resulted from an increase in the level of p70S6K1 protein, we measured the protein level using an antibody that recognizes both phosphorylated and unphosphorylated forms of p70S6K1. The results showed that the level of p70S6K1 protein did not change with H_2O_2 treatment (Fig. 2.2A&B). We conclude that H_2O_2 induces a time- and dose-dependent phosphorylation of p70S6K1, which correlates with increased activity of the enzyme.

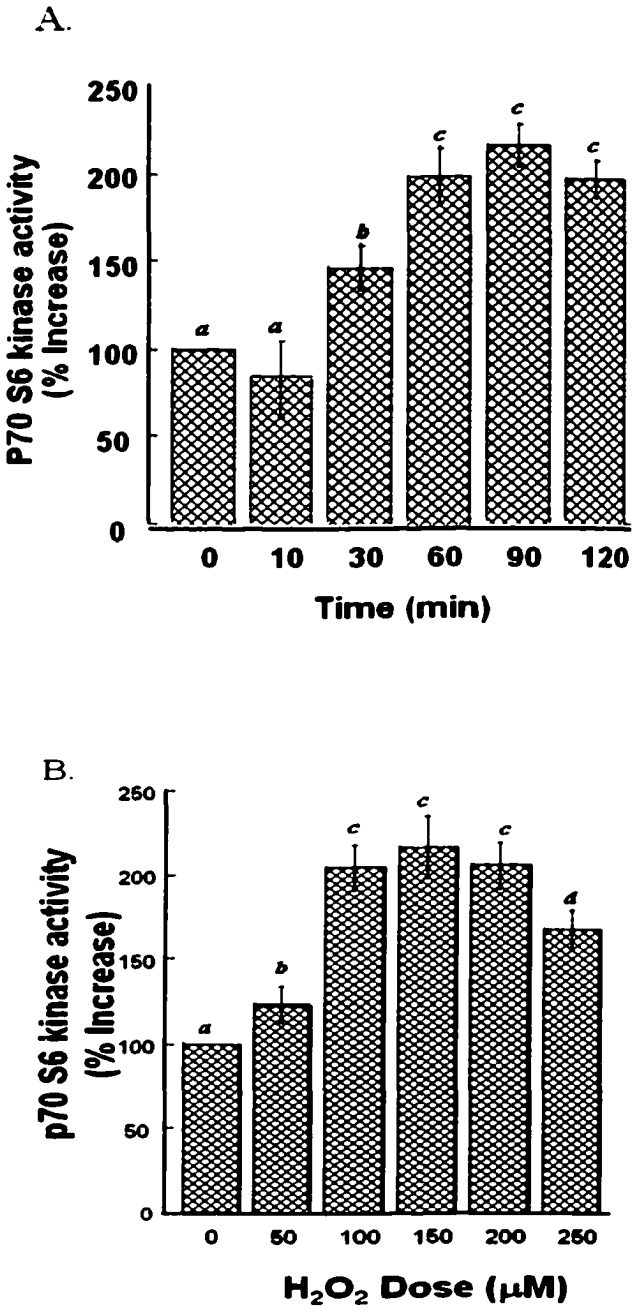


Figure 2.1 H_2O_2 Induced p70S6K1 Activation

Cardiomyocytes were treated with 200 μM H_2O_2 for the indicated time (**A**) or were treated for 60 min with various doses of H_2O_2 (**B**). The data are expressed as means ± standard deviations of percentage increase in the activity from three independent experiments. Means with a given letter designation are significantly different ($p < 0.05$) from other means with different letters as determined by ANOVA analysis of variance followed by multiple comparisons using the Student-Newman-Keuls' test.

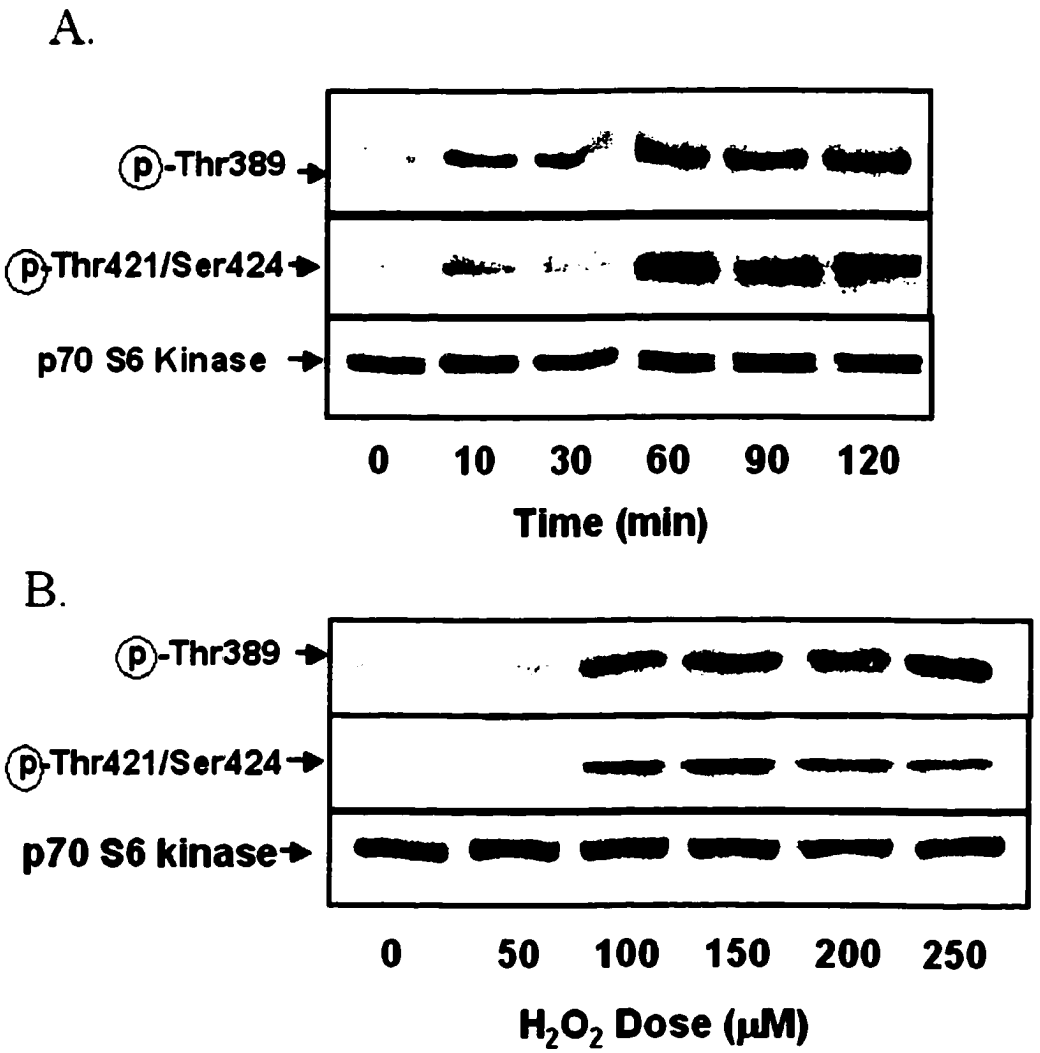


Figure 2.2 H_2O_2 Induced Phosphorylation of Thr389 and Thr421/Ser424 of p70S6K1

Cardiomyocytes were treated with 200 μM H_2O_2 for the indicated time (**A**) or were treated for 60 min with various doses of H_2O_2 (**B**). The cells were harvested for Western blots to detect phosphorylation or protein levels. The data are presented as the image from one experiment representative of three.

H_2O_2 Induces PI3K activation in cardiomyocytes.

An important upstream regulator of p70S6K1 is PI3K (Chou and Blenis 1995; Proud 1996; Dufner and Thomas 1999). H_2O_2 increases p70S6K1 activity and phosphorylation, suggesting the possibility that H_2O_2 may activate PI3K. To test whether H_2O_2 activates PI3K in cardiomyocytes, we measured PI3K activity by an *in vitro* kinase assay after isolating the PI3K complex using an antibody against the p85 subunit (Oh et al. 1998). Cardiomyocytes were treated with 200 μM H_2O_2 for 5 to 60 min. An increase in the kinase activity was detected after 5 min of treatment, reached a plateau after 10 min of treatment and remained elevated at 60 min of treatment (Fig. 2.3). One experiment indicated that the kinase activity at 120 min of H_2O_2 exposure was comparable to that at 60 min but diminished 24 h post H_2O_2 exposure (data not shown). The dose response studies of 10min H_2O_2 exposure indicated that the dose of 150 μM caused the highest activation (Fig. 2.4A). To ensure that an equal amount of PI3K protein in each sample was used for the *in vitro* kinase activity assay, the level of p85 protein was measured by Western blot (Fig. 2.3 & 2.4A, middle panels). The results showed that H_2O_2 induced PI3K activation without changing p85 protein level.

Wortmannin is a fungal metabolite that has been used widely as a selective inhibitor of PI3K. The inhibitory effect of wortmannin was confirmed by measuring PI3K activity using the p85 immunocomplex in the presence of wortmannin (Fig. 2.4A). The results showed that wortmannin at 100 nM was capable of preventing H_2O_2 from activating PI3K (Fig. 2.4A).

Activation of PI3K has been reported to result from phosphorylation of the p85 subunit at tyrosine residues (Fruman et al. 1998). To test whether H_2O_2 induces tyrosine phosphorylation of the p85 subunit, we performed Western blot using an anti-p85 antibody following immunoprecipitation of tyrosine-phosphorylated protein using an anti-phosphotyrosine antibody (Fig. 2.4B, upper panel). Immunoprecipitation using the anti-p85 antibody followed by Western blot was performed to ensure that control and H_2O_2 treated cells contained the same amount of p85 protein (Fig. 2.4B, lower panel). The results showed that H_2O_2 treatment induced tyrosine phosphorylation of the p85 subunit of PI3K (Fig. 2.4B).

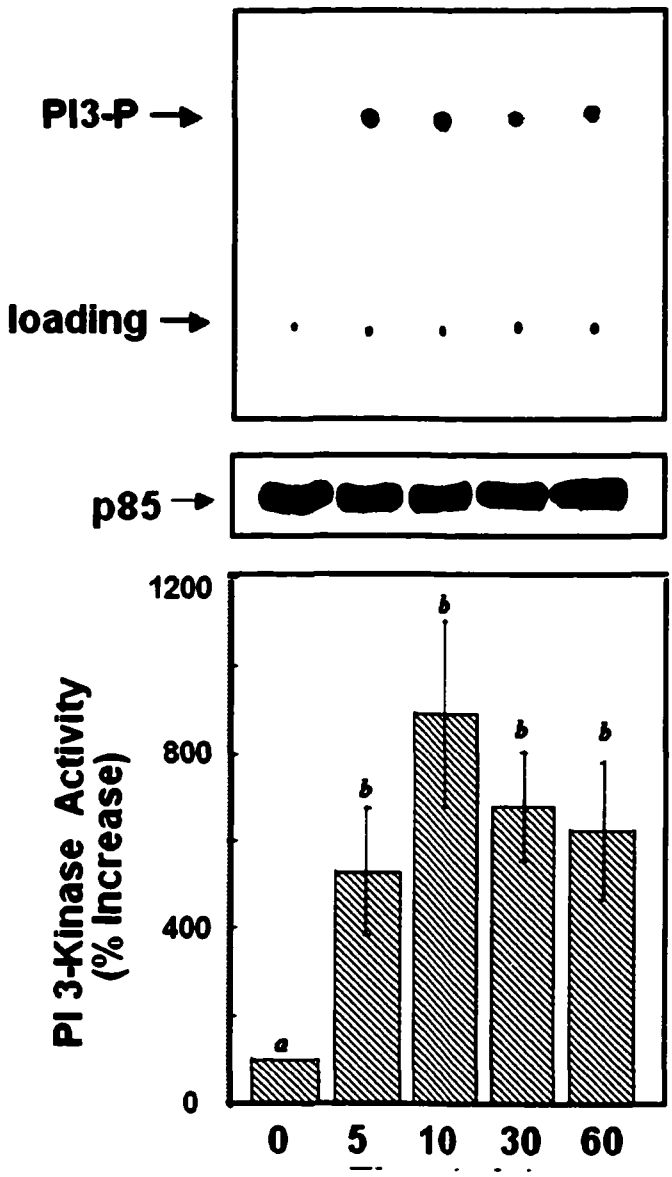
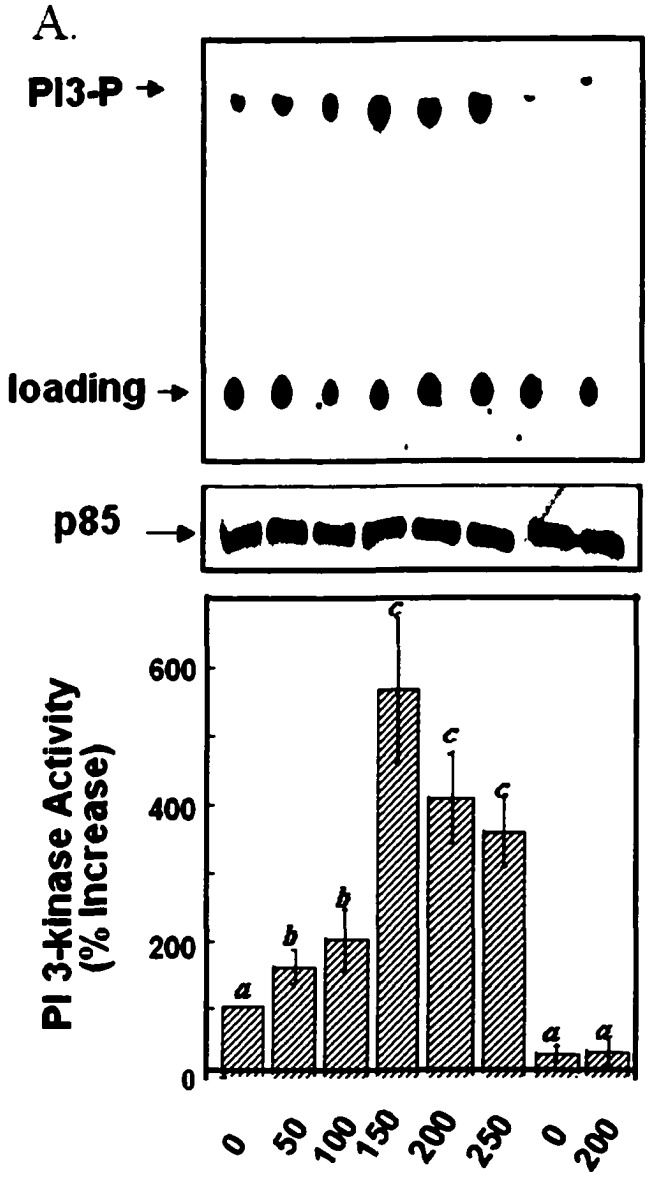


Figure 2.3 Time Course of H_2O_2 Induced PI3K Activation

Cardiomyocytes treated with 200 μM H_2O_2 for the indicated time were harvested for *in vitro* kinase activity assay. The ^{32}P-labeled lipid product (*PI3-P*) was separated by TLC from residual [^{32}P] ATP (*loading*) that remained in the aqueous phase and served as a loading control (*upper panel*). Aliquots of samples containing 20 μg protein were used for Western blots to determine the protein level of the p85 subunit (*middle panel*). The data are from one experiment representative of three (*upper and middle panels*) or are means ± standard deviation from three independent experiments (*bottom panel*). Means with a given letter designation are significantly different ($p < 0.05$) from other means with different letters determined by analysis of variance followed by multiple comparisons using the Student-Newman-Keuls' test.


A.
PI3-P
loading
p85
PI 3-kinase Activity
(% Increase)
600
400
200
0
a
b
b
c
c
c
a
a
0
50
100
150
200
250
0
200

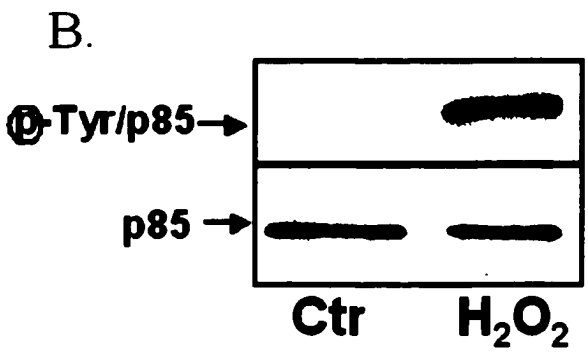


Figure 2.4 Dose Response of H_2O_2 Induced PI3K Activation (A), Inhibitory Effect of Wortmannin (WNT, A) and Tyrosine Phosphorylation of P85 Subunit (B).

Cardiomyocytes treated with H_2O_2 at various concentrations for 10 min were harvested for *in vitro* PI3K activity assay (A). The ^{32}P-labeled lipid product (*PI3-P*) was separated by TLC from residual [^{32}P] ATP (*loading*) that remained in the aqueous phase and served as a loading control (*Upper panel,* **A**). Aliquots of samples containing 20 μg protein were used for Western blots to determine the protein level of the p85 subunit (*middle panel,* **A**). Cell lysates from 10 mins, 200 μM H_2O_2 treated cardiomyocytes were used for p85 tyrosine phosphorylation assay using Western blots with an anti-p85 antibody after immunoprecipitation with an anti-phosphotyrosine antibody (**B**, *upper panel*) or an anti-p85 antibody (**B**, *lower panel*). The data presented here are either from one experiment representative of three (*upper and middle panels of* **A; B**) or are means ± standard deviation from three independent experiments (*lower panel of* **A**). Means with a given letter designation are significantly different ($p < 0.05$) from other means with different letters as determined by ANOVA analysis of variance followed by multiple comparisons using the Student-Newman-Keuls' test (**A**).

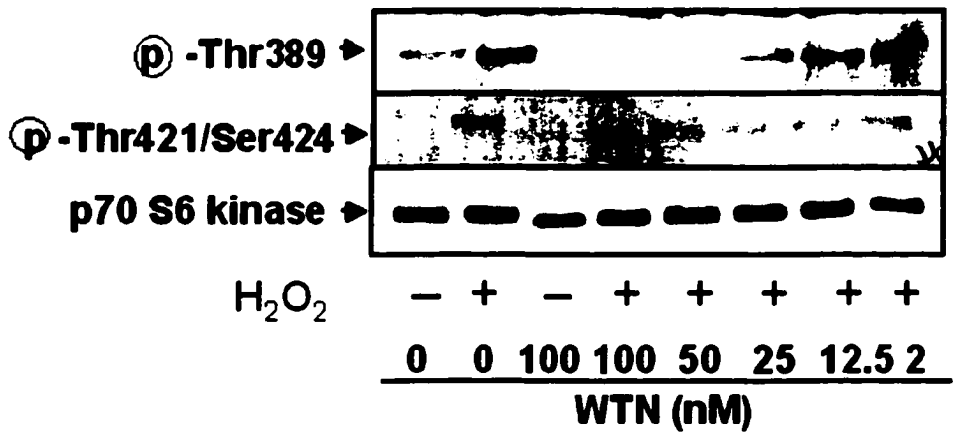
Relationship between p70S6K1 and PI3K Activation.

Our study reveals that H_2O_2 induces activation of p70S6K1 and PI3K in cardiomyocytes. The immediate question is whether PI3K is the upstream regulator of p70S6K1 under oxidative stress. A pharmacological inhibitor specific for PI3K (i.e. wortmannin) allows us to address this question. Rapamycin, an inhibitor of the key Ser/Thr kinase mTOR that regulates the activity of p70S6K1 and other cellular proteins, serves as a positive control for p70S6K1 inhibition. Induction of oxidative stress by H_2O_2 results in depletion of glutathione, which is the main source of cellular non-protein sulfhydryls. To eliminate the possibility that wortmannin and rapamycin function as antioxidants to prevent oxidative stress, we measured cellular non-protein sulfhydryl content using the Ellman's reagent (Chen and Stevens 1991). Cells were pretreated 30 min with the inhibitors and were treated with H_2O_2 in the presence of the inhibitors. We found that a 2-h treatment of H_2O_2 at 200 μM or lower failed to cause a significant loss of non-protein sulfhydryls in primary cultured rat neonatal cardiomyocytes or in the rat cardiomyocyte H9C2 cell line. Failure to detect loss of sulfhydryls is probably a result of limited sensitivity of the assay. In one representative experiment, H_2O_2 at 500 μM reduced non-protein sulfhydryls by 24.5% at the end of 2-h treatment time in H9C2 cells. In the presence of 100 nM wortmannin or 10 ng/ml rapamycin, H_2O_2 reduced non-protein sulfhydryls by 35 or 31.4%, respectively. These data indicate that the inhibitors at the concentrations used were unlikely to prevent H_2O_2 from inducing loss of non-protein sulfhydryls.

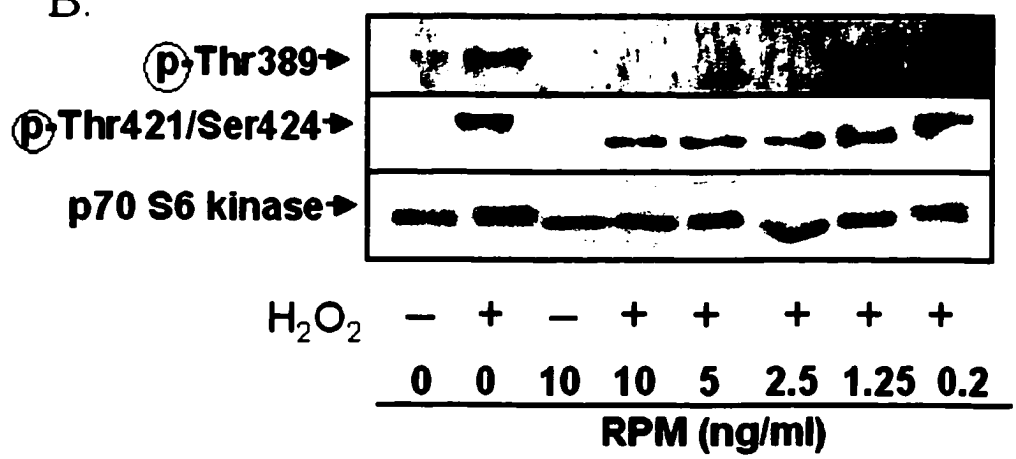
Wortmannin was tested for its effect on H_2O_2-induced p70S6K1 activation while rapamycin was used as a positive control. Cells were pretreated with these inhibitors for 30 min and were then treated with 200 μM H_2O_2 for 60 min in the presence of inhibitors. Measurements of p70S6K1 phosphorylation showed that wortmannin could inhibit Thr389 phosphorylation in a dose dependent manner and the dose 100 nM can completely abolish Thr389 phosphorylation (Fig. 2.5A). As expected, rapamycin at 5 or 10 ng/ml inhibited Thr389 phosphorylation (Fig. 2.5B). Thr421/Ser424 phosphorylation measurements showed that wortmannin or rapamycin did not prevent H_2O_2 from inducing Thr421/Ser424 phosphorylation (Fig. 2.5A&B). Overall, the effect of wortmannin on p70S6K1 is comparable to that of rapamycin.

Measurements of enzymatic activity of p70S6K1 allow us to quantify the inhibitory effect of wortmannin. Cells were harvested for immunoprecipiation and *in vitro* p70S6K1 activity assay after being treated 60 min with 200 μM H_2O_2 in the presence or absence of the inhibitors. The results showed that like rapamycin, wortmannin completely abrogated H_2O_2-induced p70S6K1 activation (Fig. 2.5C).

A.


Ⓟ -Thr389
Ⓟ -Thr421/Ser424
p70 S6 kinase
H2O2 – + – + + + + +
0 0 100 100 50 25 12.5 2
WTN (nM)

B.


ⓟThr389
ⓟThr421/Ser424
p70 S6 kinase
H2O2 – + – + + + + +
0 0 10 10 5 2.5 1.25 0.2
RPM (ng/ml)

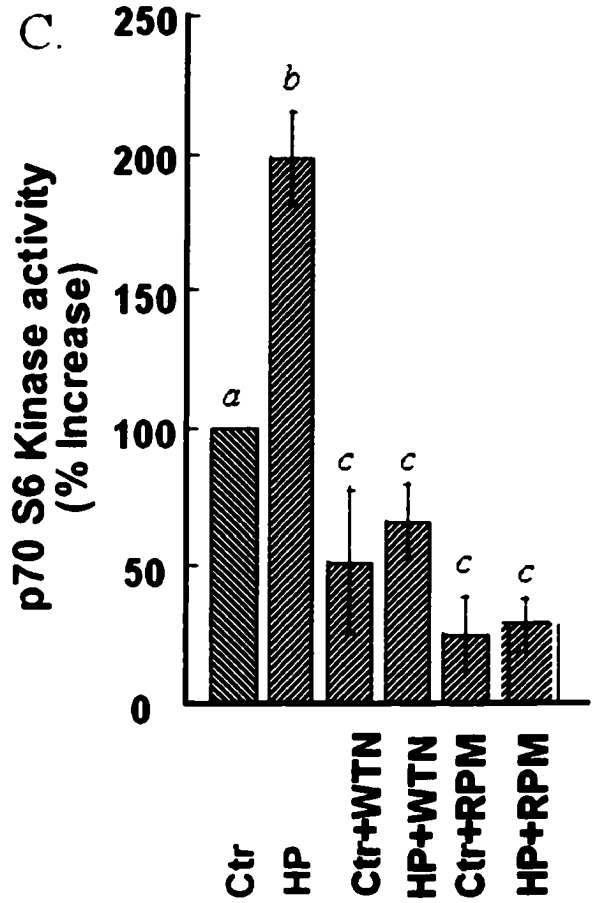


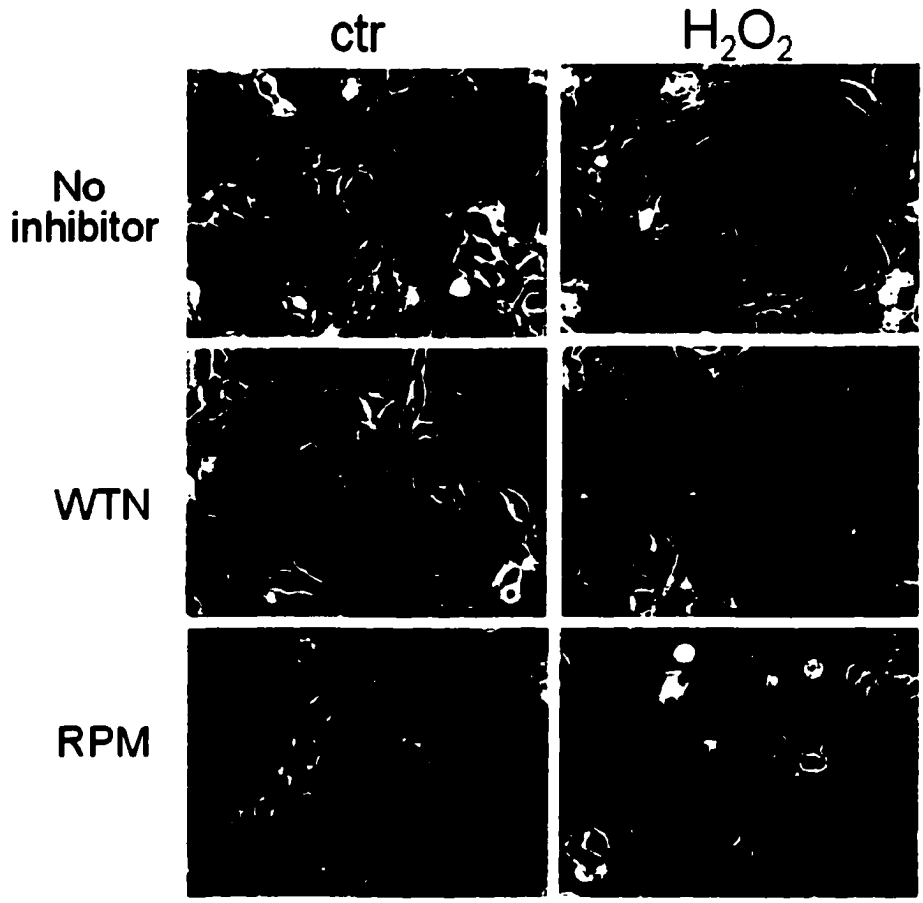
Figure 2.5 Effect of Wortamannin (WNT) and Rapamycin (RPM) on H_2O_2 Induced p70S6K1 Phosphorylation (A,B) and Activation (C).

Cardiomyocytes were pretreated 30 min with WTN (**A**) or RPM (**B**) at indicated concentrations. The cells were then treated with 200 μM H_2O_2 for 60 min in the presence of the inhibitors. Phosphorylation on Thr389 or Thr421/Ser424 and the protein level of p70S6K1 were determined by Western blot (**A, B**). Lysates harvested from cells treated with H_2O_2 in the absence or presence of 100 nM WTN or 10 ng/ml RPM were used for immunoprecipitation with anti-p70S6K1 antibody and *in vitro* p70S6K1 assay (**C**). The data are means ± standard deviation from three independent experiments (**C**). Means with a given letter designation are significantly different ($p < 0.05$) from other means with different letters as determined by ANOVA analysis of variance followed by multiple comparisons using the Student-Newman-Keuls' test (**A**)

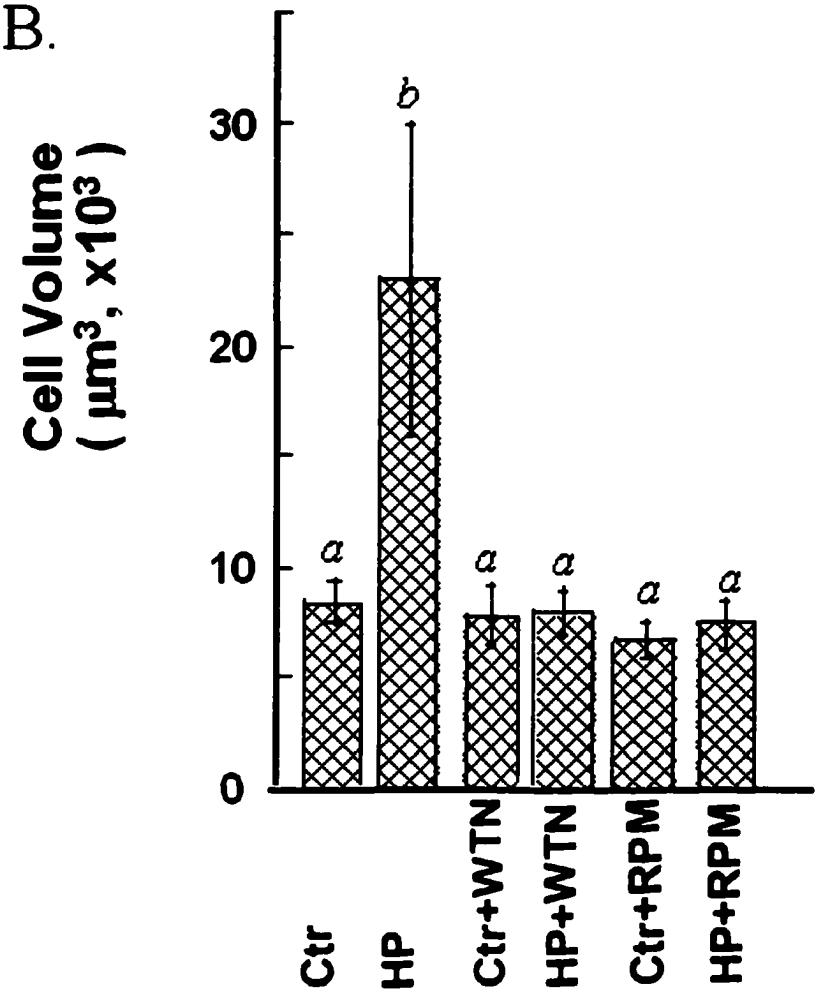
Effect of p70S6K1 and PI3K on H_2O_2 induced cell enlargement.

In our previous studies, we found that 60% of cardiomyocytes survive a 200 μM H_2O_2 treatment. The surviving cells become enlarged over the course of 5 days (Chen et al. 2000b). In these studies, cardiomyocytes were cultured in a medium containing 10% FBS and the cells were maintained in the medium containing 10% FBS during and after H_2O_2 treatment. Differing from the previous studies, here we cultured cardiomyocytes in a medium containing 0.5% FBS for 48 hr before H_2O_2 treatment. This low serum medium reduces the background for various kinase assays and prevents the complication of serum effects on cardiomyocyte hypertrophy. Cells were treated with 200 μM H_2O_2 for 90 min. H_2O_2 and oxidized medium were removed by placing cells in fresh DMEM containing 0.5% FBS. The cells were allowed to recover for 5 days in the low serum medium before examination of cell surface areas, cell volumes and the protein content per cell. To test the effect of wortmannin and rapamycin on H_2O_2 induced hypertrophy, cells were pretreated 30 min with these inhibitors and then treated with H_2O_2 in the presence of these inhibitors. During the 5-day recovery period, inhibitors were added back to the medium. Examinations of cell morphology, cell volumes and the protein content per cell showed that wortmannin and rapamycin abolished cell enlargement (Fig. 2.6A-C). These results suggest a role of PI3K and p70S6K1 in H_2O_2-induced cell enlargement.

A



B.


Cell Volume
(μm³, x10³)
30
20
10
0
a
b
a
a
a
a
Ctr
HP
Ctr+WTN
HP+WTN
Ctr+RPM
HP+RPM

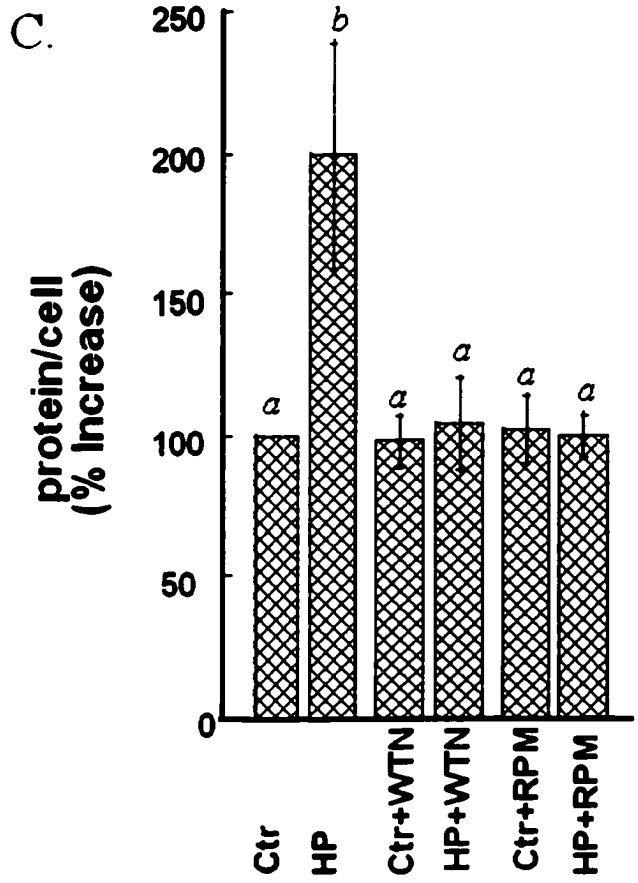


Figure 2.6 Effect of Wortmannin (WNT) or Rapamycin (RPM) on H_2O_2 Induced Cell Enlargement.

Cardiomyocytes in DMEM containing 0.5% FBS were pretreated with 100 nM WTN or 10 ng/ml RPM for 30 min. The cells were treated with 200 μM H_2O_2 for 90 min in the presence of the inhibitors and were then placed in fresh medium containing 0.5% FBS and the inhibitors. The medium was changed very 1-2 days with freshly added inhibitors. Cells at 5 days after H_2O_2 treatment were used for morphology analysis (**A**) and measurements of cell volumes (**B**) and protein content per cell (**C**). A digital camera attached to a phase-contrast microscope with 20x lens was used to acquire the images (**A**). At least 99 cells were measured for diameters randomly (**B**) and three groups of samples were measured for protein content per cell (**C**). The data are means ± standard deviations of 99 cells (**B**) or triplicates (**C**) from one experiment representative of three. Means with a given letter designation are significantly different ($p < 0.05$) from other means with different letters as determined by ANOVA analysis of variance followed by multiple comparisons using the Student-Newman-Keuls' test (**A**).

Induction of cardiomyocyte enlargement by doxorubicin and daunorubicin

The anthracycline quinones represented by doxorubicin and daunorubicin are frequently used drugs for several types of cancer. The use of these drugs is limited because of their cardiac toxicity (Singal and Iliskovic 1998; Singal et al. 2000). Although the acute toxicity, including various arrhythmias, is clinically manageable, chronic heart failure develops weeks to years after the drug administration and is a major concern. Several investigations support the conclusion that oxidative stress mediates the cardiac toxicity induced by anthracyclines (Kang et al. 1996; Singal and Iliskovic 1998; Singal et al. 2000). The chemical structures of doxorubicin and daunorubicin indicate their ability to undergo redox cycling. Doxorubicin accepts electrons from oxoreductive enzymes to form semiquinone free radicals, which may initiate a chain of redox reactions. Doxorubicin has been shown to produce superoxide and H_2O_2 with the mitochondrial fraction of heart extracts (Doroshow and Davies 1986). Daunorubicin, a structural analogue of doxorubicin, produces less reactive oxygen species (Doroshow and Davies 1986). We tested whether doxorubicin or daunorubicin induced cardiomyocyte hypertrophy by treating primary cultured neonatal rat cardiomyocytes for 6 h with the drugs at 1, 10 or 100 nM. Following the treatment, the cells were allowed to recover for 5 days in the medium containing 0.5% FBS before measurements of cell volume and protein content per cell. The results showed that doxorubicin and daunorubicin at 10 or 100 nM can both induce cell enlargement as measured by cell volume and the protein content per cell (Fig. 2.7A&B). A greater degree of cell volume increase or protein

content increase was observed with doxorubicin in comparison to daunorubicin (Fig. 2.7A&B).

To test the hypothesis that oxidative stress contributes to cell enlargement, we determined the effect of NAC and BSO on induction of cell enlargement by doxorubicin using H_2O_2 as a comparison. H_2O_2 can be detoxified by glutathione peroxidase at the expense of oxidizing glutathione to glutathione disulfide. If induction of cell enlargement by doxorubicin is mediated by formation of H_2O_2, changing the glutathione reservoir will change the dose response of doxorubicin. NAC is a thiol antioxidant and a precursor for cellular cysteine, which is a substrate for glutathione synthesis. In contrast, BSO is an inhibitor of glutathione synthesis. Primary cultured neonatal cardiomyocytes were pretreated with NAC or BSO for 48 h before 6 h treatment with 1 to 100 nM doxorubicin or 90 min treatment with 50 to 200 μM H_2O_2. NAC was added back to the cells after the treatment. Cell volume and protein content were measured 5 days after doxorubicin or H_2O_2 treatment. The results show that while NAC prevented doxorubicin or H_2O_2 from inducing cell enlargement, BSO potentiated the effect of doxorubicin or H_2O_2 (Fig. 2.8A&B). These results suggest that oxidative stress may contribute to cell enlargement induced by doxorubicin.

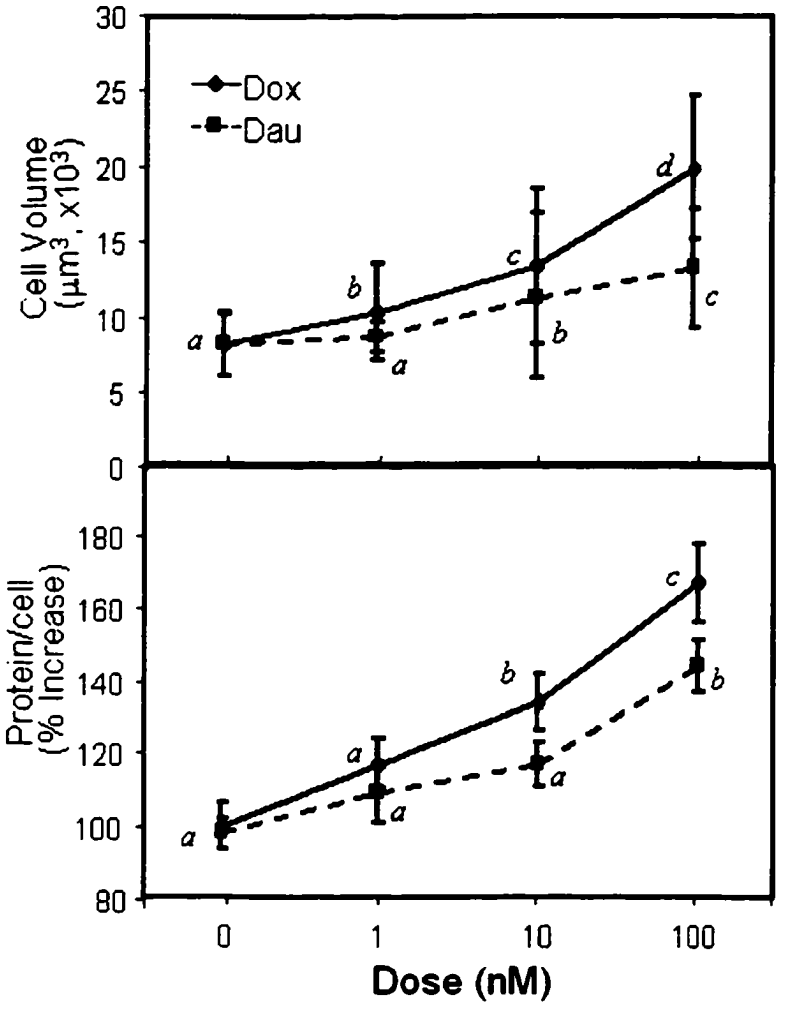
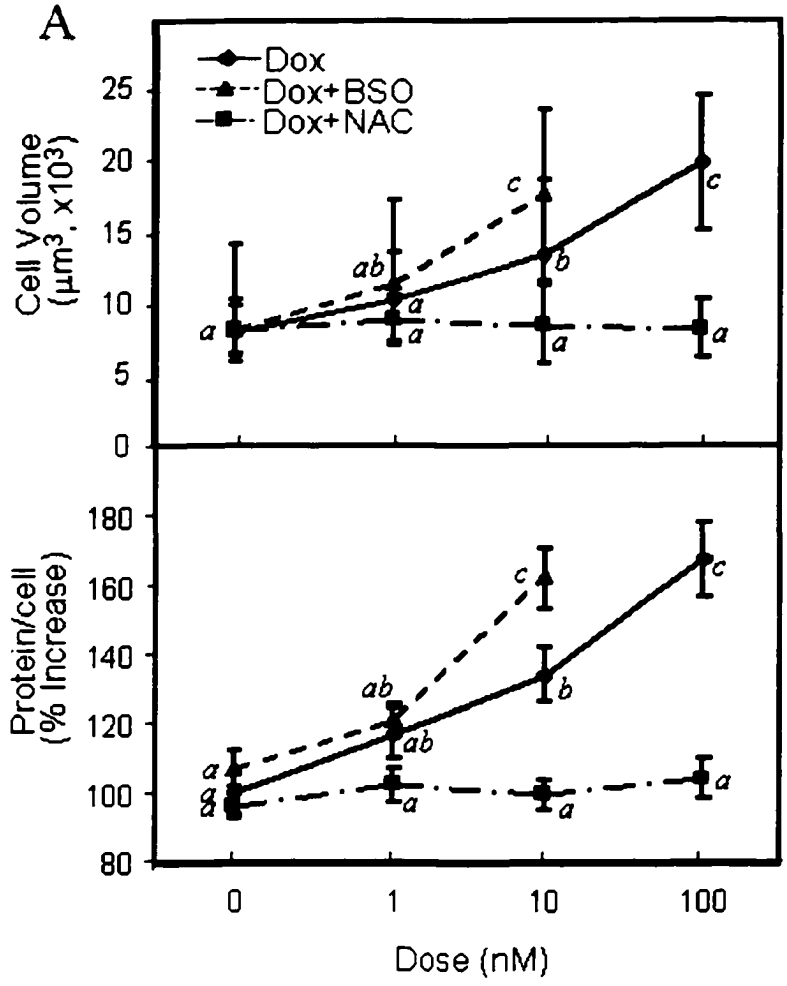


Figure 2.7 Induction of Cell Enlargement by Doxorubicin (DOX) or Daunorubecin (DAU)

Cardiomyocytes in DMEM containing 0.5% FBS were treated with 1, 10 or 100 nM Dox or Dau for 6 h. The cells were allowed to recover for 5 days before measurement of cell volume (*upper panel*) or protein content per cell (*low panel*). At least 100 cells were measured for diameter randomly and three groups of samples were measured for protein content per cell. The data are means ± standard deviations of 100 cells (*upper panel*) or triplicates (*low panel*) from one experiment representative of two. Means with a given letter designation are significantly different ($p < 0.05$) from other means with different letters as determined by ANOVA analysis of variance followed by multiple comparisons using the Student-Newman-Keuls' test.


A
Dox
Dox+BSO
Dox+NAC
Cell Volume (μm³, x10³)
25
20
15
10
5
0
Protein/cell (% Increase)
180
160
140
120
100
80
0
1
10
100
Dose (nM)

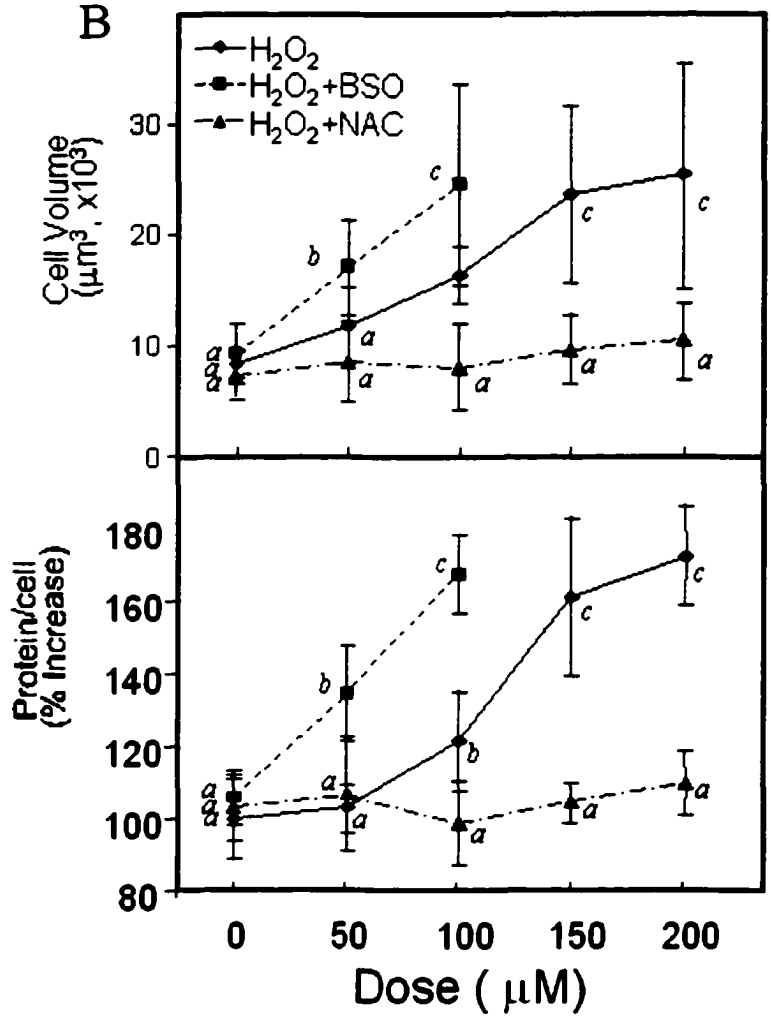


Figure 2.8 Effects of BSO and NAC on Cell Enlargement Induced by Doxorubicin (DOX) or H_2O_2.

Cardiomyocytes in DMEM containing 0.5% FBS were pretreated with 0.1 mM BSO or 5 mM NAC for 48 h before treatment with 1, 10 or 100 nM Dox for 6 h (**A**) or 50, 100, 150 or 200 μM H_2O_2 for 90 mins (**B**). The cells were allowed to recover for 5 days and NAC (2.5 mM) was added back to the cells. The data are means ± standard deviations of cell volumes measured randomly from 100 cells (*upper panels*) or of triplicate measurements of protein content per cell from one experiment representative of two (*low panels*). Means with a given letter designation are significantly different ($p < 0.05$) from other means with different letters as determined by ANOVA analysis of variance followed by multiple comparisons using the Student-Newman-Keuls' test.

Discussion

Our study indicates that H_2O_2 activates p70S6K1 and PI3K in cardiomyocytes. PI3K was activated within 5 min of 200 μM H_2O_2 treatment while p70S6K1 was not activated until 30 min of H_2O_2 exposure. These changes preceded cell enlargement. Inhibiting PI3K with wortmannin blocked p70S6K1 activation. Wortmannin and rapamycin prevented H_2O_2 from inducing cell enlargement. These data suggest that PI3K dependent activation of p70S6K1 plays an important role in cell size increases induced by oxidants.

Many of our experiments employ H_2O_2 at the dose of 200 μM. This dose may appear to be higher than the steady state concentration of H_2O_2 observed under pathophysiological conditions. With ischemia, cumulative doxorubicin treatment, or chronic heart failure, it seems that the myocardium is exposed to oxidants at low doses chronically. Evidence supports the fact that the myocardium can accumulate oxidative damage with repeated exposure to low dose oxidants. For example, cardiac toxicity of doxorubicin is usually determined by cumulative doses above 550 mg/m^2 body surface area regardless of whether the drug is administrated at a high dose once (slowly) or at low doses repetitively. Based on this phenomenon, one may postulate that chronic exposure to oxidants above certain cumulative doses may ultimately result in damage similar to that induced by 200 μM H_2O_2. However, this postulation requires experimental testing. In human fibroblasts, two treatments with 75 μM H_2O_2 result in prolonged cellular and

molecular changes similar to that induced by one treatment with 150 μM (Chen et al. 2001). Although one treatment with 200 μM H_2O_2 is convenient from the experimental prospective, it will be interesting to test whether repetitive exposures to H_2O_2 at the dose similar to that observed under pathophysiological conditions, for example 6 or 11 μM, cause enlargement of cardiomyocytes. Most importantly, whether PI3K and p70S6K1 play a role in low dose oxidant effects in cardiomyocytes remains to be determined.

An important caveat of the present study is the experimental system using neonatal rat cardiomyocytes. Cardiomyocytes *ex vivo* enable investigation of the signal transduction pathways and molecular changes induced by oxidants. However, chronic heart failure or cardiac hypertrophy is a disease that most likely develops in the later stage of adulthood. Ideally, cardiomyocytes from adult animals should be used to study the mechanism of oxidative stress and hypertrophy. The differences between neonatal rat cardiomyocytes and adult rat cardiomyocytes include the rate of DNA synthesis and cell differentiation status. Early reports reveal that about 13% of neonatal cardiomyocytes undergo DNA synthesis, but the figure is much lower in adult hearts (0.2 – 2%) (Anversa and Kajstura 1998). Adult cardiomyocytes are fully differentiated and form myofibers (Mitcheson et al. 1998). Technically it has been difficult to isolate a large quantity of cardiomyocytes from adult animals and to maintain their viability *ex vivo* over a long time course in order to study cellular and molecular changes. In contrast, isolation of neonatal rat cardiomyocytes is relatively easy and the cells can be maintained in culture for a long period of time. The large quantity of homogeneous neonatal rat

cardiomyocytes allows us to define cellular and molecular changes induced by oxidants. Because of the difference between neonatal cardiomyocytes and adult cardiomyocytes, caution should be exercised when extrapolating the results from neonatal cardiomyocytes to cases of adult cardiomyocyte hypertrophy.

Activation of p70S6K1 is a well-known mitogenic event in tumor cells (Chou and Blenis 1995). Oxidants have been shown to induce mitogenic responses and activate p70S6K1 in certain tumor cell lines (Bae et al. 1999). Differing from tumor cells, cardiomyocytes appear to grow in size instead of cell number in response to oxidant stimulation (Chen et al. 2000b). Our data are consistent with that of others, showing that activation of p70S6K1 is involved in cardiomyocyte hypertrophy. Here we have found that activation of p70S6K1 by H_2O_2 involves PI3K. PI3K dependent activation of p70S6K1 has been reported with growth factors and endocrine factors (Chou and Blenis 1995; Proud 1996; Pullen and Thomas 1997; Dufner and Thomas 1999). With these factors, phosphorylation of Thr421/Ser424 appears to be necessary but not sufficient for p70S6K1 activation. In contrast, phosphorylation of Thr389 appears to correlate with increases in p70S6K1 activity. In our experimental system, wortmannin and rapamycin can completely block Thr389 phosphorylation and p70S6KI activity. These data suggest a similarity between growth factors and oxidants in regulating p70S6K1 activity by Thr389 phosphorylation.

A few recent studies report an important role of p70S6K1 in cell size regulation. In the fruit fly and mouse, knocking-out the p70S6K1 gene results in a delay in the development and a smaller body size due to a reduction in cell sizes (Shima et al. 1998; Montagne et al. 1999). In the heart, p70S6K1 can be activated by a variety of hypertrophy inducers such as angiotensin II, α- or β-adrenergic receptor agonists, mechanical stretch *in vitro* and pressure overload *in vivo* (Sadoshima and Izumo 1995; Boluyt et al. 1997; Laser et al. 1998; Simm et al. 1998). Rapamycin is capable of inhibiting the development of cardiomyocyte hypertrophy in these experimental models. Since p70S6K1 is thought to regulate protein translation, aberrant activation of protein translation may be a key factor for cell size increases.

One of the important findings from this study is the activation of PI3K by H_2O_2 in cardiomyocytes. Although this phenomenon is novel in cardiomyocytes, oxidants have been shown to activate PI3K in other cell types. PI3K activation is usually a mitogenic response triggered by growth factor receptors or G proteins (Fruman et al. 1998). The p85 subunit of PI3K is an adapter protein that can be phosphorylated by receptor tyrosine kinases and Src family tyrosine kinases. It has been shown that oxidants activate Src family kinases in cardiomyocytes (Aikawa et al. 1997). This may explain the observed tyrosine phosphorylation of p85 in H_2O_2 treated cardiomyocytes. It is thought that phosphorylation of the p85 subunit contributes to the relocation of p85/p110 complex from the cytosol to the plasma membrane where PI3K can contact its activator ras and its substrate inositol-containing lipids (Fruman et al. 1998). Deora *et al.* (Deora et al. 1998)

demonstrated that changes in redox status led to recruitment of the p85 subunit to the plasma membrane where it associated with ras protein and the p110 catalytic subunit of PI3K. It has been reported that oxidants activate the small G-protein ras (Hardwick and Sefton 1997). Taken together, it seems that src and ras are two potential upstream factors regulating PI3K activation by oxidants. While activated src can phosphorylate p85 and cause p85 translocation to the plasma membrane, activated G-proteins contribute to changes in PI3K activities.

The signal transduction pathways of cardiomyocyte hypertrophy have attracted a great deal of attention because of the potential for developing novel pharmacological interventions against heart failure by targeting early signaling events of cardiomyocyte hypertrophy. The discovery of PI3K activation by H_2O_2 in cardiomyocytes is interesting and deserves further investigation. Whether PI3K plays a role in cardiomyocyte hypertrophy under pathophysiological conditions *in vivo* is not established. A recent study using transgenic approaches shows that PI3K activity determines the size of cardiomyocytes and the size of the heart (Shioi et al. 2000). Transgenic mice with constitutively activated PI3K develop bigger hearts and bigger cardiomyocytes compared to normal littermates. In contrast, inactivating PI3K with dominant negative mutant genes results in smaller hearts and smaller cardiomyocytes (Shioi et al. 2000). These data suggest the possibility that PI3K plays an important role in cardiomyocyte hypertrophy *in vivo*.

Chronic heart failure is often a clinically intractable disorder and lacks effective therapeutic treatment. An important morphological feature of chronic heart failure is cardiomyocyte hypertrophy. Identifying the inducers of cardiomyocyte hypertrophy and the intracellular signaling cascade of the inducers are necessary for mechanism-based drug design. This study and others suggest that oxidative stress plays a role in cardiomyocyte hypertrophy *in vitro*. Oxidative stress has been viewed as an important factor contributing to chronic heart failure, which often involves multiple pathophysiological changes. Hypertension and increased activity of the renin-angiotensin system can trigger cardiac hypertrophy and ultimately heart failure. Increased levels of angiotensin II are known to cause cardiac hypertrophy *in vitro* and *in vivo*. In vascular smooth muscle cells, oxidants appear to mediate angiotensin II-induced signaling changes of hypertrophy (Griendling et al. 2000). Angiotensin II activates a plasma membrane associated NAD(P)H oxidase (Griendling and Ushio-Fukai 2000). It is known that angiotensin II may also produce oxidants that mediate cardiomyocyte hypertrophy (Shih et al. 2001). Although further experiments will determine whether oxidative stress is involved in cardiac hypertrophy and chronic heart failure induced by ischemia, ischemia-reperfusion or pressure overload *in vivo*, several pieces of experimental evidence have already demonstrated that doxorubicin induces cardiomyocyte hypertrophy *in vivo* and oxidative stress mediates cardiac hypertrophy induced by doxorubicin (Kang et al. 1996; Sun et al. 2001). These findings suggest that antioxidants may serve as useful therapeutic agents against heart failure induced by doxorubicin and perhaps other types of cardiovascular disease. The fact that oxidants activate PI3K and p70S6K1 may provide

alternatives in developing pharmacological agents against cardiomyocyte hypertrophy and heart failure associated with oxidative stress.

Chapter 3 DISTINCT ROLES OF p42/p44ERK AND p38 MAPK IN OXIDANT INDUCED AP-1 ACTIVATION AND CARDIOMYOCYTE HYPERTROPHY

Introduction

Each year, about one million people in the U.S. have heart attacks. The attacks are fatal in one third of the cases. The hearts of survivors undergo remodeling and are at a high risk of secondary infarction and failure. Cardiac hypertrophy is often observed in association with heart remodeling (Francis et al. 1995; Muscari et al. 1996). An increase in the size of cardiomyocytes is the major characteristic of cardiac hypertrophy. Although cardiac hypertrophy can be beneficial initially to compensate for the demand on cardiac output, it is frequently a transition to heart failure. Pathological analyses of failing hearts often reveal enlarged cardiomyocytes (Colucci and Braunwald 1997). While the underlying cause of cardiomyocyte enlargement is under intensive investigation, a few clinical trials have shown promising evidence that antioxidant vitamins reduce the rate of subsequent myocardial infarction in patients with established ischemic heart disease (Stephens et al. 1996; Marchioli). In agreement with these findings, a number of studies indicate that reactive oxygen species are an important mediator of heart failure (Dhalla et al. 1996; Hill and Singal 1996; Keith et al. 1998).

AngII, ET-1 and catecholamines are molecules known to induce cardiac hypertrophy. These stimuli initiate a common cascade of molecular changes after binding to their receptors, the G protein-coupled transmembrane proteins (Homcy 1998; Force et al. 1999; Hunter and Chien 1999). Through activating G proteins, these endocrine factors activate the MAPKs (Homcy 1998). Several members of the MAPK family including p42/p44^{ERK} and p38 have been shown to play a critical role in hypertrophy, particularly in changes of gene expression associated with cell enlargement (Gillespie-Brown et al. 1995; Zechner et al. 1997; Clerk et al. 1998; Nemoto et al. 1998) (Chen and Tu 2002). Overexpressing the transgenes of the upstream activators of p42/p44^{ERK} or p38 can induce cell enlargement and expression of hypertrophy marker genes (Gillespie-Brown et al. 1995; Zechner et al. 1997; Wang et al. 1998a). Using pharmacological approaches, Clerk, et al. found that p42/p44^{ERK} plays a role in ET-1 and α-agonist phenylephrine (PE) induced increase of sarcomeric content in cultured cardiomyocytes (Clerk et al. 1998). The p38 MAPK has been shown to be the key kinase in PE induced cardiomyocyte hypertrophy by inducing marker gene expression and cell size increase (Zechner et al. 1997). These reports clearly demonstrate the importance of p42/p44^{ERK} and p38 in cardiac hypertrophy.

One of the downstream events of MAPKs is the activation of the AP-1 transcription factor. Both p42/p44^{ERK} and p38 have been reported to regulate AP-1 activation (Wisdom 1999). At least two hypertrophy marker genes, atrial natriuretic factor (ANF) and skeletal alpha-actin (SkA), contain AP-1 binding sites within their

promoter regions (Bishopric et al. 1992; Rosenzweig et al. 1991). Experimentally, AP-1 activation has been shown to play an important role in expression of these hypertrophy marker genes induced by mechanical stretch and endocrine factors (Sadoshima et al. 1993; Takemoto et al. 1999; Herzig et al. 1997). It is thought that AP-1 serves as a mediator between MAPK activation and a cascade of transcriptional events occurred with hypertrophy.

Cardiac hypertrophy involves multiple changes at the molecular level. In parallel with changes in gene expression is an increase in protein synthesis and enlargement of cell size. Besides MAPKs, activation of phosphatidylinositol 3-kinase (PI3K) and p70 S6 kinase (p70S6K1) are other important events in cardiomyocyte hypertrophy. As a lipid kinase, PI3K adds a phosphate group to the free 3-position of the inositol ring of phosphatidylinositol (PtdIns) and phosphoinositides. The products of PI3K participate in multiple signaling pathways. One of the downstream events of PI3K is p70S6K1 activation. As a kinase that phosphorylates the S6 subunit of the 40S ribosome, p70S6K1 accelerates the translation machinery and has been shown to increase cell size (Dufner and Thomas 1999).

We found that the majority (60%) of cardiomyocytes in culture undergo cell enlargement in response to mild doses of oxidants (Tu et al. 2002; Chen et al. 2000b). Recent findings in our lab have indicated that H_2O_2 is able to activate PI3K and p70 S6 kinase, and these two signaling molecules are important for oxidant-induced cell size

enlargement (Tu et al. 2002). Given the fact that H_2O_2 has been reported to activate MAPKs in a number of cell lines and MAPKs play a role in cardiac hypertrophy (Guyton et al. 1996; Deora et al. 1998; Bae et al. 1999), we investigate here the role of p42/p44^{ERK} and p38 in oxidant-induced hypertrophy and their relationship to PI3K and p70S6K1 activation. We first tested whether H_2O_2 activated p42/p44^{ERK} and p38 using primary cultures of rat cardiomyocytes and then studied the role of these MAPKs in H_2O_2-induced AP-1 activation, p70S6K1 activation and cell enlargement.

Materials and Methods

Chemicals and reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Stabilized H_2O_2 (Sigma, H-1009) was used and the concentration of the stock was verified by absorbency at 240 nm. PD98059, SB202190, wortmannin (WTN) and rapamycin (RPM) were obtained from Calbiochem (La Jolla, CA).

Cell culture and H_2O_2 treatment

Cardiomyocytes were prepared from 1 to 2-day old neonatal Sprague-Dawley rats (Harland, Indianapolis, IN) as previously described (Chen et al. 2000b; Tu et al. 2002). The myocytes were seeded at a density of 5×10^4 cells/cm^2 and plated in DMEM with 1 mM pyruvate, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin. Using this protocol, over 90% of the cells are myocytes as judged by

sarcomeric myosin content determined by immunocytochemical staining. One to two days after plating, the cells were placed in fresh DMEM containing 0.5% FBS for 48 hr before H_2O_2 treatment in the 0.5% FBS culture medium.

Assay for p38, p42/p44^{ERK} and p70S6K1 phosphorylation (activation)

After H_2O_2 exposure, cardiomyocytes were harvested in a lysis buffer [1% Triton X-100, 10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 50 mM NaCl, 50 mM NaF, 10 μg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 2 mM Na_3VO_3]. Protein concentration was measured by the Bradford method according to the manufacturer's instruction (Bio-Rad, Richmond, CA). An equal amount of proteins was loaded in each lane and separated by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for Western blot as previously described (Tu et al. 2002). After transferring the proteins to PVDF membrane, the membrane was incubated for 2 hr with an antibody that recognizes phosphorylated Thr202/Tyr204 of p42/p44^{ERK}, phosphorylated Thr180/Tyr182 of p38, or phophorylated Thr389 and Thr421/Ser424 of p70S6K1 at 1:1000 dilution (New England Biolabs, Beverly, MA). For determining the basal level of these proteins, a duplicated membrane from the same experiment was incubated with antibodies that recognize both phosphorylated and unphosphorylated forms of p42/p44^{ERK}, p38 or p70S6K1 (1:1000 dilution; New England Biolabs, Beverly, MA). Bound antibodies were detected by enhanced chemiluminescence (ECL) reaction following incubation with a secondary antibody conjugated with the horse radish peroxidase and were quantified by an Eagle Eye II Image System with installed density analysis software (Strategene, La Jolla, CA).

p38 Kinase Activity Assay - Cells were harvested in 0.5 ml kinase lysis buffer (10 mM potassium phosphates, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 0.5% Triton X-100, 1 mM Na_3VO_3, 2 mM DTT, 1 mM PMSF and 10 μg/ml aprotinin). The lysates were centrifuged for 5 min at 13,000 rpm. The supernatants were collected for protein concentration measurements using the Bradford method (Bio-Rad, Richmond, CA). An equal amount (500 μg) of proteins from different samples was used for immunoprecipitation by a 2-h incubation on ice with 5 μl anti-p38 antibody (New England Biolabs, Beverly, MA) and a subsequent 1 hr incubation in the presence of 30 μl Protein A-Sepharose. The protein A-Sepharose immunocomplexes were washed twice with kinase lysis buffer and twice with kinase reaction buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM Na_3VO_3 and 1 mM DTT). The immuocomplex was used for an *in vitro* kinase reaction using GST-ATF-2 fusion protein as a substrate. After a 20 min kinase reaction at 30°C, the Sepharose was separated from proteins by boiling for 5 min. The supernatants were loaded onto an 8% SDS-PAGE as described (Tu et al. 2002). The level of ATF-2 phosphorylation induced by p38 immunocomplex was shown by autoradiography and the incorporated radioactivity was quantified by a Packard Instant Imager (Packard Instruments, Meriden, CT).

p70S6K1 assay

Cardiomyocytes were harvested and subjected to kinase assay as described previously (Tu et al. 2002). Briefly, cells were lysed on ice in 500 μl lysis buffer. Immunoprecipitation from 500 μg cell lysate proteins were carried by 2 hr incubation on ice with 5 μl p70S6K1 antibody (Santa Cruz Biotechnology, CA) and subsequent 1-hr incubation in the presence of Protein A-Sepharose (30 μl per reaction tube). After washing with lysis buffer and subsequently reaction buffer, the immunocomplexes were resuspended in 30 μl kinase reaction buffer containing 20 μM S6 kinase substrate peptide (RRRLSSLRA, Santa Cruz Biotechnology, CA). The kinase reaction was initiated by addition of a mixture of 10 μCi [γ- ^{32}P]ATP (final 40 μM) plus $MgCl_2$ (final 10 mM). After a 10-min incubation at 30°C, 20 μl of supernatants were spotted onto P81 phosphocellulose discs (Whatman, Clifton, NJ). The discs were then washed and the radioactivity of phosphorylated products on the discs were measured by a liquid scintillation counter (Tu et al. 2002).

Electrophoretic mobility shift assay (EMSA)

Cardiomyocytes were harvested and nuclear extracts were prepared as described by Peng, et al. (Peng et al. 1995). Briefly, cardiomyocytes were rinsed with ice cold PBS and lysed on ice with lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 0.15% NP-40, 10 mM HEPES, pH 7.9, 0.5 mM DTT, 0.5 mM PMSF) for 10 min with gentle shaking. After microscopic verification for the completion of cell lysis, cells were scraped off the culture

plates. The nuclei were collected by centrifugation at 2000xg for 10 min and suspended in an ice-cold buffer [1.5 mM $MgCl_2$, 0.2 mM EDTA, 26% Glycerol (v/v), 5 mM HEPES, pH 7.9, 0.5 mM DTT, 0.5 mM PMSF]. NaCl was then added to a final concentration of 0.3 M. After incubation on ice for 30 min, samples were centrifuged at 20,000×g for 10 min and supernatants were collected for subsequent DNA binding assay. Binding reactions were performed for 20 min on ice in a reaction volume of 20 μl containing 5 μg of nuclear protein, 4 % Glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 50 mM NaCl, 10 mM Tris.HCl, pH 7.5, 0.05 mg/ml poly(dI-dC) and about 30,000 cpm of ^{32}P-labeled oligonucleotide probes (CGCTTGATGAGTCAGCCGGAA). DNA-protein complexes were separated on nondenaturing 5% polyacrylamide gels. Gels were vacuum dried and exposed to X-ray film for autoradiography. The radioactive oligonucleotide bound to AP-1 protein was quantified by a Packard Instant Imager (Packard Instruments, Meriden, CT).

AP-1 luciferase assay

Cardiomyocytes were seeded at 0.2 x 10^6 per well in 12-well plates (4 cm^2 culture area per well). The next day, cells were transfected with 0.5 μg AP-1 luciferase plasmid pAP-1-luc (Strantagene, La Jolla, CA) and 0.025 μg pRL-TK plasmids per well using FuGene-6 liposomes (Roche, Mannheim, Germany) for 5 hr and then placed in 0.5% FBS/DMEM for 36 to 48 hr. pRL-TK plasmid has a Renilla luciferase gene under the control of the thymidine kinase (TK) promoter, allowing us to correct for transfection

efficiency using dual luciferase assay. Cells were pretreated with inhibitors for 30 min and then treated with 200 μM H_2O_2 for 1 hr. After H_2O_2 treatment, oxidized medium was changed to fresh 0.5% FBS/DMEM and the cells were harvested at various time points. Firefly luciferase encoded by pAP-1-luc plasmid and Renilla luciferase encoded by pRL-TK were measured by a luminometer (Tuner Designs, Sunnyvale, CA) with a duel luciferase assay kit purchased from Promega (Madison, WI). Results were expressed as the ratio of the relative light unit (RLU) from the two luciferase readings.

Measurements of cell size increase

Cell volume and protein content per cell were determined as previously described (Tu et al. 2002). For measurement of cell volume, the adherent cells were detached at 4-5 days after H_2O_2 treatment by trypsin treatment and rounded cells were loaded onto a microslide field finder (Fisher Scientific, Pittsburgh, PA) for measurement of cell diameters, which were then used to calculate cell volume using the equation of $4/3 \times \pi \times Radius^3$. Protein concentrations were measured by bicinchoninic acid (BCA) method according to the manufacturer's instruction (Pierce, Rockford, IL). Protein content per cell was determined by dividing the total amount of protein by the cell number, which was determined by a Coulter Counter or hemocytometer after trypsin treatment.

Statistics

One-way analysis of variance was used to compare groups of means followed by the Student-Newman-Keuls' method for multiple comparisons. Groups of means that are not different are indicated in the figure by a common letter symbol. Any mean with a letter designation different from others is significantly different from the others.

Results

H_2O_2 activates p42/p44^{ERK} and p38 in cardiomyocytes

Phosphorylation of Thr202/Tyr204 residues in p42/p44^{ERK} or Thr180/Tyr182 residues in p38 is a signature indicating activation of these kinases. Measurements of phosphorylation of these residues are now widely used for determining endogenous activation of these kinases. Using antibodies against phosphorylated Thr202/Tyr204 of p42/p44^{ERK}, we measured the level of phosphorylation by Western blot. Cardiomyocytes were treated with H_2O_2 at 200 μM, a dose that induces maximal hypertrophy response without killing the majority of the cells (Chen et al. 2000b). We found that phosphorylation of p44^{ERK} and p42^{ERK} was first detected within 10 min of 200 μM H_2O_2 exposure and reached a plateau at 60 min (Fig. 3.1A). Both p44^{ERK} and p42^{ERK} remained highly phosphorylated at 60 to 120 min (Fig. 3.1A). Western blot using an antibody that recognizes both unphosphorylated and phosphorylated forms of p42/p44^{ERK} indicated no changes in the basal level of these proteins by H_2O_2 treatment (Fig. 3.1A). To determine whether activation of p42/p44^{ERK} is sustained, we tested the phosphorylation level of these kinases 24 hr after H_2O_2 treatment. The results showed no significant difference between control and H_2O_2 treated cells (Fig. 3.1A). Therefore, although phosphorylation (activation) of p42/p44^{ERK} was detected from 10 to 120 min of H_2O_2 treatment, the level of phosphorylation returned to base line within 24 hr after H_2O_2 treatment.

An inhibitor of p42/44^{ERK} is useful for testing the functional significance of p42/p44^{ERK} activation. However, the inhibitory effect needs to be confirmed. PD95089

has been widely used as a specific inhibitor for p42/p44^{ERK}. Since PD95089 inhibits the kinase MEK1/2 upstream of p42/p44^{ERK}, the inhibitory effect of PD95089 in cardiomyocytes can be verified by p42/p44^{ERK} phosphorylation assay. The results show that PD95089 at 50 μM prevented H_2O_2 from inducing p42/p44^{ERK} phosphorylation (Fig. 3.1B).

Activation of p38 was also determined by Western blot for increased phosphorylation. Phosphorylation of p38 was elevated within 10 min of 200 μM H_2O_2 exposure and reached the highest level at 60 min but started to decline at 90 min of treatment (Fig. 3.2A). At 24 hr after H_2O_2 treatment, the phosphorylation was no longer detectable (Fig. 3.2A). The basal level of p38 protein did not change with H_2O_2 treatment (Fig. 3.2A). Therefore, like p42/p44^{ERK}, p38 is rapidly phosphorylated (activated) by H_2O_2 treatment. However the window of p38 phosphorylation (activation) is narrower than that of p42/p44^{ERK}.

The inhibitory effect of SB202190 was verified by measurement of p38 kinase activity *in vitro*. SB202190 is a new generation of p38 inhibitor that has a higher efficacy than the widely used compound SB203580, which inhibits p38 kinase activity by occupying its ATP binding site (Gum et al. 1998; Lisnock et al. 1998; Salituro et al. 1999). Since p38 activation leads to phosphorylation of the transcription factor ATF-2 (Force et al. 1996), we verified the inhibitory effect of SB202190 by measurement of ATF-2 phosphorylation *in vitro* using the immunocomplex of p38 isolated from

cardiomyocytes treated with H_2O_2 in the absence or presence of SB202190. The results show that SB202190 at 10 μM prevented H_2O_2 from inducing p38 activation (Fig. 3.2B).

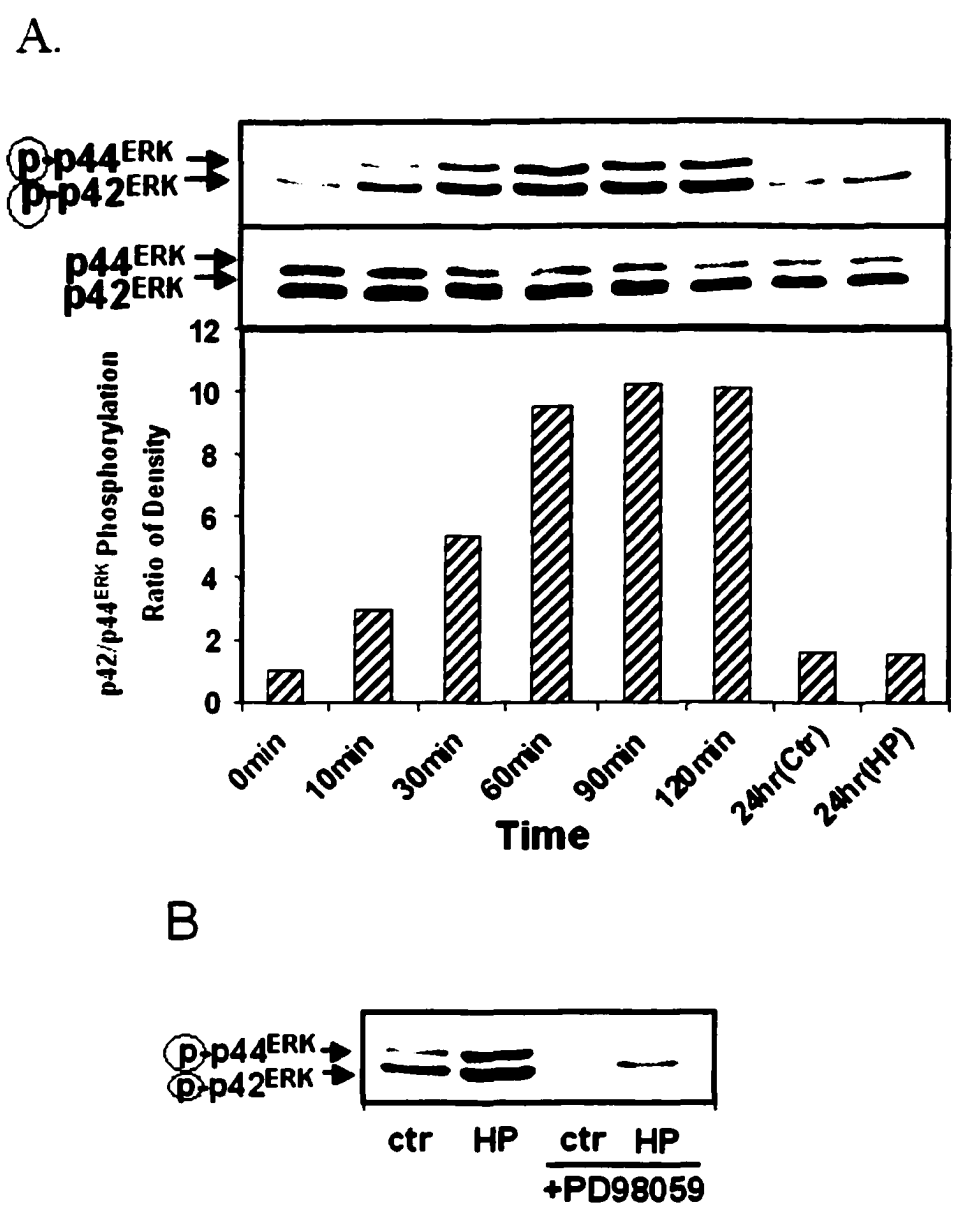
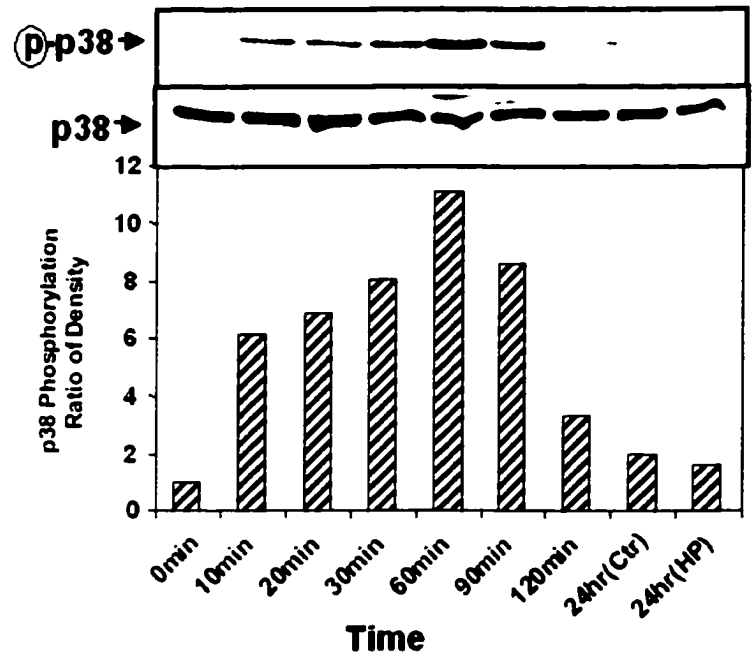


Figure 3.1 H_2O_2 Induces Phosphorylation (Activation) of p42/p44ERK.

Cardiomyocytes were treated with 200 μM H_2O_2 for the indicated time (**A**) or were pretreated 30 min with 50 μM PD98059 before 30 min treatment with 200 μM H_2O_2 in the presence of 50 μM PD98059 (**B**). An equal amount of proteins (20 μg) was loaded to SDS-PAGE for the Western blot to determine the level of Thr202/Tyr204 phosphorylation (**A**, *upper panel* and **B**) or the level of $p42/p44^{ERK}$ protein (**A**, *middle panel*). The densities of phosphorylated or total $p42/p44^{ERK}$ were determined by an Eagle Eye II Image System for quantitative expression of $p42/p44^{ERK}$ phosphorylation using the ratio. The data are from one experiment representative of three.

A.



B.

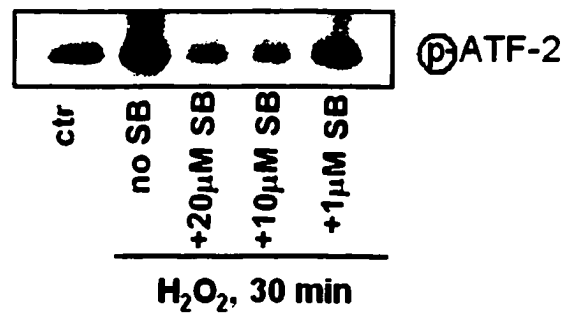
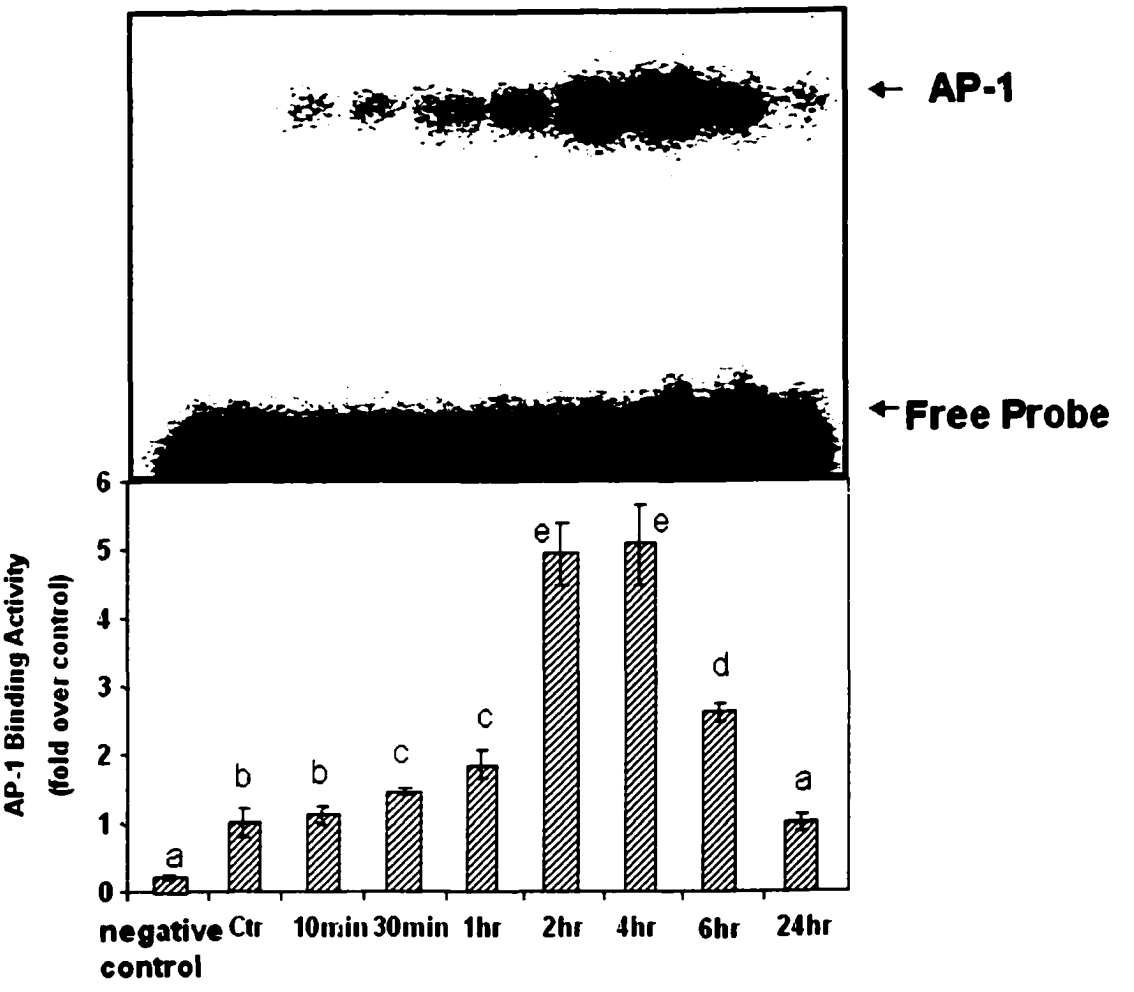


Figure 3.2 H_2O_2 Induces Phosphorylation (Activation) of p38 MAPK.

Cardiomyocytes were treated with 200 μM H_2O_2 for the indicated time (**A**). The cells were harvested for Western blot to determine the phosphorylation of Thr180/Tyr182 residues (**A**, *upper panel*) or the level of p38 protein (**A**, *upper panel*). The densities of phosphorylated and total p38 were determined by an Eagle Eye II Image System for quantitative expression of p38 phosphorylation using the ratio. SB202190 was added to cells 30 min prior to the 30 min treatment with 200 μM H_2O_2 (**B**). Cell lysates were collected for p38 immunoprecipitation and *in vitro* kinase assay using ATF-2 protein as a substrate (**B**). The data are from one experiment representative of three.

A


AP-1
Free Probe
AP-1 Binding Activity
(fold over control)
0
1
2
3
4
5
6
a
b
b
c
c
e
e
d
a
negative control
Ctr
10min
30min
1hr
2hr
4hr
6hr
24hr

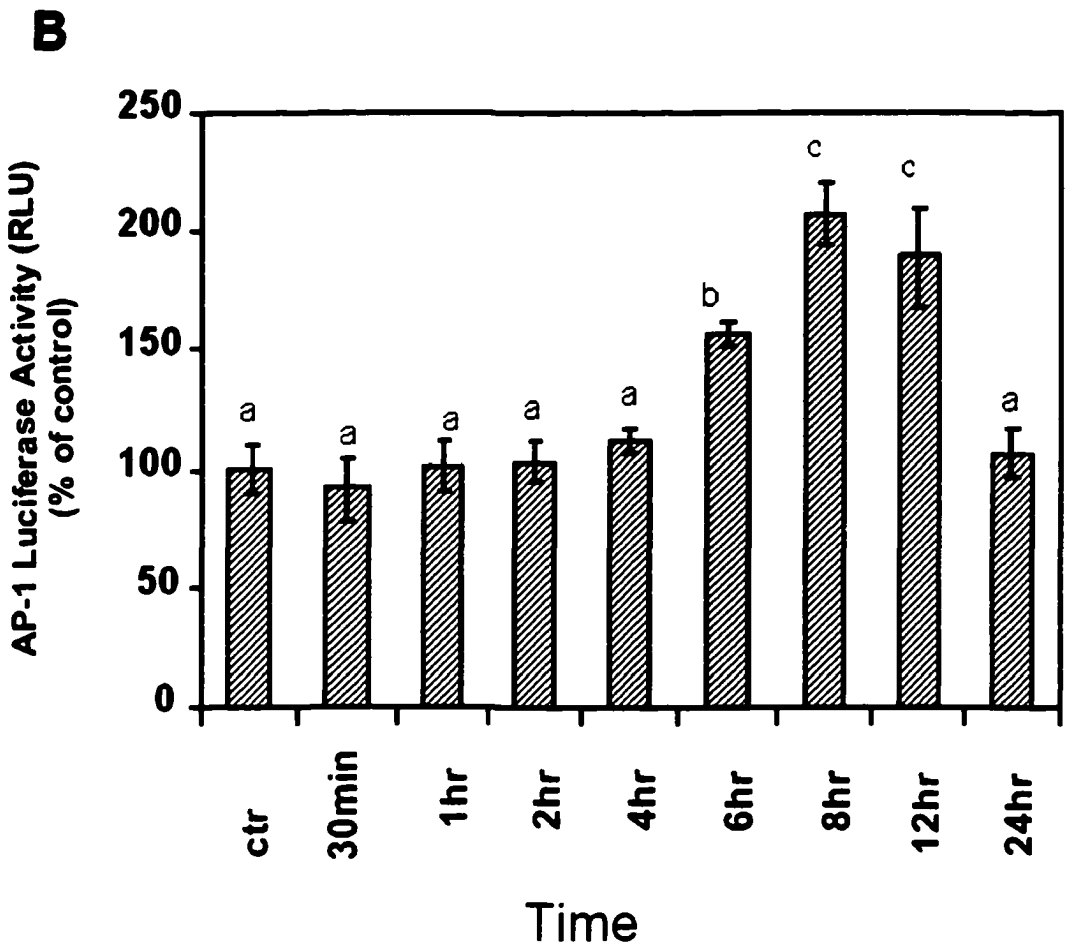


Figure 3.3 H_2O_2 Induces Activation of AP-1 Transcription Factor.

Cardiomyocytes were treated with 200 μM H_2O_2 for 60 min and then placed in fresh DMEM with 0.5%FBS. Samples were collected at indicated time points after the treatment and nuclear proteins were extracted for gel shift assay as described in the Methods (**A**, *upper panel*). Protein bound radioactivity was quantified by a Packard Instant Imager and is expressed as means ± standard deviation from three independent experiments (**A**, *lower panel*). Cardiomyocytes were transfected with pAP-1-lu and pRL-TK-lu plasmids. At 24 to 48 hours after transfection, cells were treated with 200 μM H_2O_2 for 60 min and then placed in fresh medium. Samples were collected at indicated time points for dual luciferase assay (B). The data are means ± standard deviation of the ratios of AP-1 luciferase over the Ranilla luciferase in relative light unit (RLU) (**B**). A letter indicates a significant difference ($p<0.05$) from other means with different letter designations as determined by ANOVA followed by multiple comparisons with Student-Newman-Keuls test.

Role of p42/p44^{ERK} and p38 in H_2O_2 induced AP-1 activation.

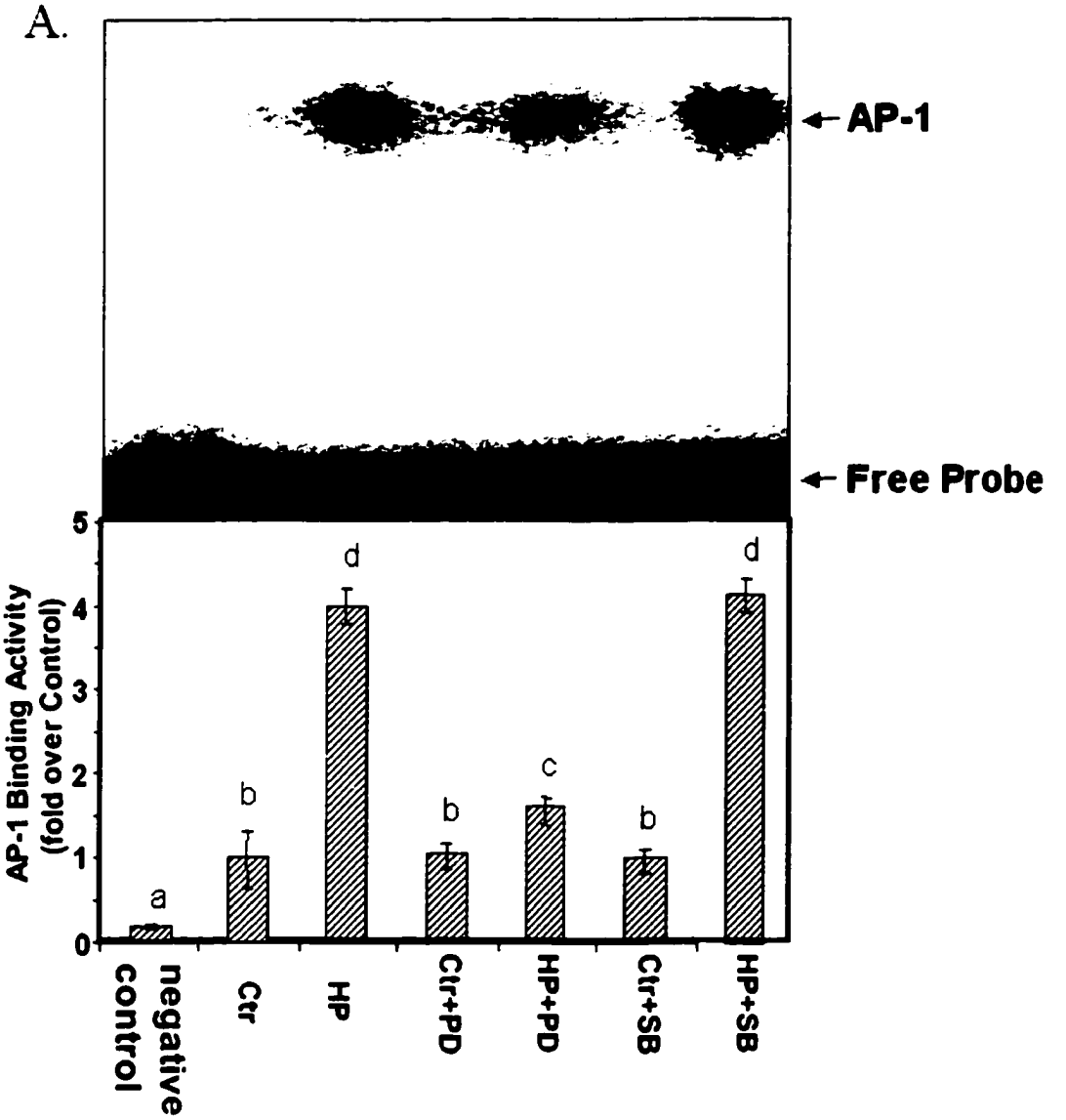
Activation of the AP-1 transcription factor has been indicated in both mechanical and endocrine factor induced hypertrophy and contributes to the change of gene expression associated with hypertrophy in cardiomyocytes (Sadoshima et al. 1993; Herzig et al. 1997; Takemoto et al. 1994). A number of studies have reported that p42/44^{ERK} or p38 regulates AP-1 activation (Wisdom 1999). To determine whether AP-1 is activated by H_2O_2, both DNA binding and AP-1 promoter activity were measured. Gel shift assays (i.e. EMSAs) showed that the binding ability of AP-1 proteins to the consensus DNA sequence was not detectable within 60 min of 200 μM H_2O_2 treatment (Fig. 3.3A). After the 60-min treatment, the DNA binding activity was increased at 30 min, reached a peak at 4 hr and started to decline at 6 hr.

AP-1 activation was verified by measuring the activity of the promoter using a luciferase reporter construct under the control of AP-1 promoter. Cardiomyocytes were transfected with the pAP-1-lucreporter gene construct. After treating the transfected cells with 200 μM H_2O_2 for 60 min, cardiomyocytes were harvested and subjected to luciferase activity assay at various time points. The results show that luciferase activity first increased at 4 hr and reached a peak of 2-fold induction at 8 hr. The activity declined at 12 hr and the induction was no longer detected at 24 hr after H_2O_2 treatment (Fig. 3.3B).

Inhibitors of p42/p44^{ERK} and p38 are tools for testing the contribution of these kinases in H_2O_2 induced hypertrophy. Since many chemicals can function as antioxidants, we first eliminated the possibility that PD95089 and SB202190 serve as antioxidants and prevent oxidative stress under our experimental conditions. Induction of oxidative stress by H_2O_2 results in depletion of glutathione, which is a main component of cellular non-protein sulfhydryls. We measured cellular non-protein sulfhydryl content using Ellman's reagent after pretreating the cells 30 min with the inhibitors and treating the cells with H_2O_2 in the presence of these inhibitors (Sedlak and Lindsay 1968; Chen and Stevens 1991). We found that a 2-hr treatment of H_2O_2 at 200 μM or lower could not cause significant loss of non-protein sulfhydryls in primary cultured cardiomyocytes or in the H9C2 cell line of rat cardiomyocytes. In one representative experiment, H_2O_2 at 500 μM reduced non-protein sulfhydryls by 24.5% at the end of a 2 hr treatment time in H9C2 cells. In the presence of 50 μM PD95089 or 10 μM SB202190, H_2O_2 at 500 μM reduced non-protein sulfhydryls by 32.7 or 29.3%. These data indicate that the inhibitors at the concentrations used were unlikely to prevent oxidative stress *per se* under our experimental conditions.

Whether p42/p44^{ERK} or p38 plays a role in the AP-1 activation induced by H_2O_2 was investigated using their inhibitors. Cardiomyocytes were pretreated with PD95089 or SB20219 for 30 min before a 60 min H_2O_2 treatment. Samples were collected at 4 hr for gel shift assays or at 8hr for AP-1 promoter activity using cells transfected with pAP-1-luc and pRL-TK (see Methods). Pretreatment of 50 μM PD95089 inhibited about 80-

90 percent of DNA binding and AP-1 luciferase activity induced by H_2O_2 (Fig. 3.4A&B). In contrast, SB20019 had no inhibitory effect on AP-1 activation. These data suggest that $p42/44^{ERK}$ but not p38 plays an important role in H_2O_2 induced AP-1 activation in cardiomyocytes.


A.
AP-1
Free Probe
AP-1 Binding Activity
(fold over Control)
0
1
2
3
4
5
a
b
d
b
c
b
d
control
negative
Ctr
HP
Ctr+PD
HP+PD
Ctr+SB
HP+SB

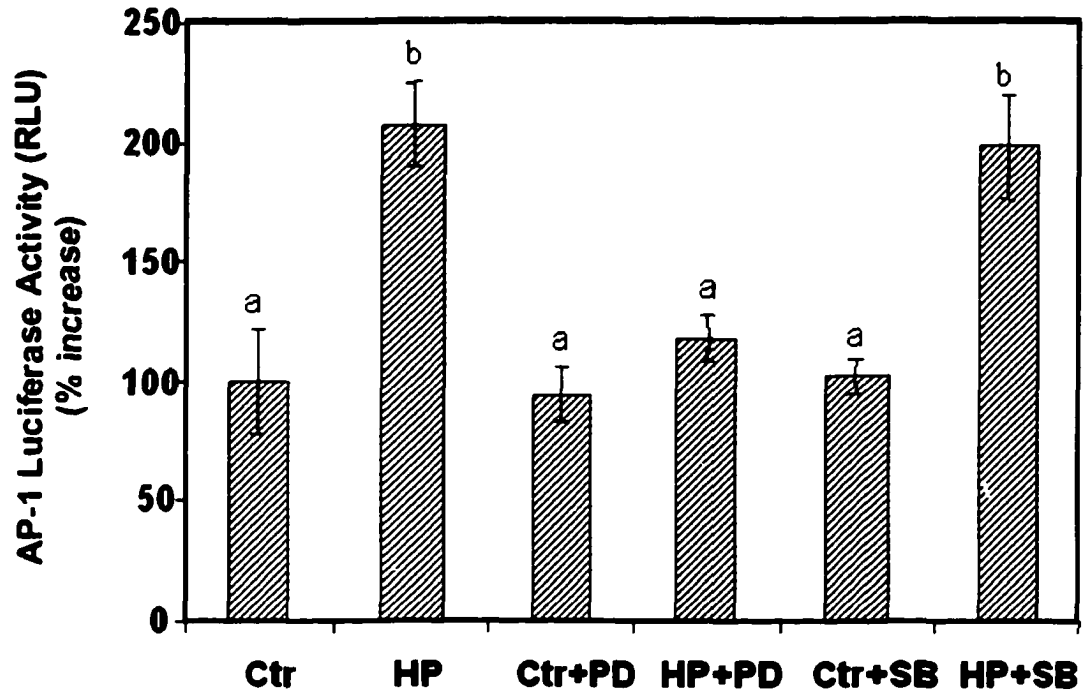


Figure 3.4 Effect of PD98059 (PD) and SB202190 (SB) on H_2O_2 Induced AP-1 Activation.

Cardiomyocytes were pretreated with 50 μM PD or 10 μM SB for 30 min before 60 min treatment with 200 μM H_2O_2. The cells then placed in fresh DMEM with 0.5%FBS. Samples were harvested at 4 hr after H_2O_2 treatment for nuclear extraction and gel shift assay (**A**, *upper panel*). Protein bound radioactivity was quantified by a Packard Instant Imager and is expressed as means ± standard deviation from three independent experiments (**A**, *lower panel*). Cells were transfected with pAP-1-luc and pRL-TK plasmids. At 24 to 48 hr after transfection, cells were preteated with PD or SB and treated with H_2O_2 as described above. Samples were collected at 8 hr after H_2O_2 treatment for duel luciferase assay. The data are presented as means ± standard deviation of the ratio of two luciferases in RLU from these experiments (**B**). A letter indicates a significant difference ($p<0.05$) from other means with different letter designations as determined by ANOVA followed by multiple comparisons with Student-Newman-Keuls test.

Role of p42/p44^{ERK} and p38 in H_2O_2 induced p70S6K1 activation and cell size enlargement

Our previous studies revealed that H_2O_2 induces activation of p70S6K1, which contributes to cell size enlargement (Tu et al. 2002). Activation of p70S6K1 occurs downstream of PI3K activation (Tu et al. 2002). To first eliminate the possibility that activation of p42/p44^{ERK} and p38 is downstream of PI3K or p70S6K1, we determined the level of phosphorylation of these two MAP kinases when cells were treated with the PI3K inhibitor wortmannin or the p70S6K1 inhibitor rapamycin. The results show that wortmannin and rapamycin did not affect the activation of p42/p44^{ERK} and p38 by H_2O_2 (Fig. 3.5). PD95089 and SB202190 were tested for their effects on H_2O_2-induced p70S6K1 activation. Cells were pretreated with these inhibitors for 30 min and were then treated with 200 μM H_2O_2 for 1 hr in the presence of inhibitors. The p70S6K1 activation was measured by phosphorylation at Thr389 and Thr421/Ser424. Measurements of p70S6K1 phosphorylation showed that neither PD98059 nor SB202190 could inhibit Thr389 or Thr421/Ser424 phosphorylation (Fig. 3.6A&B).

Measurements of enzymatic activity of p70S6K1 allow us to generate quantitative data. Cells were harvested for immunoprecipiation and *in vitro* kinase activity assay after being treated 1 hr with 200 μM H_2O_2 in the presence or absence of PD98059 or SB202190. The results showed that PD98059 did not affect p70S6K1 activity (Fig. 3.6C). Interestingly, SB202190 partially prevented H_2O_2 from inducing p70S6K1 activation in cardiomyocytes (Fig. 3.6C). Since SB202190 inhibited p70S6K1 activity

without inhibiting Thr389 or Thr421/Ser424 phosphorylation, it is possible that SB202190 may function as a non-specific inhibitor for p70S6K1. If SB202190 is an inhibitor for p70S6K1, we would expect an inhibition of the kinase activity *in vitro*. To test this possibility, we isolated p70S6K1 by immunoprecipitation and added SB202190 to the kinase reaction mixture in test tubes. One experiment showed that when p70S6K1 activity from control untreated cells was set to 100%, H_2O_2 treated cells had 198% p70S6K1 activity. The reaction mixtures of the control or H_2O_2 treated cells with 10 μM SB202190 added had 104% and 196% p70S6K1 activity respectively. Similar results were obtained with an independent experiment of the same design. Therefore SB202190 at a concentration of 10 μM failed to inhibit p70S6K1 activity *in vitro*. The fact that SB202190 inhibits p70S6K1 activity in cardiomyocytes treated with H_2O_2 suggests the possibility that p38 may participate in the regulation of p70S6K1 in cells.

Cardiomyocytes can survive a 2-h treatment of mild doses of H_2O_2 but develop hypertrophic morphology over a course of 4-5 days (Chen et al. 2000b; Tu et al. 2002). Increases in cell volume and protein content per cell were detected with H_2O_2 treated cells (Fig. 3.7A&B). To test the effect of PD98059 and SB202190 on H_2O_2 induced cell enlargement, the cells were pretreated 30 min with these inhibitors and then treated with H_2O_2 in the presence of these inhibitors. After removing H_2O_2 and oxidized medium, the cells were incubated in fresh culture medium containing 0.5% FBS and the inhibitors. The results showed that while PD98059 had no effect on H_2O_2 induced increases in cell

volume and protein content, SB202190 partially inhibited the increases (Fig. 3.7A&B). These results suggest a role of p38 in H_2O_2 induced cell enlargement.

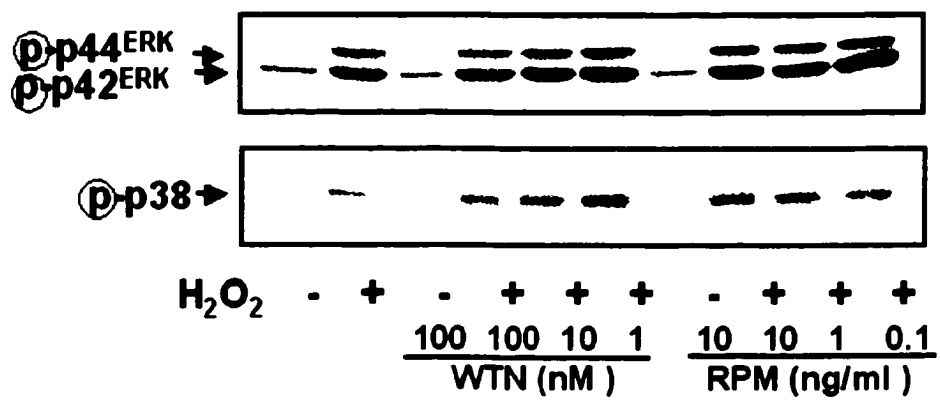
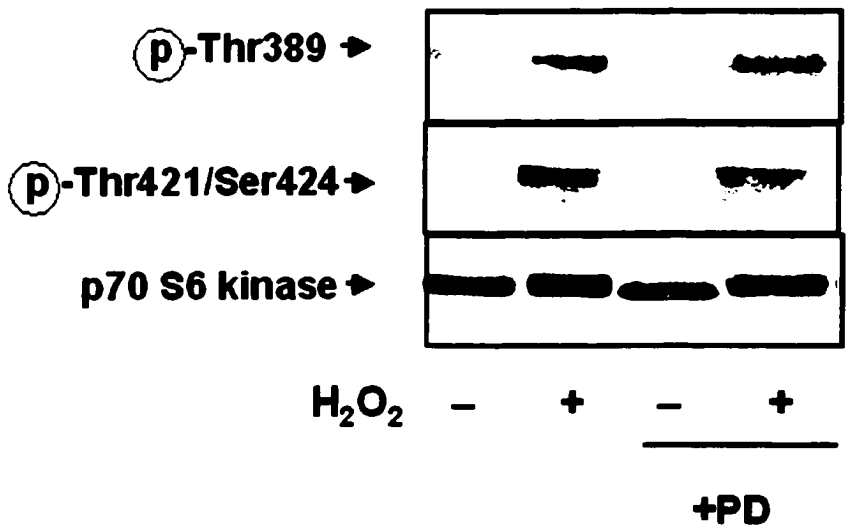


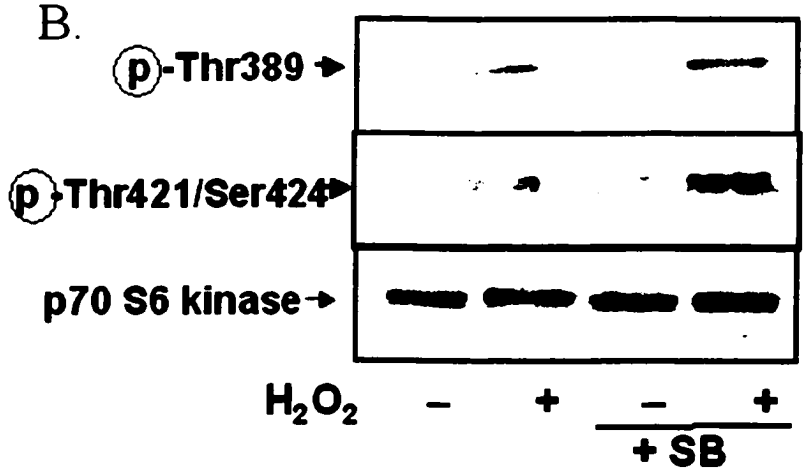
Figure 3.5 Wortmannin (WNT) or Rapamycin (RPM) Does Not Affect H_2O_2 Induced p42/p44ERK or p38 Phosphorylation (Activation).

Cardiomyocytes were pretreated with WTN or RPM at indicated doses for 30 min. The cells were treated with 200 μM H_2O_2 in the presence of the inhibitors for 60 min. Cell lysates (20 μg protein) were used to determine the phosphorylation of Thr202/Tyr204 residues in p42/p44^{ERK} (*upper panel*) or Thr180/Tyr182 residues in p38 (*lower panel*) by Western blot. The data are from one experiment representative of three.

A.


(p)-Thr389
(p)-Thr421/Ser424
p70 S6 kinase
H2O2 – + – +
+PD

B.


(p)-Thr389
(p)-Thr421/Ser424
p70 S6 kinase
H2O2 – + – +
+ SB

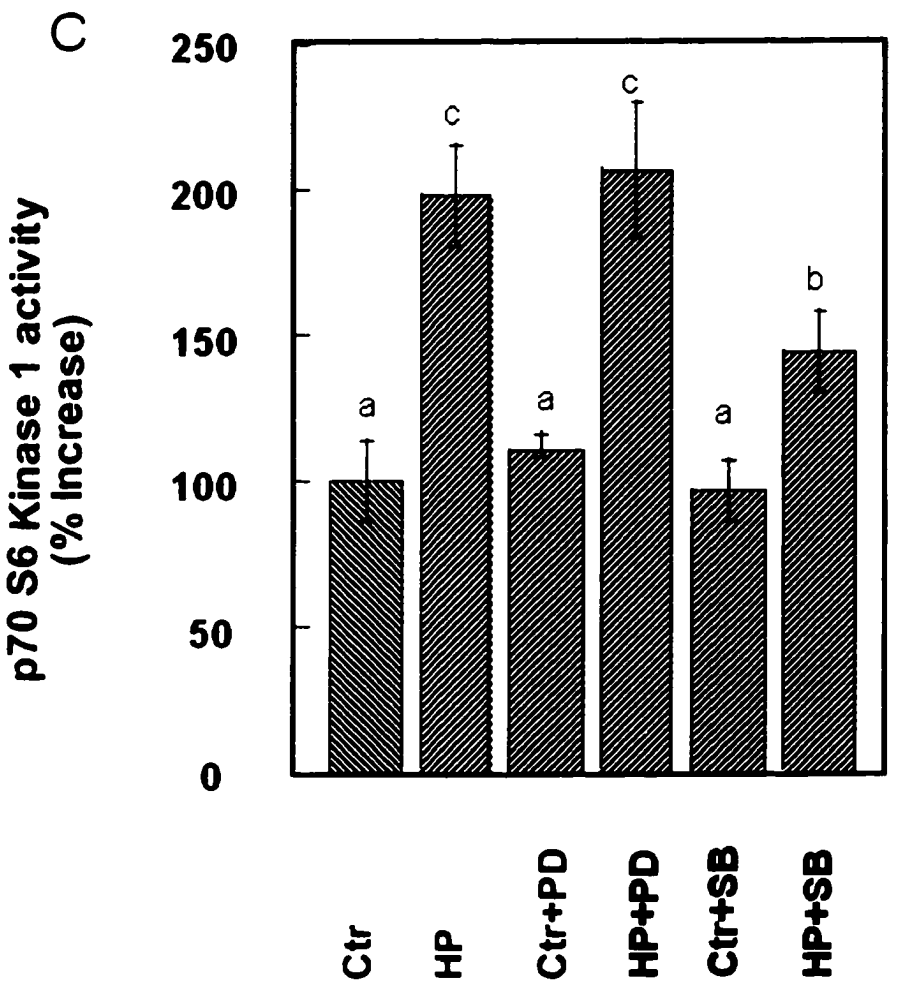
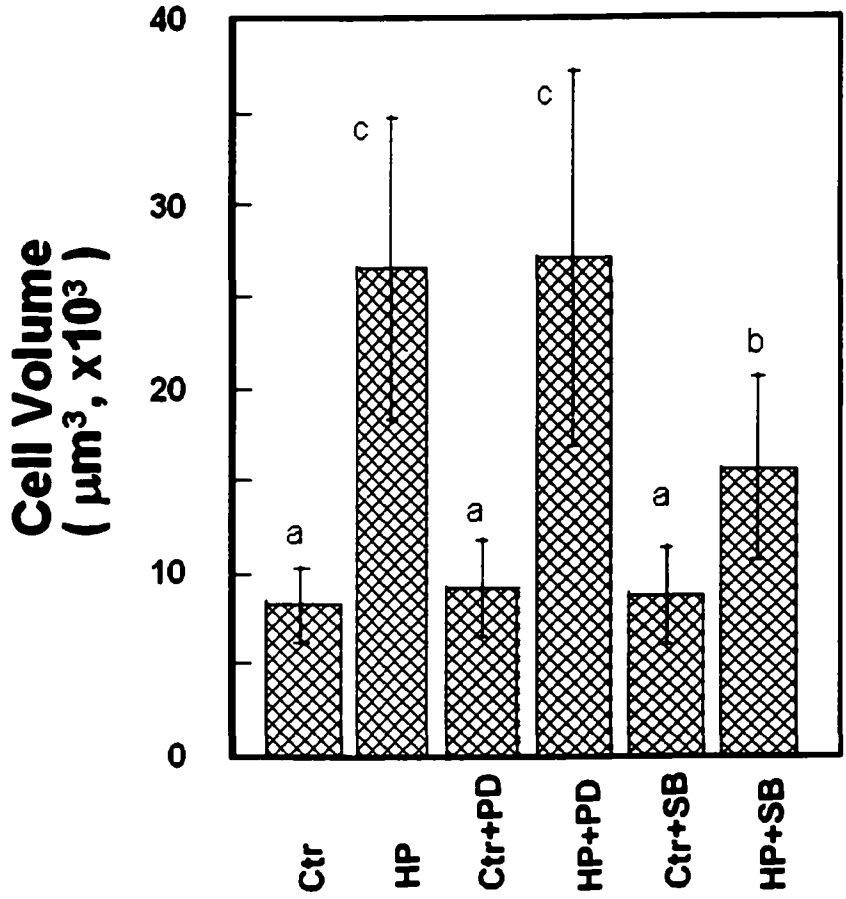


Figure 3.6 Effect of PD98059 (PD) and SB202190 (SB) on H_2O_2 Induced p70S6K1 Activation.

Cardiomyocytes were pretreated 30 min with 50 μM PD (**A, C**) or 10 μM SB (**B, C**). The cells were then treated with 200 μM H_2O_2 for 60 min in the presence of the inhibitors. Phosphorylation on Thr389 or Thr421/Ser424 and the protein level of p70S6K1 were determined by Western blot (**A, B**). Cell lysates were used for immunoprecipitation with anti-p70S6K1 antibody and *in vitro* p70S6K1 assay as described in the Methods (**C**). The data are means ± standard deviation from three independent experiments (**C**). A letter indicates a significant difference ($p<0.05$) from other means with different letter designations as determined by ANOVA followed by multiple comparisons with Student-Newman-Keuls test.

A.


Cell Volume
(μm³, x10³)
40
30
20
10
0
a
c
a
c
a
b
Ctr
HP
Ctr+PD
HP+PD
Ctr+SB
HP+SB

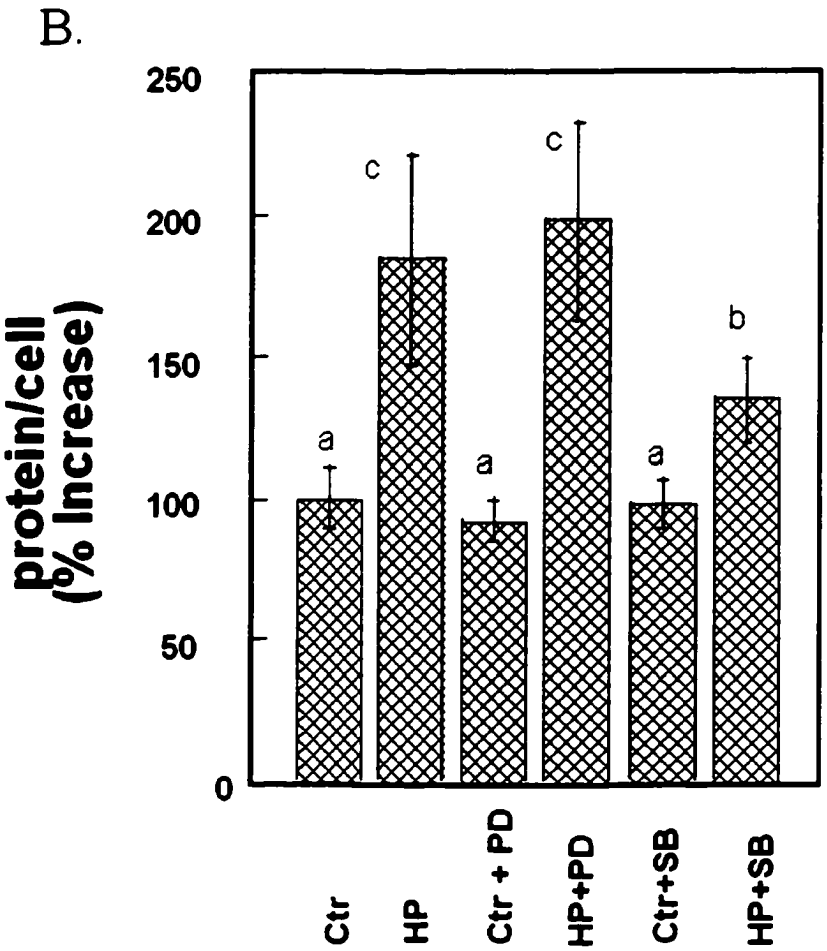


Figure 3.7 Effects of PD98059 (PD) and SB202190 (SB) on H_2O_2 Induced Hypertrophy.

Cardiomyocytes were pretreated with 50 μM PD or 10 μM SB for 30 min. The cells were treated with 200 μM H_2O_2 for 90 min in the presence of the inhibitors and were then placed in fresh medium containing the inhibitors for 5 days before measurement of cell volume (**A**) or protein content (**B**). At least 99 cells were measured for diameters randomly (**A**) and three groups of samples were measured for protein content per cell (**B**). The data are means ± standard deviations from 99 cells (**A**) or triplicate measurements (**B**) of one representative experiment. A letter indicates a significant difference ($p<0.05$) from other means with different letter designations as determined by ANOVA followed by multiple comparisons with Student-Newman-Keuls test]

Discussion

This study shows that H_2O_2 can activate p42/p44^{ERK} and p38 in cardiomyocytes. These two kinases were activated within 10 min of H_2O_2 exposure and the activation reached a peak at 60 min of H_2O_2 exposure for both kinases. Following these early changes in MAPKs, the AP-1 transcription factor was activated when the cells were allowed to recover for 30 min or longer from H_2O_2 treatment. The p42/p44^{ERK} inhibitor PD59089 but not p38 inhibitor SB202190 was able to prevent H_2O_2 from inducing AP-1 activation. However, SB202190, at the dose completely inhibiting p38 activity, partially blocked p70S6K1 activation and cell enlargement induced by H_2O_2. These data suggest that p42/p44^{ERK} and p38 control different aspects of oxidant induced cardiomyocyte hypertrophy. While p42/p44^{ERK} plays a critical role in AP-1 activation, p38 likely contributes to activation of p70S6K1 and cell size increase (Fig. 3.8).

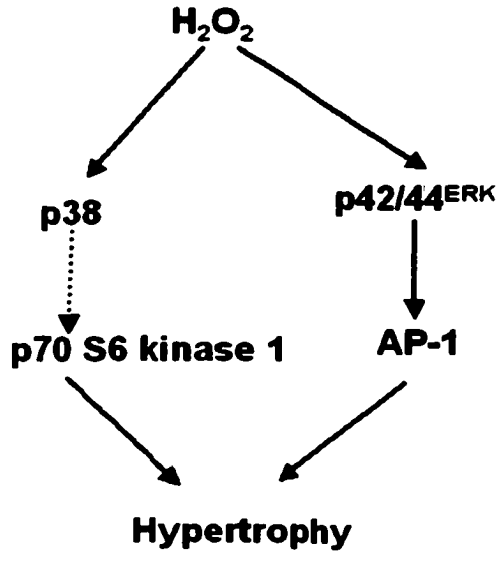


Figure 3.8 Schemetic of the Proposed Roles of p42/p44ERK and p38 in Oxidant-Induced Cardiomyocyte Hypertrophy.

Hypertrophy is a complex process involving a cellular increased protein accumulation as well as changes in gene expression. Activation of MAPKs kinase, a common event of hypertrophy induced by endocrine factors and mechanical stretch, contributes to reactivation of fetal gene program including expression of ANF, SkA and β-myosin heavy chain (βMHC) genes (Copper 1997; Clerk et al. 1998; Homcy 1998; Sugden and Clerk 1998; Force et al. 1999). AP-1 is one of several transcription factors known to regulate the expression of some of these hypertrophy marker genes such as ANF and SkA (Rosenzweig et al.; Bishopric et al. 1992; Sadoshima et al.; Herzig et al.; Takemoto et al.). Our finding on $p42/p44^{ERK}$ is consistent with the report that overexpressing $p44^{ERK}$ or an upstream kinase of $p42/p44^{ERK}$ in cultured cardiomyocytes causes expression of hypertrophy marker genes in the absence of cell enlargement (Gillespie-Brown et al. 1995). This effect of $p42/p44^{ERK}$ in regulating the expression of hypertrophy marker genes without affecting protein synthesis or cell size increase has also been reported with other hypertrophy inducers (Thorburn et al. 1995; Aoki et al. 2000; Ono et al. 2000; Xiao et al. 2001). In contrast to $p42/p44^{ERK}$, overexpressing the β-isoform of p38 gene or a constitutively active upstream kinase of p38 can produce cell enlargement *in vitro* (Zechner et al. 1997; Wang et al. 1998a). Inhibiting p38 by its chemical inhibitor SB203580 prevents phenylephrine from inducing cell enlargement and expression of hypertrophy marker genes *in vitro* (Zechner et al. 1997). Although our data failed to identify a role of p38 in AP-1 activation, the fact that $p42/p44^{ERK}$ influences AP-1 activity and SB202190 inhibits 50% of the p70S6K1 activation and cell enlargement

suggests the importance of transcription-dependent and -independent mechanisms in cardiomyocyte hypertrophy induced by oxidants.

AP-1 activation is thought to be an important event in hypertrophy induced by endocrine factors and mechanical stress. The AP-1 transcription factor is a dimeric protein composed of Jun-Jun, Jun-Fos or Jun-ATF-1 (Wisdom 1999). Although $p42/p44^{ERK}$ and p38 both have been reported to activate AP-1 in several cell lines, the activation mechanism is different between these two MAPKs. p38 can phosphorylate ATF-2 and cause activation of AP-1. Such transcription-independent activation usually occurs immediately following p38 activation (Wisdom 1999). Although there is no evidence that $p42/p44^{ERK}$ can directly phosphorylate c-jun, c-fos or ATF-2, $p42/p44^{ERK}$ can phosphorylate Elk-1 at Ser283 and cause activation of the transcription factor (Liao et al. 1997; Wisdom 1999). Activated Elk-1 binds to the serum response element (SRE) in the promoter region of c-Fos or JunB genes and increases the transcription of these genes (Hodge et al. 1998; Paradis et al. 1996). Babu et al. (Babu et al. 2000) have demonstrated that phosphorylation of Elk-1 by the MEK/ERK pathway is necessary for c-fos gene expression in cardiomyocyte hypertrophy. With H_2O_2 treatment, while activation of $p42/p44^{ERK}$ was first observed within 10 min, DNA binding activity of AP-1 was not detected until 30 mins after the 60 min treatment of H_2O_2. The delay in the time courses of AP-1 activation versus $p42/p44^{ERK}$ activation are consistent with the literature, suggesting a possible transcription-dependent mechanism of AP-1 activation.

Our data with SB202190 suggest a potential role of p38 in cell enlargement by H_2O_2. The mechanism of SB202190 action remains to be elucidated. SB202190 is a pyridinyl imidazole-like compound and a selective inhibitor of p38α and p38β, the two major isoforms of p38 MAPK in the heart (Sugden and Clerk 1998). The compound competes for the ATP binding site of p38. The nonconserved regions within or near the ATP binding pocket of p38 determine the selectivity of SB202190 (Gum et al. 1998; Lee et al. 1999). As with any kinase inhibitors that compete for ATP binding site, it is possible that SB202190 may exert a non-specific inhibitory effect on kinases other than p38, since all kinases contain ATP binding sites. SB202190 partially inhibits the enzymatic activity of p70S6K1 induced by H_2O_2 in cardiomyocytes without affecting phosphorylation at Thr389 or Thr421/Ser424 further supports this possibility of non-specificity. We have tested this possibility by adding SB202190 to p70S6K1 kinase reaction mixture in test tubes. Failure of SB202190 to inhibit p70S6K1 activity *in vitro* argues against the possibility that SB202190 inhibits p70S6K1 activity *per se*. Although phosphorylation of Thr389 or Thr421/Ser424 by PI3K and PKC has been known to be important for p70S6K1 activation, p70S6K1 protein contains 21 Thr and 29 Ser residues. Some of these Thr or Ser residues can be phosphorylated by yet unknown kinases and their phophorylation influences p70S6K1 activation (Ming et al. 1994). This leads us to postulate that p38 may participate in regulating p70S6K1 activity by mechanisms other than Thr389 or Thr421/Ser424 phosphorylation.

Cardiac hypertrophy is a consequence of a large number of cardiovascular diseases. Although hypertrophy is initially compensatory and enhances cardiac output to meet the workload, prolonged hypertrophy is decompensatory and eventually leads to heart failure (Chen and Tu 2002). Oxidative stress has been viewed as an important process leading to cardiac disease and heart failure. Our studies provide evidence that oxidants may be involved in the development of cardiomyocyte hypertrophy by activating signal transduction pathways, changing gene expression and increasing cell size. Many changes induced by oxidants, such as activation of MAPKs, activation of AP-1, and cell enlargement, resemble changes induced by endocrine hypertrophy inducers such as AngII, ET-1 and α-adrenergic agonists. If oxidants cause these changes *in vivo*, it may explain in part the benefit of antioxidant therapy to prevent or delay heart failure.

Chapter 4 ACTIVATION OF NFAT TRANCRIPTION FACTOR BY H_2O_2 IN CARDIOMYOCYTES: ASSISTANCE OF AP-1

Introduction

The Nuclear Factor of Activated T cells (NFAT) transcription factors are a group of proteins that play an important role in the immune response of mammals. They belong to the Rel domain protein family, which includes the transcription factors NF-κB and Rel. NFATs were first characterized as transcription factors that bind to the interleukin-2 (IL-2) promoter. Four subfamilies of NFAT have been identified: NFAT1 (NFATp/NFATc2), NFAT2 (NFATc/NFATc1), NFAT3, and NFAT4 (NFATx/NFATc3). NFAT1 and NFAT2 are expressed predominantly in lymphoid tissues. NFAT2 is also expressed in muscle cells. NFAT3 is expressed primarily in non-lymphoid tissues, while NFAT4 is mainly expressed in the thymus. These four NFAT proteins are coded by four different genes and each NFAT gene appears to have splice variants. However, all four NFAT members share sequence similarity in NFAT-homology region (NHR) and Rel-homology domain (RSD). All of them are able to interact with AP-1 and bind to the IL-2 NFAT binding site. In immune cells, a group of important inflammatory response genes are regulated by NFATs. Besides IL-2, NFATs also control the expression of cytokines such as IL-3, IL-4, and IL-5, interferon (IFN)-γ, tumor necrosis factor (TNF-α), and cell surface receptors including CD40L, CTLA-4, and FasL (Masuda et al. 1998).

An important signal molecule that regulates the activity of NFATs is Ca^{2+}. A sustained increase in intracellular Ca^{2+} level activates calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase (Timmerman et al. 1996). Calcineurin then dephosphorylates NFATs and induces their translocation from the cytoplasm to the nucleus (Clipstone and Crabtree 1992). The immunosuppressive drugs cyclosporin A and FK506 are inhibitors of calcineurin and inhibit nuclear translocation of NFATs (Flanagan et al. 1991). Nuclear NFATs bind to their cis-element in the promoter region of genes and activate these genes' transcription. An important character of NFAT binding is that it is often facilitated by the interaction with AP-1 transcription factor. For example, the NFAT consensus site in the promoter region of IL-2 is **GGAAA**ATT. Adjunct to this site is a weak AP-1 binding site TGTTTCA. In addition to AP-1, NFATs interact with other transcription factors such as GATA-4 (Macian et al. 2001).

The calcineurin and NFAT signaling pathway has been recently implicated to play an important role in the hypertrophic response of the heart (Molkentin et al. 1998; Molkentin 2000). Transgenic mice constitutively expressing a NFAT3 mutant that lacks regulatory region but retains the Rel homology and transactivation domains develop cardiac hypertrophy (Molkentin et al. 1998). Calcineurin/NFAT3 pathway can be activated by all three major types of hypertrophy inducers: AngII, ET-1 and catecholamines (Molkentin 2000; Zhu et al. 2000). Presumably, these hypertrophy inducers activate PLC after binding to their receptors on the cellular membrane. Activation of PLC then leads to an increase of cytoplasmic Ca^{2+} concentration and

NFAT3 activation. In chapter II, we showed that H_2O_2 activates PI3K. Since PI3K is a kinase crosstalking with multiple signaling pathways including PLC, it is likely that H_2O_2 may increase intracellular Ca^{2+} concentration through PI3K leading to NFAT3 activation. In addition, previous studies indicated that H_2O_2 as oxidative stress can lead to Ca^{2+} overload inside cardiomyocytes, which may be responsible for the cardiac damages caused by ischemic reperfusion (Tones et al. 1985; Dixon et al. 1990; Josephson et al. 1991). Taken together, these lines of information suggest that NFAT3 is likely activated in oxidant-induced hypertrophy. The aim of the study in this chapter was to investigate whether and how NFAT3 can be activated by H_2O_2.

Materials and Methods

Chemicals and reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Stabilized H_2O_2 (Sigma, H-1009) was used and the concentration of the stock was verified by absorbency at 240 nm (OD_{240}=1.0 for 0.023 Molar of H_2O_2). PD98059, SB202190, Cyclosporin A and LY249002 were obtained from Calbiochem (L a Jolla, CA). Polyclonal antibody against NFATc1 (K-18) that recognizes NFAT3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and H_2O_2 treatment

Cardiomyocytes were prepared from 1 to 2-day old neonatal Sprague-Dawley rats (Harland, Indianapolis, IN) as previously described (Tu et al. 2002). The cardiomyoctes were seeded at a density of 5×10^4 cells/cm^2 and plated in DMEM with 1 mM pyruvate, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin. Cells were placed in 0.5% FBS/DMEM for 18 to 24 hr before H_2O_2 treatments.

NFAT luciferase assay

Cardiomyocytes were seeded at 0.5 x 10^6 per well in 6-well plates (963 mm^2 culture area per well). At 24 hr after plating, cells were transfected with 0.8 μg NFAT luciferase plasmids pNFAT-luc (Stratagene, La Jolla, CA), 0.4 μg NFAT3 expression vector RSV-NFAT3 (a gift from Dr. Jeffery Molkentin) and 0.04 μg pRL-TK plasmids per well by 3 μl FuGene-6 liposomes (Roche, Mannheim, Germany) for 5 hr and then placed in 10 % FBS/DMEM for 24 hr and subsequently in 0.5% FBS/DMEM for 18-24 hr before H_2O_2 treatment. When cells were cotransfected with hisC-Tam67 or dnNFAT, the amount of pNFAT-luc, RSV-NFAT3 or pRL-TK DNA was reduced to 50%. pRL-TK plasmid has a Renilla luciferase gene under the control of a thymidine kinase (TK) promoter and was used to correct for transfection efficiency in a dual promoter luciferase assay. Cells were pretreated with inhibitors for 1 hr and then treated with H_2O_2 for 10 min. After H_2O_2 treatment, oxidized medium was changed to fresh 0.5% FBS/DMEM with or without inhibitors added and the cells were harvested at various time points. Dual

luciferase activities were measured with a dual luciferase assay kit purchased from Promega (Madison, WI) by a luminometer (Turner Designs, Sunnyvale, CA). Results were espressed as ratios of the relative light unit (RLU) from the readings of firefly over that of Ranilla luciferase.

Electrophoretic mobility shift assay (EMSA)

Cardiomyocytes were harvested and nuclear extracts were prepared as described in Chapter III. Binding reactions were performed for 30 min at 4 °C in a reaction volume of 20 μl containing 8 μg of nuclear protein, 4 % Glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 50 mM NaCl, 10 mM Tris.HCl, pH 7.5, 0.05 mg/ml poly(dI-dC) and about 30,000 cpm of ^{32}P-labeled oligonucleotide probes (GGA**GGAAAAACTGTTTCA**TACAGAAGGCGT). When supershift assay was performed, nuclear extract was preincubated with a polyclonal antibody against NFATc1(K-18) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr in a reaction buffer at 4 °C before the addition of oligo nucleotide probe. DNA-protein complexes were separated on nondenaturing 5% polyacrylamide gels. Gels were vacuum dried and exposed to X-ray film for autoradiography.

Assay for c-Fos protein level

At different time points after H_2O_2 exposure, cardiomyocytes were harvested in a lysis buffer (1% Triton X-100, 10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 50 mM NaCl,

50 mM NaF, 10 μg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 2 mM Na_3VO_3). Protein concentration was measured by the Bradford method according to the manufacturer's instruction (Bio-Rad, Richmond, CA). An equal amount of proteins was loaded in each lane and separated by a 7% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then the proteins were transferred to a PVDF membrane. c-Fos protein was detected by incubating the membrane with a c-Fos antibody (Santa Cruz Technology, Santa Cruz, CA) for 2 hours and subsequently a horse radish peroxidase conjugated secondary antibody for 45 min. Bound antibodies were detected by enhanced chemiluminescence (ECL) reaction.

Results

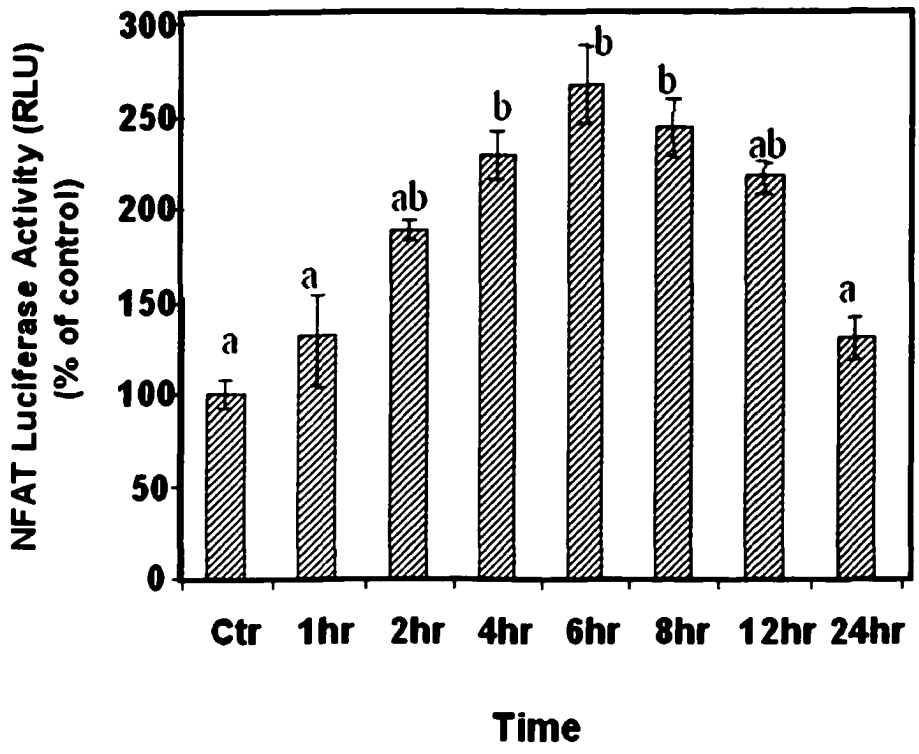
Increase of NFAT activity by H_2O_2

NFAT3 is a major form of NFAT family member expressed in cardiomyocytes (Rao et al. 1997). Recent evidence indicates that the expression of B-type natriuretic prptide (BPN), a hypertrophic marker gene, in cardiomyocytes is regulated by NFAT3, and a potential binding site has been founded in the BNP promoter (Molkentin et al. 1998). A luciferase reporter gene construct containing tandem repeats of a NFAT binding site derived from the IL-2 promoter has been used in studying NFAT activation (Ichida and Finkel 2001). Due to the low expression level of NFAT3 in cardiomyocytes and low transfection efficiency of cardiomyocytes, NFAT3 expression vector pRSV-NFAT3 was cotransfected with the NFAT luciferase reporter construct to augment the promoter response. To test whether H_2O_2 is able to induce NFAT-promoter activation, cardiomyocytes were transfected with pNFAT-luc, pRSV-NFAT3, and pRL-TK as described in the Methods. Transfected cardiomyocytes were treated with 100 μM H_2O_2 for 10 min and harvested at different time points. Measurements of luciferase activities indicated a rapid increase of NFAT promoter activity within 2 hours after treatment. Luciferase activity reached its peak of 2.5 fold elevation at 6 hours and started to decline at 8 hours after treatment. At 24 hr after treatment, the activity returned to the basal level (Fig. 4.1A). Dose response experiments were also performed. Five groups of cardiomyocytes were treated with H_2O_2 of various doses for 10 min and harvested at 6 hours after treatment. A dose-dependent increase of luciferase activity was observed. The optimal dose was 100 μM. At 200 μM, luciferase activity began to decline (Fig.

4.1B). These results implicate that H_2O_2 is able to induce NFAT3-dependent transcription activation.

To verify the binding of NFAT to its specific site, the same IL-2 NFAT binding site in pRL-NFAT was used for gel shift assay. The result indicated that the binding was first detected at 1 hr after treatment, reached a plateau at 2 hr and declined at 6 hr. To test the specifity of the binding band we observed, we used an antibody that presumably recognizes all forms of NFATs to perform supershift assay. Preincubation of nuclear extract with the antibody weakened the intensity of the band and a new band appeared above the original band. This result proved the specificity of the binding (Fig. 4.2).

A


NFAT Luciferase Activity (RLU)
(% of control)
300
250
200
150
100
50
0
a
a
ab
b
b
b
ab
a
Ctr
1hr
2hr
4hr
6hr
8hr
12hr
24hr
Time

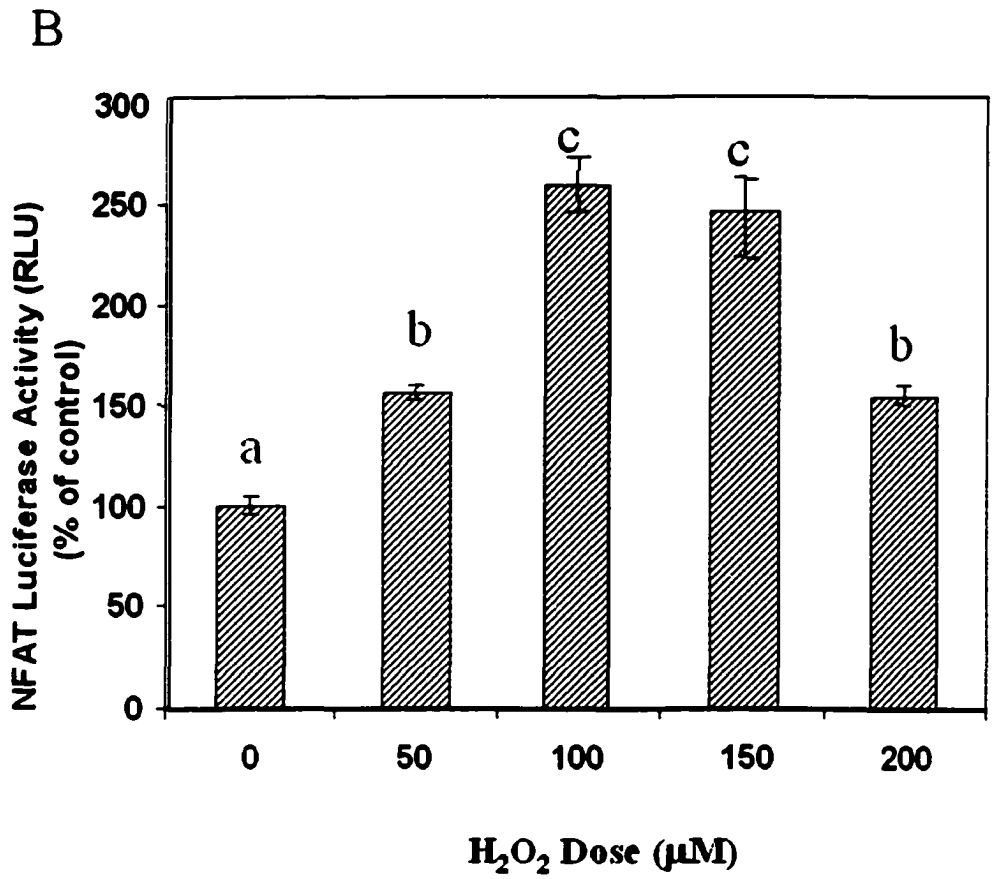


Figure 4.1 H_2O_2 Induces Activation of NFAT3 Transcription Factor

At 24 to 48 hours after transfection, cells were treated with 100 μM H_2O_2 (A) or H_2O_2 at various doses (B) for 10 min and then placed in fresh medium. Samples were collected at indicated time points (A) or at 6 hr after treatment (B) for dual luciferase assay. The data are means ± standard deviation of the ratios of NFAT luciferase over the Renilla luciferase in relative light unit (RLU). A letter indicates a significant difference ($p<0.05$) from other means with different letter designations as determined by ANOVA followed by multiple comparisons with Student-Newman-Keuls test.

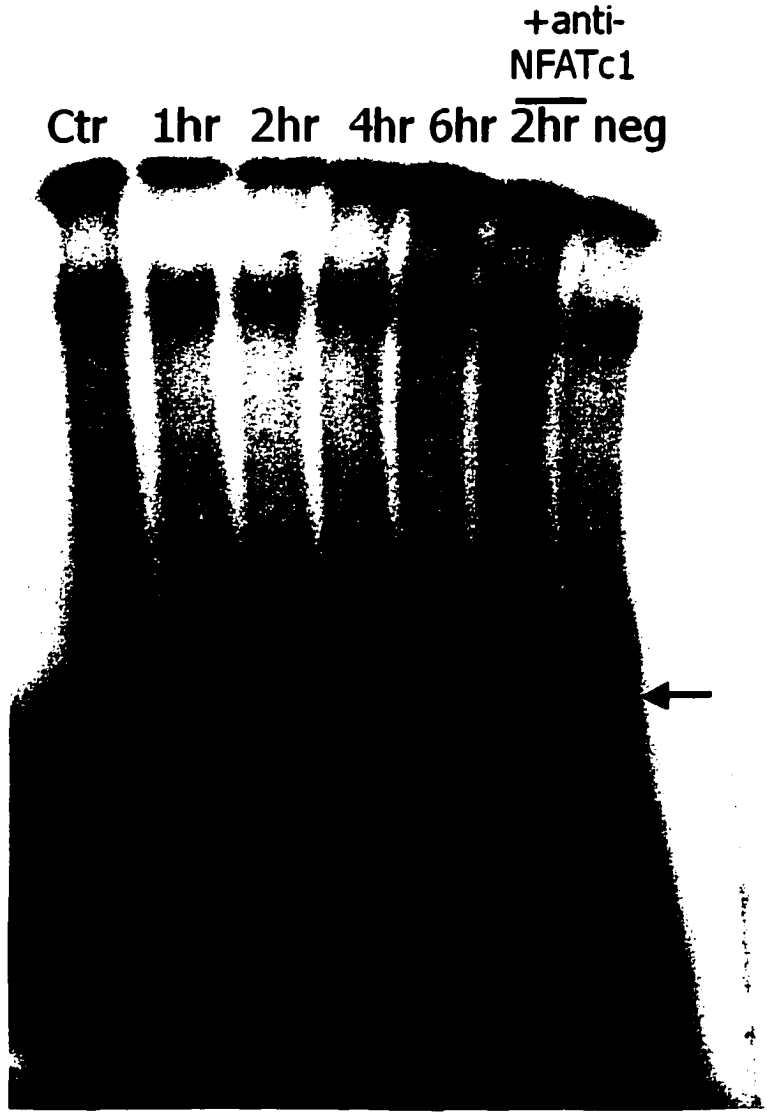


Figure 4.2 Time Course of NFAT Binding to the NFAT Site in the IL-2 Promoter.

Cardiomyocytes were treated with 100 μM H_2O_2 for 10 min and then placed in fresh 0.5% FBS medium. Samples were harvested at indicated time points after H_2O_2 treatment for nuclear extraction and gel shift assay.

The role of calcineurin in H_2O_2-induced NFAT3 activation

Calcineurin removes phosphatase of NFATs and causes nuclear translocation and subsequent activation of NFATs. In cardiomyocytes, activation of NFAT3 has been shown to be dependent on calcineurin activation. Therefore, we examine whether H_2O_2-induced NFAT3 activation is mediated by calcineurin.

Cyclosporin A (CysA) is a calcineurin-specific inhibitor. This drug forms a complex with cyclophilin in the cytoplasm that competitively binds to and inhibits calcineurin (Liu et al. 1991). To test whether CysA is able to block H_2O_2-induced NFAT3 activation, transfected cardiomyocytes were preincubated with CysA at various concentrations for 1 hour before 100 μM H_2O_2 treatment for 10 minutes. Cells were harvested for dual luciferase assay at 6 hours after treatment. CysA of 0.5 μg/ml has been shown to inhibit calcineurin activation and NFAT3 translocation induced by PE or AngII in cardiomyocytes (Molkentin et al. 1998). This concentration failed to inhibit H_2O_2 induced NFAT3 activation. At the concentration of 10 μg/ml, which is ten times higher than the usual concentration for inhibiting calcineurin in cells, CysA was only able to inhibit NFAT3 luciferase activity by less than 20% (Fig.4.3A).

To further investigate the role of calcineurin in H_2O_2-induced NFAT3 activation, a dominant negative NFAT3 plasmid (a gift from Dr. Roger Davis) was cotransfected with pNFAT-luc. This dnNFAT3 is a truncated form of NFAT3 with remaining amino

acid 1-130. Chow *et al.* indicated that this dnNFAT blocks nuclear translocation of NFAT proteins by interfering with calcineurin (Chow et al. 1999). When dnNFAT was cotransfected with pNFAT-luc at a DNA amount ratio of 3:1 (1.2μg dnNFAT per transfection), it had no effect on H_2O_2 induced NFAT luciferase activation (Fig. 4.3B). Data from Cys A and dnNFAT experiments suggest that H_2O_2-induced activation of NFAT3 may not be mediated by calcineurin.

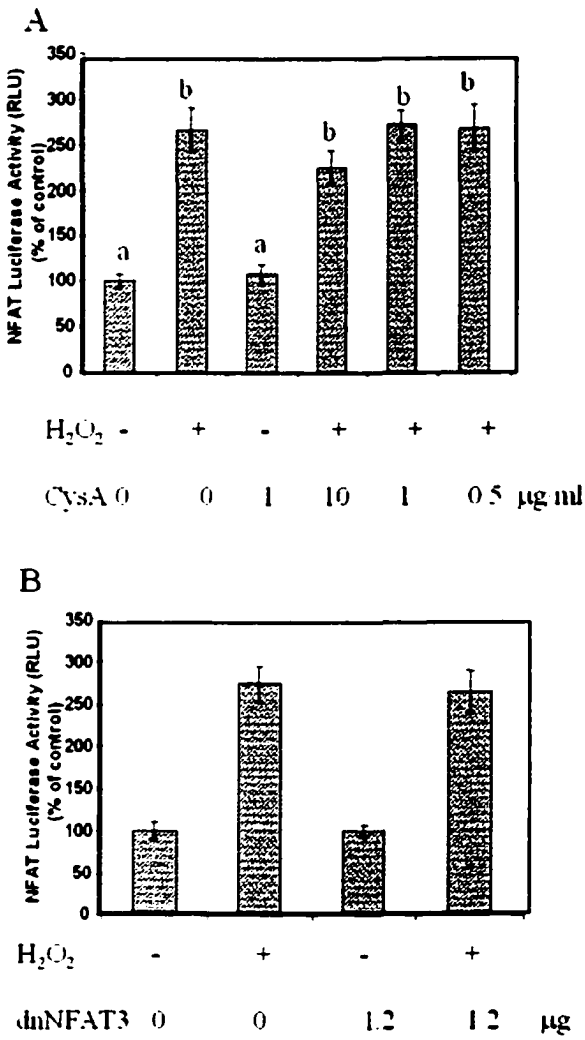


Figure 4.3 Cyclosporin A and dnNFAT Failed to Block H_2O_2-Induced NFAT3 Activation.

Cardiomyocytes were transfected with pNFAT-luc and pRL-TK without (A) or with (B) cotransfection of dnNFAT3. At 24 to 48 hours after transfection, cells were pretreated with indicated doses of cyclosporin A for 1 hr (A) before treatment of 100 μM H_2O_2 for 10 min and then placed in fresh medium. Samples were collected at 6 hr after treatment (A, B) for dual luciferase assay. The data are means ± standard deviation of the ratios of NFAT luciferase over the Ranilla luciferase in relative light unit (RLU).

The role of PI3K and MAPKs in H_2O_2 induced NFAT3 activation

Previous studies described in Chapter III demonstrate that ERK, p38 MAPK and PI3K were activated by H_2O_2 and contributed to the development of hypertrophy (Tu et al. 2002). Since calcineurin is not the major contributor to H_2O_2-induced NFAT3 activation, we explored whether ERK, p38 or PI3K participates in NFAT3 regulation.

Transfected cells were pretreated with MEK1/ERK inhibitor PD98059 at 50μM, p38 MAPK inhibitor SB210190 at 10μM, or PI3K inhibitor LY249002 at 20μM before H_2O_2 treatment. Luciferase assay demonstrated that neither SB nor LY showed any inhibitory effect. In contrast, PD completely blocked the activation of NFAT3 induced by H_2O_2 (Fig.4.4A&B). Various doses of PD were tested on their effect on NFAT activation. Data showed that PD prevented NFAT3 activation in a dose-dependent manner with inhibition observed at 20 μM PD pre-incubation (Fig. 4.4B). Since PD prevents ERK activation, these results implicate the ERK pathway playing an important role in H_2O_2-induced NFAT activation. On the other hand, p38 or PI3K does not seem to be involved in H_2O_2-induced NFAT dependent activation.

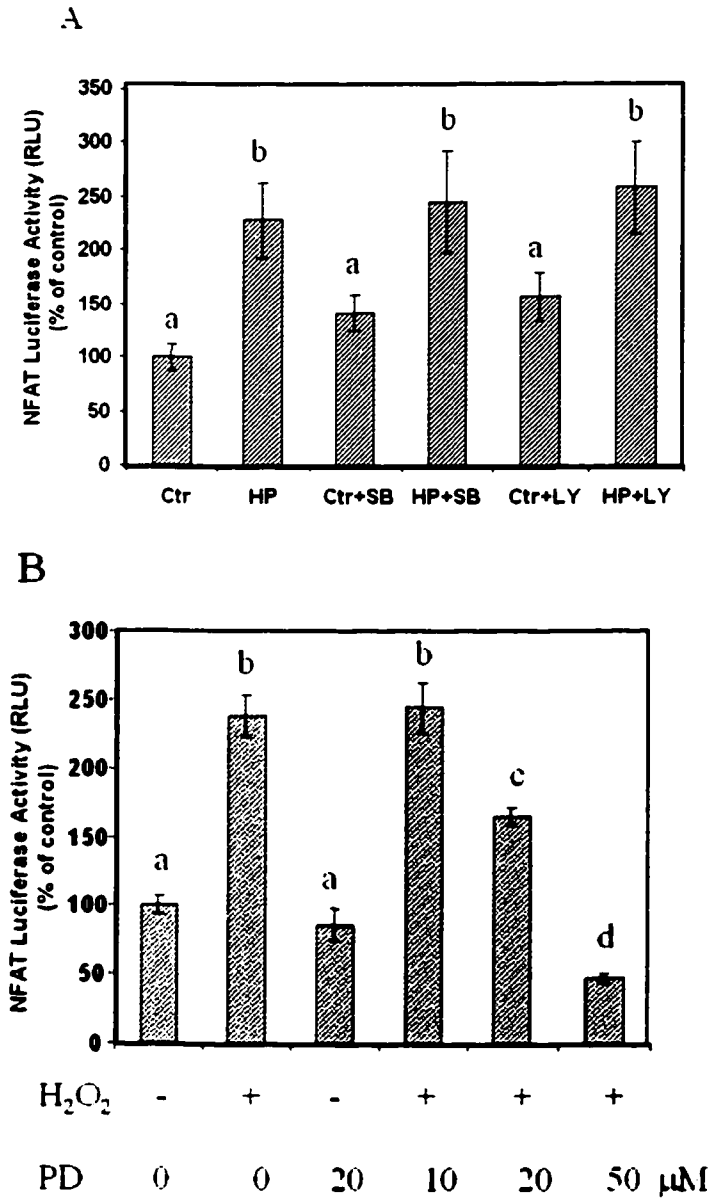


Figure 4.4 Effects of Inhibitors on H_2O_2-Induced NFAT3 Activation.

At 24 to 48 hours after transfection, cells were pretreated with 10μM SB, 20 μM LY (A) or indicated PD (B) for 1hr before treatment of H_2O_2 at 100 μM for 10 min and then placed in fresh medium. Samples were collected at 6 hr after treatment for dual luciferase assay. The data are means ± standard deviation of the ratios of NFAT luciferase over the Renilla luciferase in relative light unit (RLU). A letter indicates a significant difference ($p<0.05$) from other means with different letter designations as determined by ANOVA followed by multiple comparisons with Student-Newman-Keuls test.

AP-1 mediates H_2O_2-induced NFAT activation

NFAT binding site in IL-2 promoter is known to be adjacent to a weak AP-1 binding site. The cooperation between NFAT and AP-1 has been shown to be an important mechanism of NFAT-dependent gene expressions in immune cells. Many genes related to the immune response have one or more NFAT/AP-1 composite binding sites in their promoter regions. In fact, full transcriptional activation of these genes requires the presence of a Jun-Fos heterodimer, which binds to the AP-1 site immediately next to the NFAT site. The cooperativity of NFAT and Jun-Fos to DNA binding greatly enhances the stability of the ternary complex as compared to the DNA binding affinity of either NFAT or Fos-Jun alone (Rao et al. 1997; Macian et al. 2001). Therefore, we tested whether NFAT activation induced by H_2O_2 is AP-1 mediated.

It has been known that the AP-1 transcription factor binding to the NFAT/AP-1 composite site is limited to be the heterodimer of c-Jun and c-Fos (Macian et al. 2001). c-Fos has been shown to increase with H_2O_2 treatment in tumor cells. An increase of c-Fos protein level is usually an early and transient event of AP-1 activation. c-Fos then dimerizes with c-Jun and binds to the AP-1 promoter of c-Jun to enhance c-Jun expression and AP-1 activity (Wisdom 1999). A detailed review of AP-1 can be found in chapter 1. We therefore examined c-Fos protein level after H_2O_2 treatment. Western Blot experiments indicated that c-Fos protein level increased rapidly after H_2O_2

treatment. An induction was observed as early as 1 hr after the treatment. The induction reached its peak at 2 hr and started to decline at 4 hours. At 8 hours after treatment, the level of c-Fos returned to the basal level (Fig. 4.5). This result suggests that the induction of c-Fos protein level is likely an important mechanism of AP-1 activation in cardiomyocytes.

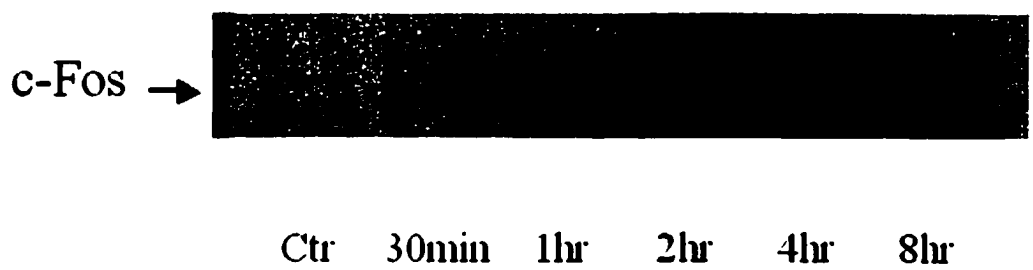


Figure 4.5 H_2O_2 Increases c-Fos Protein Level.

Cardiomyocytes were treated with 100 μM H_2O_2 for 10 min and then placed in fresh medium. Samples were collected at indicated time points for Western Blot analysis using a c-Fos antibody.

To investigate the role of AP-1 in H_2O_2-induced NFAT activation, we used a dominant negative mutant c-jun, TAM67. TAM67 lacks the transactivation domain of c-Jun but is able to dimerize with c-Jun or c-fos family members to inhibit AP-1 activity. This construct has been shown to inhibit AP-1 in various cellular responses (Dong et al. 1994; Dong et al. 1995; Alani et al. 1991). We cotransfected a TAM67 expression vector with pNFAT-luc. H_2O_2-induced NFAT luciferase activity was completely blocked by TAM67 cotransfection (Fig. 4.6). This inhibitory effect was TAM67 dose dependent and was absent with the control vector (Fig. 4.6). To further verify that the increased NFAT3 luciferase activity observed with H_2O_2 treatment is AP-1 dependent, we used another

NFAT luc construct kindly provided by Dr. Molkentin. This construct has a NFAT driven minimal promoter composed by three repeats of consensus NFAT binding site but no weak AP-1 binding site is adjacent to any of them (Molkentin et al. 1998). After transfection, cardiomyocytes were treated with H_2O_2, AngII or PE and harvested for luciferase assay. Interestingly, H_2O_2 was not able to induce NAFT3 luciferase activity any more. In addition, the NFAT3 activity induced by AngII or PE was not as significant as that obtained using pRL-NFAT plasmid (Fig. 4.7). In our previous study, we have shown that AP-1 is activated by H_2O_2 in cardiomyocytes. This evidence combined with TAM67 experiments supports that AP-1 activation is crucial for NFAT3 activation induced by H_2O_2.

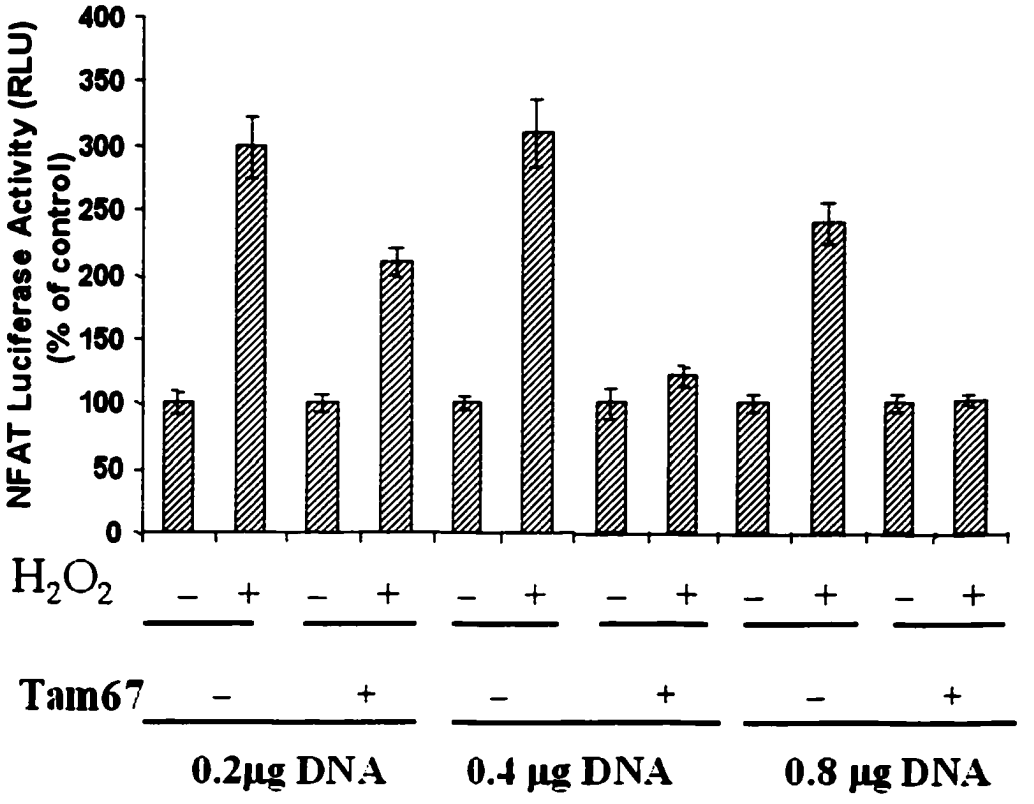


Figure 4.6 TAM67 Blocks H_2O_2-Induced NFAT3 Activation.

HisC-Tam67 or HisC at indicated amounts of DNA was cotransfected with pNFAT-luc. At 24 to 48 hours after transfection, cells were treated with 100 μM H_2O_2 for 10 min and then placed in fresh medium. Samples were collected at 6 hr after treatment for dual luciferase assay. The data are means ± standard deviation of the ratios of NFAT luciferase over the Ranilla luciferase in relative light unit (RLU).

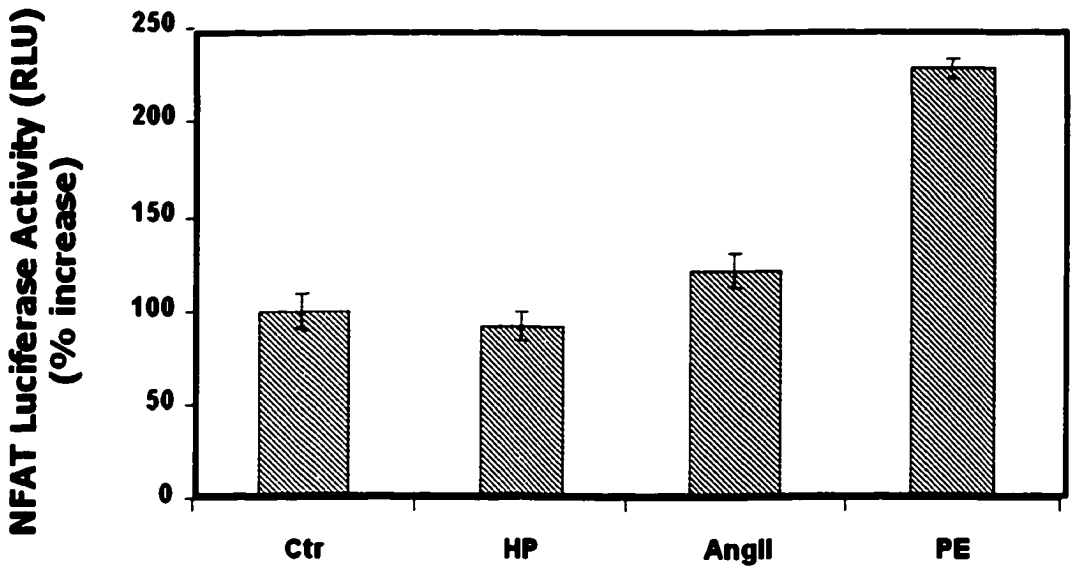


Figure 4.7 H_2O_2 Does Not Induce NFAT3 Activation Using a NFAT Minimal Construct.

Cardiomyocytes were transfected with Dr. Molkentin's p NFAT-luc construct and pRL-TK. At 24 to 48 hours after transfection, cells were treated with 100 μM H_2O_2 for 10 min and then placed in fresh medium, or 1 μM AngII, or 100 nM PE. H_2O_2 treated samples were collected at 6 hr after treatment for dual luciferase assay. AngII or PE treated samples were collected at 24 hr after beginning of the treatment. The data are means ± standard deviation of the ratios of NFAT luciferase over the Renilla luciferase in relative light unit (RLU).

Discussion

Our results demonstrate an essential role for ERKs and AP-1 in the H_2O_2-induced NFAT3 dependent gene expression in cardiomyocytes. We first show that H_2O_2 is able to stimulate NFAT3 dependent gene expression in a both time- and dose- dependent manner. At an optimal time point and H_2O_2 dose, NFAT luciferase activity increases by 2.5 fold. Interestingly, calcineurin seems to play a minimal role in our NFAT3 dependent gene expression, given the fact that CysA or dnNFAT is unable to prevent NFAT3 activation. On the other hand, the ability of H_2O_2 to stimulate NFAT activation is significantly inhibited by treatment with the MEK1 inhibitor, PD98059, suggesting that activation of the MEK1/ERK pathway is necessary for NFAT3 dependent gene expression. Neither p38 inhibitor SB202190 nor PI3K inhibitor LY249002 show any inhibitory effect on the H_2O_2-induced NFAT3 activation. In addition to ERKs, our data also support a crucial role for AP-1 in NFAT3 promoter activation stimulated by H_2O_2. A dominant negative form of c-Jun with an ability to block AP-1 activation appears to be a strong inhibitor of NFAT dependent gene expression. In chapter III, I have shown that ERKs, instead of p38, are responsible for H_2O_2-induced AP-1 activation in cardiomyocytes. Hill *et al.* (Hill et al. 1995) indicated that ERK activation is able to enhance c-Fos expression by activating/phosphorylating the c-fos regulation transcription factor TCF. In consistent with these findings, a rapid increase of c-Fos protein level was observed. Our results clearly support the central role of ERK and AP-1 in the H_2O_2-induced NFAT3 dependent gene expression, and suggest that this pathway is different from the traditional calcineurin/NFAT route reported in cardiomyocyte hypertrophy.

In recent years, multiple signaling pathways leading to cardiac hypertrophy have been identified (Molkentin and Dorn 2001). The development of therapeutic strategies against hypertrophy is undoubtedly dependent on the thorough understanding of the crosstalk and overlap among these pathways. Our data place MEK1/ERK in the network of NFAT3 regulation in cardiomyocytes. Still, the mechanism through which MEK1/ERK leads to NFAT activation needs further investigation. NFATs are traditionally regarded as effecters of calcium signaling through calcineurin. However, recent evidence suggested that MAPK activation contribute to NFAT activation. Tsatsanis *et al.* (Tsatsanis et al. 1998) reported that PD98059 significantly blocked the Tpl-2 kinase induced NFAT activation in T cells, while CysA only showed partial inhibitory effect. Ichida *et. al.* demonstrated results that activating Ras/MEK1/ERK pathway results in NFAT activation in cardiomyocytes (Ichida and Finkel 2001). These are consistent with what we observed in the H_2O_2-induced NFAT3 activation.

Cooperation of NFAT with other transcription factors is an important mechanism of NFAT activation. In immune cells, NFAT and AP-1 cooperation is required for IL-2 gene expression, as supported by studies using a NFAT1 mutant unable to interact with Fos/Jun heterodimers (Macian et al. 2000). In fact, the binding of AP-1 and NFAT to their individual binding sites in the composite elements is characterized by relatively high dissociation rates, but a strong cooperative complex with greatly increased stability is formed when all three proteins bind to the composite DNA site. In cardiomyocytes, a

NFAT3-GATA4 cooperation has been described (Molkentin et al. 1998). Our data evidently demonstrate that NFAT3 activation observed with H_2O_2 treatment is AP-1 dependent. This activation probably occurs in the absence of NFAT3 nucleus translocation. Green fluorescent protein-conjugated NFAT3 (GFP-NFAT3) transfection indicated that NFAT3 has a combined nuclear and cytosolic distribution in cardiomyocytes (Ichida and Finkel 2001). Therefore, activated AP-1 could possibly recruit the existing nuclear NFAT3 onto the NFAT/AP-1 composite site and cause transcriptional activation. This finding may present an important mechanism of NFAT dependent gene expression in cardiomyocytes.

Although this study increases our understanding of the mechanism of NFAT3 activation in cardiomyocytes, many questions remain. First, it is unclear to what degree this AP-1 dependent NFAT3 activation happens in cardiomyocytes. The cooperation of NFAT3 with GATA transcription factor has been implicated in regulating the hypertrophy marker gene BNP (Molkentin et al. 1998). NFAT3 or other isoforms of NFAT are likely to cooperate with other transcription factors such as AP-1 and NFκB to regulate gene expression in cardiomyocytes. Therefore, the proposed mechanism of cooperative- transcription-factor-assistant activation of NFAT may also occur in other forms of NFAT cooperation. Second, where and to what extent this finding fits into advancing our understanding of cardiac hypertrophy and heart failure needs to be answered. Pro-inflammatory interleukins, which are often regulated by NFAT transcription factors, have been indicated to play a role in the myocardial remodeling

after cardiac infarction where oxidants are often overproduced (Hwang et al. 2001). Therefore, the oxidant induced NFAT activation is likely to be a mechanism of enhanced interleukin expression during ischemia and ischemic reperfusion. In fact, it has been known that interleukins, such as IL-6 and IL-1, represent a group of hypertrophy inducers and heart failure mediators (Hirota et al. 1995; Palmer et al. 1995; Thaik et al. 1995; Harada et al. 1999). Their plasma levels are elevated in hypertension and heart failure patients (Palmieri et al. 2002; Wollert and Drexler 2001). If oxidants are able to induce or mediate the expression of these interleukins through NFAT3 or other NFATs in cardiomyocytes, the hypothesis that oxidants contribute to cardiac hypertrophy is further supported and complicated.

Taken together, we demonstrate that H_2O_2, a representative oxidant, is able to activate NFAT3 through a calcineurin independent mechanism. The cooperation of NFAT3 with AP-1 seems be important in H_2O_2 induced cardiomyocyte hypertrophy. This format of NFAT activation may contribute to the oxidant-induced hypertrophy by altering the expression of cytokine or other immune response genes.

Chapter 5 SUMMARY STATEMENT

This dissertation has tested the hypothesis that H_2O_2 induces cardiomyocyte hypertrophy through the activation of multiple signaling pathways. Two major signaling pathways have been studied in this dissertation: the signaling pathways are responsible for cardiomyocyte enlargement and the signaling pathways that lead to altered gene expression in hypertrophic cardiomyocytes. These two sets of signaling pathways are summarized below with discussion on future research opportunities.

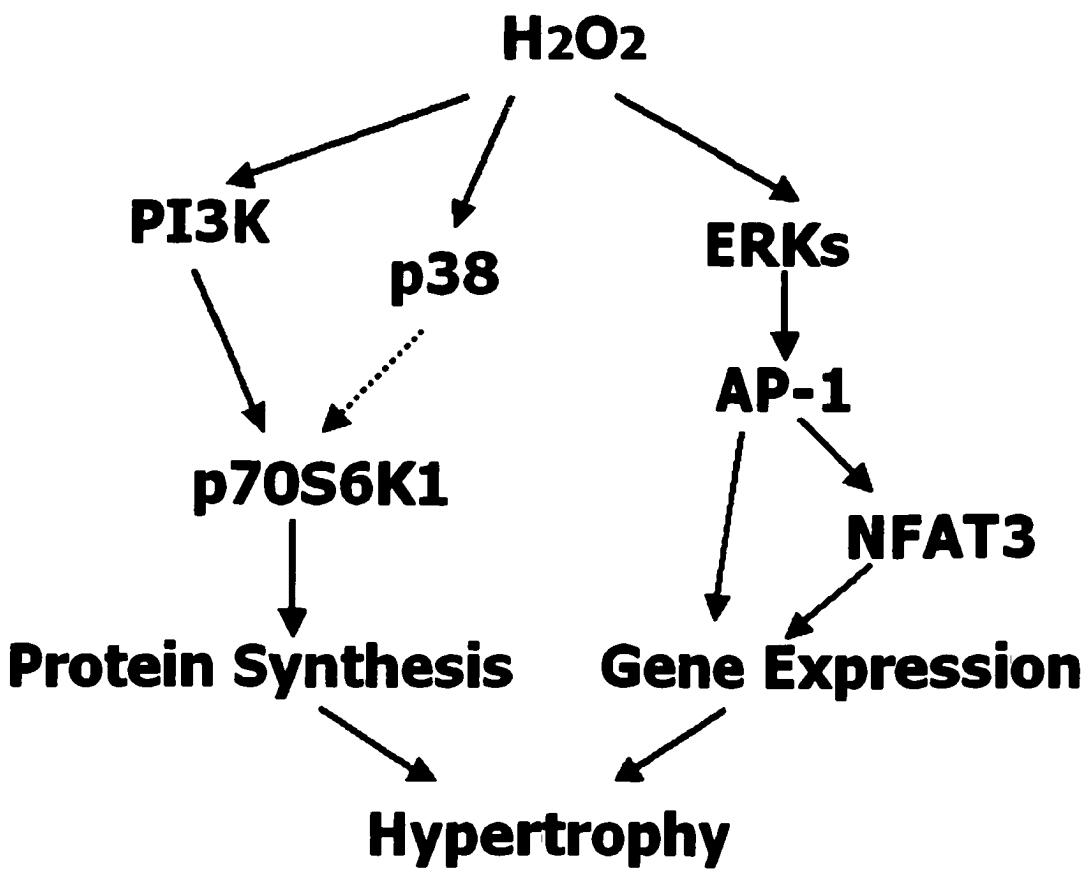


Figure 5.1 Summary of Research

In this study, we chose to use the H_2O_2 dose of 100 μM and treat cardiomyocytes for less than two hours. We believe that this treatment protocol represents pathological situations in the heart such as ischemic reperfusion, which produces a surge of oxidants in ischemic region. In clinics, these pathological conditions frequently result in cardiac hypertrophy. Previous studies in our lab indicated that a high-dose and short-time treatment of H_2O_2 can produce the same cellular response as low-dose and repeated treatments of H_2O_2 (Chen et al. 2001). This finding further justifies our treatment model as a valid *in vitro* model to study cardiac hypertrophy. However, possible differences in cardiomyocyte's response to an acute and high-dose treatment *versus* a chronic and low-dose treatment should be investigated.

To avoid the interference of FBS to the cardiomyocyte's response to H_2O_2, all experiments studying signaling pathways were performed under a low FBS condition (0.5% FBS). FBS consists of several growth factors and has been shown to induce hypertrophy at a concentration higher than 1% (Molkentin et al. 1998). However, we found that cardiomyocytes become extremely sensitive to H_2O_2 and tend to die by apoptosis at 3 days or more after treatment when they are kept in serum free medium. Therefore, we chose to use low FBS medium to minimize the FBS influence while keeping cardiomyocytes healthy.

In this study, we report that H_2O_2 is able to activate several signaling pathways. How does H_2O_2 initiate these signaling cascades? Through literature search, we found that this

is still a poorly studied research area. Identifying the signaling molecule upstream of PI3K and MAPKs will add a key piece to the whole picture of H_2O_2 induced cardiomyocyte hypertrophy. Another question regarding our multi-pathway model of hypertrophy is whether some pathways are more important than others. Cardiomyocyte hypertrophy is a complicated event. More than one signaling pathways have been found to contribute to endocrine factor-induced hypertrophy. So far, which one of these pathways is the most important is still controversy (Molkentin and Dorn 2001). Likewise, for H_2O_2 induced hypertrophy, the involved signaling pathways probably act in a synergistic manner to achieve the largest magnitude of hypertrophic response. For example, although the PI3K inhibitor inhibits the cell enlargement, it fails to regulate NFAT3 activation. Instead, it is regulated by MAPK pathways. Therefore, both pathways are important for H_2O_2 induced hypertrophy. The next question is whether it is necessary to block all involved signaling pathways in order to prevent hypertrophy? Our answer is no. Because some of the pathways are responsible for many critical molecular functions in the cell, blocking all of them will probably result in cell death instead of rescuing the cells from hypertrophy. Theoretically, the downstream hypertrophy effecters of these signaling pathways should be a better choice of therapeutic targets. Molkentin's discovery of the important role of NFAT3 in endocrine factor-induced hypertrophy represents such a case, although whether NFAT3 is the sole solution of curing hypertrophy remains questionable. In the rest of the summary, the signaling pathways contributing to either cell enlargement or gene expression are discussed separately.

1. H_2O_2-induced signaling events

Previous studies using endocrine hypertrophy inducers implicate a significant role of PI3K and its downstream p70S6K1 in protein synthesis and development of hypertrophic morphology. Inhibiting this signaling pathway results in losing the morphology of AngII, PE and ET-1 induced hypertrophy. My experiments demonstrate that both PI3K and p70S6K1 are strongly activated by H_2O_2 treatment. The relationship between these two kinases was further identified by using pharmacological inhibitors. With PI3K being upstream of p70S6K1, the same pharmacological inhibitor approach also proved that this signaling pathway is necessary for H_2O_2 induced hypertrophic morphology. The finding of p38 kinase's contribution to p70S6K1 regulation implies the complexity of H_2O_2 induced cardiomyocyte hypertrophy. Although these findings advanced our knowledge in the response of cardiomyocytes to oxidative stress, several questions remain unanswered.

First of all, how PI3K is activated is unclear. Ras is known to activate PI3K and has been reported to be activated by H_2O_2 (Lander et al. 1995; Fuller et al. 1998). Other small GTP proteins such as Rac, Rho, cdc42 could also be upstream of PI3K activation. Currently, plasmids expressing dominant negative mutants of these GTP proteins have been developed. These constructs will be helpful to answer whether G proteins mediate H_2O_2 induced PI3K activation. Tyrosine kinases are another possible upstream regulators

for PI3K. A group of tyrosine kinses such as Src and focal adhesion kinase (FAK) were reported regulating PI3K under certain circumstances. There are several general tyrosine kinase inhibitors such as genestein that are commercially available. Using these inhibitors will define the involvement of tyrosine kinase in PI3K activation. In addition, specific inhibitor of individual tyrosine kinase can be used to identify the involvement.

The fact that p70S6K1 has multiple potential phosphorylation sites make it a possible substrate of multiple kinases. p70S6K1 protein contains 21 Thr and 29 Ser residues that are potential phosphorylation sites. Our knowledge of the regulation and function of p70S6K1 is still limited. Our result indicates a role of p38 for p70S6K1 regulation. Whether p38 directly phosphorylates p70S6K1 or there are other kinases between them is an interesting question to address.

2. H_2O_2 induced gene regulation

The selective expression of genes is an important feature of cardiomyocyte hypertrophy. Reactivation of the fetal gene program results in increased expression of ANF, βMHC and SkA. A group of constitutively expressed contractile proteins such as MLC-2v and troponin C also enhance their expression in hypertrophic myocardium. This change of gene expression profile is under the control of upstream signaling pathways through certain transcription factors. Multiple transcription factors contribute to the

expression of hypertrophy important genes. Our research focused on two of them, AP-1 and NFAT3.

a. H_2O_2 induces AP1 activation through ERKs but not p38

First, activation of ERKs and p38 MAPK was observed with H_2O_2 treatment. Another important observation is that JNKs, the third member of MAPK family, are also activated by H_2O_2 (data not shown). However, due to lack of pharmacological inhibitor for JNKs at the time of this study, we did not further investigate the role of JNK in AP-1 activation. Whether H_2O_2 activated AP-1 downstream of MAPKs was addressed here. Gel shift assays using AP-1 consensus binding sequence as the probe indicates that H_2O_2 increases AP-1 binding in a time dependent manner with a peak of 5 fold increase. In parallel with gel shift experiments, promoter reporter activation analysis using an AP-1 luciferase plasmid demonstrates that H_2O_2 is able to induce AP-1 dependent transcriptional activation in cardiomyocytes. Both of these experiments proved that H_2O_2 induces of AP-1 activation in cardiomyocytes. Pretreatment of the MEK1 inhibitor PD98059 but not p38 inhibitor SB202190 prevents the increase of both AP-1 binding and AP-1 luciferase activity, suggesting that ERK is the key kinase upstream of H_2O_2-induced AP-1 activation in cardiomyocytes. The above experiments demonstrate that H_2O_2 induces AP-1 activation through ERK instead of p38 kinase. Whether JNKs affect AP-1 activation by H_2O_2 demands further study.

augments the luciferase readings of samples. Our luciferase assay results support the hypothesis that H_2O_2 induces NFAT activation. To investigate the mechanism of H_2O_2 induced NFAT3 activation, a number of pharmacological inhibitors and dominant negative expression vectors were used. The result that cyclosporin A and dnNFAT failed to prevent H_2O_2 from inducing NFAT3 activation argues against a role of calcineurin in H_2O_2 induced NFAT3 activation, and raises the question of how NFAT3 was activated without calcineurin. We found that ERKs and AP-1 are necessary for NFAT3 activation. This finding suggests a new mechanism of NFAT activation in cardiomyocytes depending on cooperation with AP-1 transcription factor. This mechanism could have a broad impact on the general picture of NFAT activation since NFAT binding sites in the promoter regions of many genes are composite sites with other transcription factors.

b.H_2O_2 induces NFAT3 Activation Mediated by ERK and AP-1

NFAT3 represents a newly discovered pathway for cardiomyocyte hypertrophy. Its role in regulating hypertrophy marker genes was first established by Molkentin *et al.* in 1998. The significance of NFAT3 and its upstream regulator calcineurin in cardiomyocyte hypertrophy was further demonstrated by transgenic studies. However, to what degree this pathway contributes to hypertrophy with cardiac diseases often observed in clinics requires further study, owing to conflicting data on the effect of cyclosporinA on hypertension induced hypertrophy and the activity of calcineurin in various models of heart failure involving hypertrophy. Evidence from studying gene expression in immune cells has indicated that activation of calcineurin/NFAT pathway is Ca^{2+} dependent. A sustained increase of Ca^{2+} level in a cell triggers calcineurin activation and in turn NFAT activation. Oxidants were reported capable of increasing the cytosolic level of Ca^{2+} in cardiomyocytes (Tones et al. 1985; Dixon et al. 1990; Josephson et al. 1991). Although these lines of evidences suggest a calcineurin dependent NFAT3 activation following H_2O_2 treatment. Our data disapproved this hypothesis.

A pNFAT-luc plasmid with a luciferase gene under the control of IL-2 promoter containing NFAT binding site is frequently used in the literature studying NFAT regulation. We cotransfected a NFAT3 expression vector RSV-NFAT3 to ensure the luciferase activity is indeed from the binding of NFAT3. This cotransfection also

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