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Extracellular Ubiquitin: Role in Cardiac Myocyte Apoptosis and Myocardial Remodeling

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A dissertation

presented to

the faculty of the Department of Biomedical Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

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by

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May 2014

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Keywords: Ubiquitin, Heart,  $\beta$ -AR, CXCR-4, Ischemia/reperfusion

## ABSTRACT

Extracellular Ubiquitin: Role in Cardiac Myocyte Apoptosis and Myocardial Remodeling

by

Christopher Ray Daniels

Activation of sympathetic nervous system is a key component of myocardial remodeling that generally occurs following ischemia/reperfusion (I/R) injury and myocardial infarction. It induces cardiac myocyte apoptosis and myocardial fibrosis, leading to myocardial dysfunction. Intracellular ubiquitin (UB) regulates protein turnover by the UB-proteasome pathway. The biological functions of extracellular UB in the heart remain largely unexplored. Previously, our lab has shown that  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation increases extracellular UB levels, and extracellular UB inhibits  $\beta$ -AR-stimulated apoptosis in adult rat ventricular myocytes (ARVMs). This study explores the role of extracellular UB in myocyte apoptosis, fibroblast phenotype and function, and myocardial remodeling following  $\beta$ -AR stimulation and I/R injury. First, left ventricular (LV) structural and functional remodeling was studied 7 days after chronic  $\beta$ -AR-stimulation in the presence or absence of UB infusion. Echocardiographic analyses showed UB infusion decreases  $\beta$ -AR-stimulated increases in percent fractional shortening and ejection fraction. It decreased cardiac myocyte apoptosis and myocardial fibrosis. UB activated Akt, and inhibition of Akt inhibited  $\beta$ -AR-stimulated increases in matrix metalloproteinase-2 expression. Second, using cardiac fibroblasts, we provide evidence that extracellular UB interacts with the cell surface and co-immunoprecipitates with CXCR4. UB treatment increased expression of  $\alpha$ -smooth muscle actin (myofibroblast marker), and induced rearrangement of actin into stress fibers. It inhibited lamellopodia and filopodia formation, and cell migration into the

wound. Third, using isolated mouse heart and I/R injury as a model, we provide evidence that UB treatment decreases I/R-mediated increases in infarct size. UB treatment improved functional recovery of the heart as measured by increased % LV developed pressure. Activation of proapoptotic proteins, p-STAT-1 and caspase-9, was significantly lower in UB I/R hearts versus I/R alone. In ARVMs, UB treatment decreased simulated I/R-induced apoptosis. It activated Akt (anti-apoptotic kinase) and inhibited activation of GSK-3 $\beta$  (pro-apoptotic kinase). It decreased I/R-induced oxidative stress and protected anoxia-induced mitochondrial polarization. In fibroblast and ARVMs, CXCR4 antagonism negated the effects of UB, while mutated UBs (unable to interact with CXCR4) had no effect. Thus, extracellular UB, most likely acting via CXCR4, modulates myocardial remodeling with effects on heart function, fibroblast phenotype and function and myocyte apoptosis.

## DEDICATION

To my grandparents Daniel Joseph and Nita Ann Smith: From a very young age you have taught me the importance of an education and hard work and that I could be anything I wanted if I set my mind to it. I appreciate the never ending encouragement throughout my undergraduate and graduate career and for sacrificing so much to provide the means for me to reach whichever level of education I chose to pursue. Thank you for your unwavering patience and love. Words cannot express how thankful I am to the both of you.

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## TABLE OF CONTENTS

	Page
ABSTRACT .....	2
DEDICATION .....	4
ACKNOWLEDGEMENTS .....	5
LIST OF FIGURES .....	11
 Chapter	
1. INTRODUCTION .....	13
Cardiac Remodeling.....	13
Ischemia/Reperfusion and Myocardial Infarction .....	15
$\beta$ -Adrenergic Receptor Stimulation and Heart Disease.....	17
$\beta$ -AR-Stimulation and Cardiac Myocyte Apoptosis.....	18
$\beta$ -AR-Stimulation and Myocardial Fibrosis .....	20
Ubiquitin .....	21
Extracellular UB and $\beta$ -AR-Stimulated Myocyte Apoptosis .....	23
Specific Aims.....	24
2. EXOGENOUS UBIQUITIN MODULATES CHRONIC $\beta$ -ADRENERGIC RECEPTOR STIMULATED MYOCARDIAL REMODELING: ROLE IN AKT ACTIVITY AND MATRIX METALLOPROTEINASE EXPRESSION	25
Abstract .....	26
Introduction.....	27
Methods and Material .....	28
Experimental Animals .....	28
Mice Treatment.....	28
Echocardiography .....	29

Morphometric Analysis .....	30
Measurement of Serum UB Levels.....	30
Myocyte Cross-Sectional Area .....	30
TUNEL Staining .....	30
Fibroblast Isolation and Treatment .....	31
Western Analysis .....	31
In-gel zymography .....	32
Statistical Analysis.....	32
Results.....	32
Morphometric Studies.....	32
Serum UB Levels .....	33
Echocardiographic Measurements .....	34
Fibrosis, Apoptosis and Hypertrophy .....	35
Activation of Akt and GSK-3 $\beta$ .....	37
Activation of JNKs and ERK1/2.....	38
Expression and Activation of MMP-2 and MMP-9.....	39
Expression of TIMP-2 and TIMP-4.....	40
UB Modulates Expression of MMPs and Timp-2 in Cardiac Fibroblast .....	41
UB Activates Akt, and Inhibition of Akt Inhibits MMP-2 Expression in Cardiac Fibroblast.....	43
Discussion .....	44
Perspective .....	48
Grants.....	49
Disclosures .....	49
References.....	50
<b>3. EXTRACELLULAR UBIQUITIN MODULATES CARDIAC FIBROBLAST</b>	
<b>PHENOTYPE AND FUNCTION: ROLE OF CXCR-4 RECEPTOR.....</b>	<b>59</b>



Abstract .....	60
Introduction.....	61
Materials and Methods.....	62
Vertebrate Animals .....	62
Fibroblast Isolation and Treatment .....	63
Immunocytochemical Analysis.....	63
Actin Polymerization Assay .....	64
Co-immunoprecipitation Assay .....	64
DuoLink In Situ Proximity Ligation Assay .....	65
Migration Assay.....	65
Western Blot Analysis .....	66
Statistical Analysis.....	66
Results.....	66
Extracellular UB Interacts with Fibroblast and Induces Cytoskeletal Reorganization .....	66
UB Interacts with CXCR-4 Receptors.....	69
Extracellular UB Inhibits Migration of Fibroblast into the Wound.....	71
Activation of Intracellular Signals by Extracellular UB, and Involvement of CXCR-4 Receptor.....	72
Extracellular UB Affects Expression of $\beta$ 3-Integrin and VEGF-A.....	74
Discussion.....	76
Grants.....	80
Disclosures.....	81
References.....	82
<b>4. EXOGENOUS UBIQUITIN MODULATES ISCHEMIA/REPERFUSION</b>	
<b>INJURY IN ISOLATED HEARTS AND CARDIAC MYOCYTES .....</b>	<b>92</b>
Abstract.....	93

Introduction.....	94
Methods and Materials.....	96
Vertebrate Animals .....	96
Isolated Heart Perfusion System (Langendorff) .....	96
Infarct Size .....	97
Cardiac Myocyte Isolation, Treatment and Hypoxia/Reoxygenation.....	97
Apoptosis .....	98
Measurement of Mitochondrial Membrane Polarization .....	98
ROS Detection .....	98
Western Analysis .....	99
Statistical Analysis.....	100
Results.....	100
UB Treatment Decreases I/R-Induced Infarct Size and Improves Functional Recovery of the Heart .....	100
UB Decreases Activation of p-STAT-1 and Caspase-9 in I/R Hearts .....	101
UB Treatment Decreases Hypoxia/Reoxygenation-Induced Apoptosis in ARVMs.....	102
Activation of Akt and GSK-3 $\beta$ in ARVMs .....	103
UB Treatment Decreases the Number of ROS and Superoxide-Positive Myocytes.....	105
UB Treatment Protects Mitochondrial Polarization .....	107
Discussion .....	108
Perspectives.....	112
Grants.....	113
Disclosures .....	113
References.....	114
5. CONCLUSION.....	124

REFERENCES .....	129
VITA.....	143

## LIST OF FIGURES

Figure	Page
1.1.	Cascade of Events Due to Ischemia/Reperfusion Injury ..... 17
1.2.	Summary Diagram Illustrating Signaling Pathways Involved in $\beta$ -AR-Stimulated Cardiac Myocyte Apoptosis ..... 20
2.1.	Serum UB Levels ..... 33
2.2.	Myocardial Remodeling 7 Days after ISO-Infusion ..... 36
2.3.	Activation of Akt and GSK-3 $\beta$ in the Heart ..... 38
2.4.	Activation of JNKs and ERK1/2 in the Heart ..... 39
2.5.	Expression and Activation of MMP-2 in the Heart ..... 40
2.6.	Expression of MMP-9 and TIMP-2 in the Heart ..... 41
2.7	Expression and Activity of MMP-2 in Cardiac Fibroblast ..... 42
2.8	Expression of MMP-9 and TIMP-2 ..... 43
2.9	Activation of Akt and its Role in MMP-2 Expression ..... 44
3.1.	Cellular Interaction of Extracellular UB ..... 67
3.2	Extracellular UB Induces Cytoskeleton Rearrangement, and Increases Expression of $\alpha$ -Smooth Muscle Actin ..... 68
3.3	Interaction of UB with CXCR-4 Receptor ..... 70
3.4	Extracellular UB Inhibits Migration of Cardiac Fibroblast ..... 72
3.5	UB Activates ERK1/2, Not Akt ..... 74
3.6	UB Decreases $\beta$ 3-Integrin Expression, and Mutated UBs (UBV70A and

	UBF4A) Have no Effect .....	75
3.7	UB Increases VEGF-A Expression, While Mutated UBs (UBV70A and UBF4A) Have no Effect .....	76
4.1.	UB Decreases Infarct Size and Improves LVDP Recovery.....	101
4.2.	UB Decreases I/R-Induced Activation of STAT-1 and Caspase-9.....	102
4.3.	UB Inhibits H/R-Induced Apoptosis in ARVMs .....	103
4.4.	UB Activates Akt in ARVMs .....	104
4.5.	UB Inactivates GSK-3 $\beta$ in ARVMs... ..	105
4.6.	UB Decreases the Number of H/R-Induced ROS-Positive ARVMs .....	106
4.7.	UB Decreases the Number of H/R-Induced Superoxide –Positive ARVMs.....	107
4.8.	UB Treatment Helps Maintain Mitochondrial Membrane Polarization .....	108
5.1	Signal Transduction Pathways for Extracellular UB in $\beta$ -AR-Stimulated Apoptosis and Fibrosis .....	126

## CHAPTER 1

### INTRODUCTION

Cardiovascular disease is the leading cause of death in men and women in the United States and is responsible for over 29% of all deaths world-wide. Cardiovascular disease is the number 1 cause of deaths among people over the age of 65 years and the number 2 cause of deaths in ages 45-64 years. The disease kills more women every year than cancer and stroke.

Over one million people in the United States sustain a myocardial infarction (MI), or heart attack, every year. An MI occurs when the supply of blood, oxygen, and nutrients to an area of the heart muscle is blocked. This results in a decrease in function of the heart and causes sudden death in about half of all victims. For those who survive, the affected heart muscle will be replaced by scar tissue. However, 19% of women and 26% of men die within 1 year post-MI. The estimated annual incidence of MI is 515,000 new attacks and 205,000 recurrent attacks, and the average age at first MI is 64.9 years in men and 72.3 years for women. (Statistic provided by the American Heart Association and the National Heart Lung and Blood Institute.)

#### Cardiac Remodeling

Myocardial remodeling refers to the changes in the structure and function of the heart that commonly occur as a result of heart disease. Adverse myocardial remodeling associates with poor patient outcomes in the context of ischemic heart disease and/or MI, myocardial hypertrophy, and other cardiomyopathic disease states. Cardiac remodeling generally associates with a number of cellular changes including loss of myocytes due to apoptosis (Sharov and others 1996; Olivetti and others 1997), fibroblast proliferation, and fibrosis (Weber and others 1990; Villarreal and others 1993). These changes clinically manifest as changes in size, shape,

and function of the heart after myocardial injury (Kehat and Molkenin 2010). The remodeling process acts as a compensatory mechanism in early stage but becomes deleterious. Cardiac myocyte apoptosis plays a role in MI (Cohn and others 2000). Apoptosis, programmed cell death, is a highly organized process. After MI or ischemic event, left ventricular (LV) dysfunction occurs, in part, as a result of ongoing cardiac myocyte apoptosis (Sharov and others 1996). Cardiac myocyte apoptosis occurs at an increased rate after injury due to ischemia, reperfusion, and MI (Olivetti and others 1997).

Fibrosis is the excess production and accumulation of extra cellular matrix (ECM) structural proteins and results in enhanced stiffness of the myocardium and impedes ventricular contraction and relaxation, leading to distorted architecture and function of the heart (Fan and others 2012). Cardiac fibroblast play a critical role in the remodeling process of the heart that occurs in response to MI, hypertension, and heart failure (Souders and others 2009). Activated fibroblasts are critical in both the reparative and fibrotic processes. In normal tissues resident fibroblasts are quiescent, producing limited amounts of extracellular matrix proteins and exhibiting few actin-associated cell-matrix and cell-cell contacts (Tomasek and others 2002). Tissue injury induces alterations in the microenvironment, resulting in differentiation of fibroblasts into myofibroblasts (Hinz 2007). The matrix metalloproteinases (MMPs) and the endogenous tissue inhibitor of metalloproteinase inhibitors (TIMPS) play a major role in remodeling of the ECM. Plasma levels of MMP2 and MMP9 are elevated in MI patients, but only the MMP9 levels exhibit a biphasic profile that peaked within the first 12 hours and then fall to a plateau (Webb and others 2006). For example, mice with global deletion of MMP-9 develop normally in the absence of pathophysiological stress, but they show a reduction in the degree of ventricular dilation and adverse matrix remodeling after myocardial infarction

(Ducharme and others 2000). Similarly, MMP-2–null mice exhibited a reduction in the rupture rate after myocardial infarction (Matsumura and others 2005). However, pressure overload induced by aortic constriction in MMP-2–null mice have shown blunting of the hypertrophic response (Matsumura and others 2005). Therefore, gene deletion of either MMP-9 or MMP-2 is associated with significant effects on myocardial matrix remodeling, fibrosis, and whole organ geometry (Kehat and Molkentin 2010). These findings support a mechanistic role for both MMP-2 and -9 in adverse myocardial remodeling processes.

### Ischemia/Reperfusion and Myocardial Infarction

Myocardial ischemia is the reduction of blood flow and nutrients, and reperfusion is the return of blood flow to the myocardium. If the period of acute myocardial ischemia is prolonged, more than 20min, a “wave front” of cardiomyocyte death occurs in endocardium and extends over time to the epicardium (Reimer and others 1977). Many biochemical and metabolic changes occur within the myocardium during ischemia. The absence of oxygen prevents oxidative phosphorylation, leading to mitochondrial membrane depolarization, ATP depletion, and inhibition of myocardial contractile function (Hausenloy and Yellon 2013).

Reperfusion is a prerequisite for the survival of ischemic myocardium. Reperfusion or restoration of blood flow to the ischemic heart limits infarct size and reduces mortality. However, reperfusion can also result in additional cardiac damage, referred as reperfusion injury. Reperfusion injury is a complex process that includes rapid restoration of physiological pH, oxidative stress, intracellular  $\text{Ca}^{++}$  overload, and mitochondrial damage. During reperfusion, physiological pH is rapidly restored by the removal of lactate and the activation of the  $\text{Na}^+ - \text{H}^+$  exchanger as well as the  $\text{Na}^+ - \text{HCO}^-$  symporter (Hausenloy and Yellon 2013). The change in pH



leads to cardiomyocyte apoptosis by allowing mPTP opening and cardiomyocyte hypercontracture in the first few minutes of reperfusion (Lemasters and others 1996). Shortly after reperfusion the electron transport chain is reactivated causing an increase in reactive oxygen species (ROS). ROS is also produced by xanthine oxidase in endothelial cells and NADPH oxidase released during an influx of neutrophils (Brechard and Tschirhart 2008; Frey and others 2009). This leads to oxidative damage of DNA, proteins, and the sarcoplasmic reticulum causing intracellular  $\text{Ca}^{++}$  overload (Dejeans and others 2010). Intracellular and mitochondrial  $\text{Ca}^{++}$  overload starts during ischemia. However, reperfusion accelerates the process due to disruption of the plasma membrane, oxidative stress-induced damage to the sarcoplasmic reticulum, and mitochondrial re-energization (Hausenloy and Yellon 2013; Rodrigo and others 2013). Mitochondrial re-energization is the recovery of the mitochondrial membrane potential that causes the entry of  $\text{Ca}^{++}$  into the mitochondria and induces the opening of the mPTP (Fig 1.1). A severe microvascular dysfunction may limit adequate perfusion after reperfusion. This ‘no flow’ phenomenon occurs in about 30% of patients and associates with an increased incidence of acute MI, myocardial rupture, and death (Moens and others 2005).

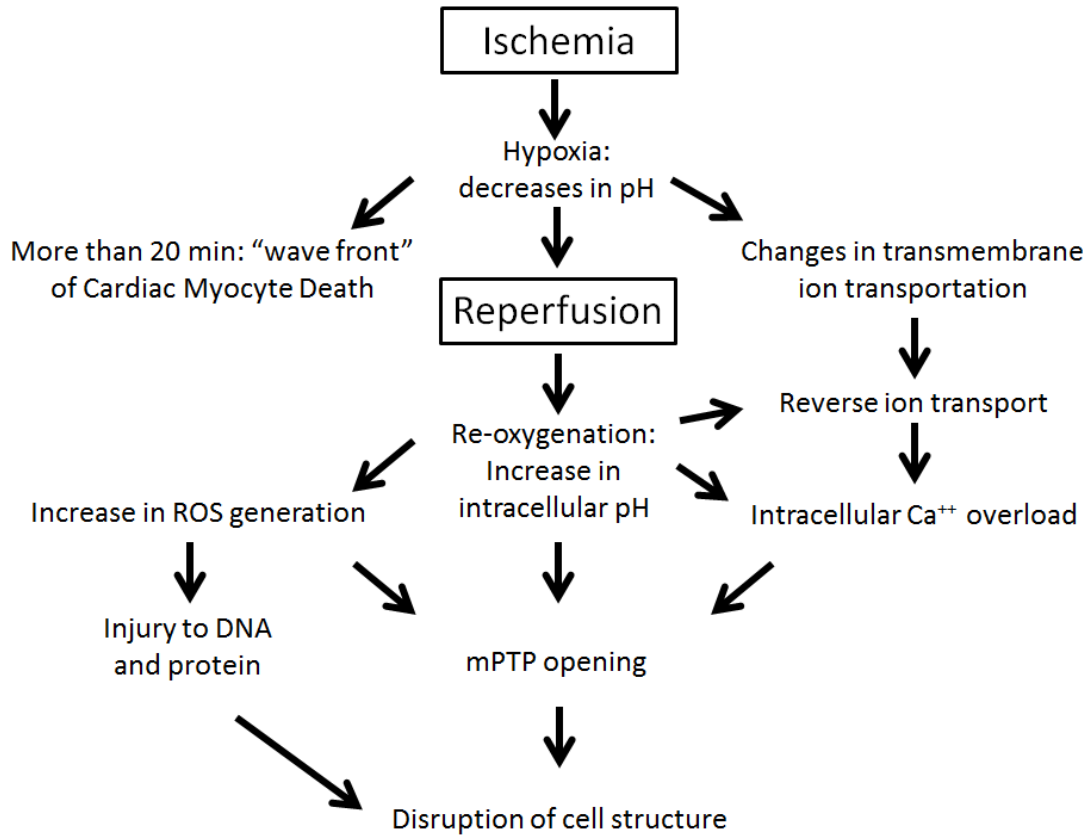


Figure 1.1. Cascade of Events Due to Ischemia/Reperfusion Injury. ROS, reactive oxygen species; mPTP, mitochondrial membrane permeability transition pore.

### $\beta$ -Adrenergic Receptor Stimulation and Heart Disease

Increased sympathetic nerve activity to the myocardium is a central feature in patients with heart failure (Hasking and others 1986), and chronic exposure to catecholamines is known to be toxic to cardiac myocytes (Rona 1985). Chronic  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation potentiates myocardial ischemia/reperfusion (I/R) injury (Hu and others 2006). Initial release of catecholamines by the sympathetic nervous system exerts important tonic effects on the biology of cardiac myocytes leading to increased contractility. However, chronic increase in sympathetic activity is known to have adverse effects in the heart. The efficacy of  $\beta$ -AR antagonist in improving the clinical outcome as well as benefit in long-term morbidity and mortality of

patients with chronic heart failure (Bristow 2000) has confirmed the importance of sympathetic nerve activity in the pathological remodeling, a process that leads to progressive left ventricular dilation and contractile dysfunction. Norepinephrine, a primary neurotransmitter of sympathetic nervous system, signals via its interaction with  $\alpha$ - and  $\beta$ -AR, a family of G protein-coupled receptors. Specific stimulation of  $\beta$ -AR using norepinephrine or isoproterenol ( $\beta$ -AR agonist) induces apoptosis in cardiac myocytes *in vitro* and *in vivo* (Singh and others 2001; Goldspink and others 2004; Krishnamurthy and others 2007). Increased myocyte apoptosis may influence the development of heart failure.

#### $\beta$ -AR-Stimulation and Cardiac Myocyte Apoptosis

Stimulation of  $\beta$ -AR induces apoptosis in cardiac myocytes *in vitro* and *in vivo* (Shizukuda and others 1998; Iwai-Kanai and others 1999; Zaugg and others 2000; Singh and others 2001). Three subtypes of  $\beta$ -AR have been identified ( $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ) (Hadcock and Malbon 1993), and all 3 types are expressed on cardiac myocytes (Devic and others 2001).  $\beta_1$ -AR is the most abundant receptor type in cardiac myocytes. However, the ratio of  $\beta_1$  to  $\beta_2$ -AR varies among species and under various pathophysiological states (Bristow 2000). Both  $\beta_1$ - and  $\beta_2$ -AR couple to the stimulatory G protein ( $G_{as}$ ) (Amin and others 2011). This coupling leads to activation adenylyl cyclase and production of cAMP a common second messenger.  $\beta_2$ -AR can also couple to a  $G_i$ , a different heterodimeric G-protein whose  $G_\alpha$  subunit inhibits adenylyl cyclase and synthesis of cAMP. However, this coupling may require phosphorylation of  $\beta_2$ -AR by cAMP-dependent protein kinase A (Daaka and others 1997). Stimulation of  $\beta_1$ -AR- $G_s$  and activation of PKA induces myocyte apoptosis (Communal and others 1999; Shizukuda and

Buttrick 2002), while stimulation of  $\beta_2$ -AR- $G_i$  and activation of phosphatidylinositol 3-kinase (PI3-kinase) and Akt inhibit apoptosis (Xiao 2001).

Mitogen activated protein kinases (MAPKs), a large family of serine-threonine kinases, have important functions as mediators of intracellular signal transduction (Figure 1.2). Three subgroups of MAPKs have been identified: c-jun N-terminal kinases (JNKs), p38 kinase, and ERK1/2 (Cargnello and Roux 2011).  $\beta$ -AR stimulation has been shown to activate these 3 subgroups of MAPKs (Communal and others 2000). Activation of JNKs plays a proapoptotic role (Remondino and others 2003). Activation of p38 kinase plays a protective role in  $\beta$ -AR-stimulated myocyte apoptosis, and inhibition of ERK1/2 using PD98059 had no effect on  $\beta$ -AR-stimulated apoptosis (Communal and others 2000). Superoxide dismutase/catalase-mimetics or catalase overexpression inhibited JNK activation and  $\beta$ -AR-stimulated apoptosis (Remondino and others 2003). Inhibition of mitochondrial permeability transition pore opening or caspase activation decreases  $\beta$ -AR-stimulated apoptosis (Remondino and others 2003). These studies suggest that  $\beta$ -AR-stimulated apoptosis in adult rat ventricular myocytes involves reactive oxygen species/JNK-dependent activation of the mitochondrial death pathway.  $\beta$ -AR stimulation also activates glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), and activation of GSK-3 $\beta$  plays a proapoptotic role in  $\beta$ -AR-stimulated apoptosis via the involvement of mitochondrial death pathway (Novikoff and others 1975). Adenoviral-mediated overexpression of constitutively active GSK-3 $\beta$  increased JNK phosphorylation, suggesting that GSK-3 $\beta$  may also act upstream in the activation of JNKs (Singh and others 2010).

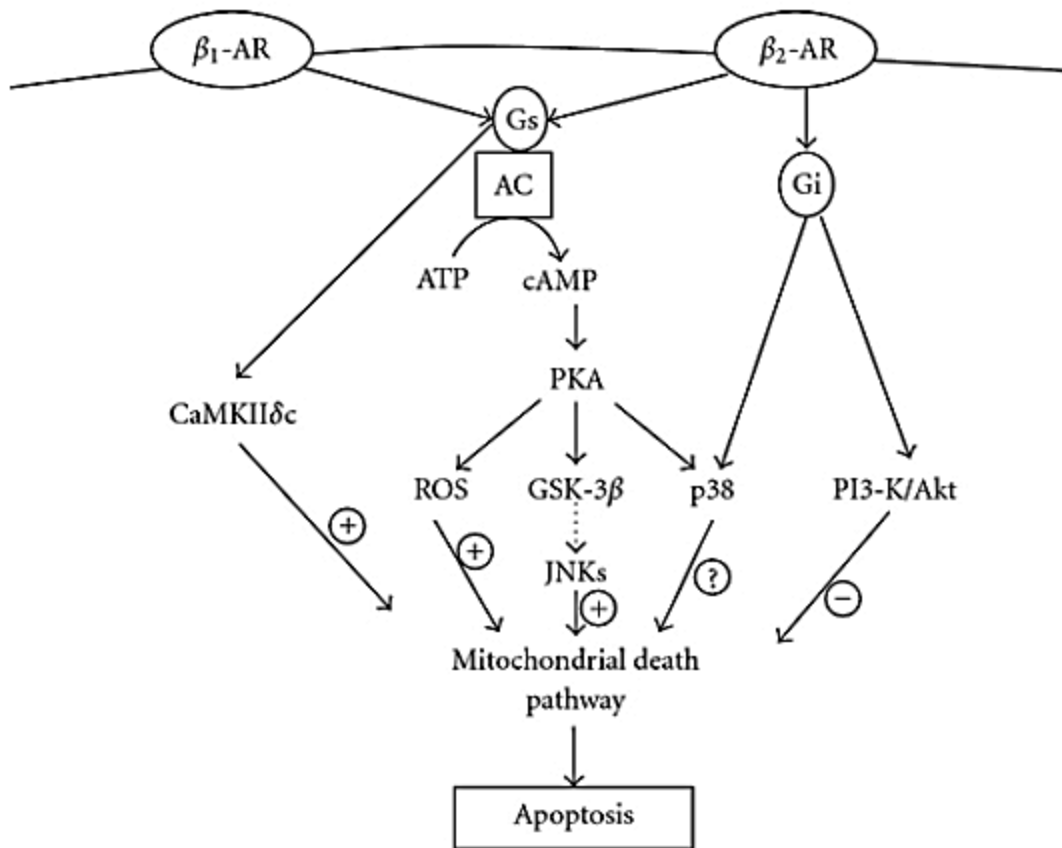


Figure 1.2 Summary Diagram Illustrating Signaling Pathways Involved in  $\beta$ -AR-Stimulated Cardiac Myocyte Apoptosis. AR: adrenergic receptors; JNKs: c-Jun-N-terminal kinase; GSK-3 $\beta$ : glycogen synthase kinase-3 $\beta$ ; ROS: reactive oxygen species; PKA: protein kinase A; AC: adenylyl cyclase; Gs: stimulatory G protein; Gi: inhibitory G protein; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; CaMKII $\delta$ : calcium calmodulin kinase II $\delta$ ; PI-3K: phosphatidylinositol 3-kinase. (Source Amin 2011)

### $\beta$ -AR-Stimulation and Myocardial Fibrosis

In heart failure the sympathetic system is overactivated and leads to formation of cardiac fibrosis, which contributes to the aggravation of cardiac function (Bos and others 2005). Cardiac fibrosis results from proliferation of interstitial fibroblasts and concomitant increased biosynthesis of ECM components. Isoproterenol ( $\beta$ -AR agonist) has been shown to produce increased fibrosis and hypertrophy (Krishnamurthy and others 2007). Greater cardiac fibrosis in

Brown Norway rats treated with isoproterenol correlates with the early and higher expression of proinflammatory factors (Copaja and others 2008). Isoproterenol-induced myocardial fibrosis is initiated by the appearance of interstitial edema and fibrillar collagen disruption and is followed soon thereafter by the formation of thinner collagen fibers that extend across muscle fibers and into which thicker collagen fibers become entwined in a crisscrossing pattern. Once formed, this mesh of collagen fibers encircles cardiac muscle (Pick and others 1989). It is also interesting to note that stimulation of  $\beta$ -AR increases expression and activity of MMP-2 and MMP-9 in cardiac myocytes and in the heart (Menon and others 2005; Krishnamurthy and others 2007). Thus, deposition of fibrosis may represent a complex function of both collagen synthesis and degradation.

### Ubiquitin

Ubiquitin is a small heat-stable protein (~ 8.5 kDa) found in all eukaryotic cells (Ciechanover and others 1980; Ozkaynak and others 1984). Its amino acid sequence is highly conserved between animal species with only 2-3 amino acid residues differing between yeast and human (Wilkinson and others 1986). Ubiquitin is a well-known posttranslational protein modifier and covalent ligation of ubiquitin to intracellular proteins is known as ubiquitination or ubiquitylation. This process is catalyzed by ubiquitin protein ligase systems (Bairoch 2000). The ubiquitin protein ligase system consist of 3 enzymes and leads to the degradation of proteins through the ubiquitin proteasome pathway (Hershko and Ciechanover 1998). In the ubiquitin-proteasome pathway ubiquitination is the signal for degradation of the target protein by the 26S proteasome (Hershko and Ciechanover 1998; Schwartz and Ciechanover 2009). Ubiquitination leads to a polyubiquitin tag and is required for protein degradation by the proteasome (Hicke

2001). Following phosphorylation, ubiquitination is the second most common posttranslational protein modification (Weissman 2001; Haataja and others 2002).

Ubiquitin is a normal plasma protein. Normal ubiquitin concentrations in human plasma and serum lies in the low nanomolar range ( $<10\text{nM}$  ;  $<100\text{ng/mL}$ ) (Okada and others 1993; Okada and others 1995). Ubiquitin is also detectable in cerebral spinal fluid (CSF), bronchoalveolar lavage fluid (BALF), seminal plasma, and urine. Ubiquitin concentrations are 3 to 5 folds lower in CSF than what is found in circulation. However, normal urine concentrations are slightly lower than serum concentrations (Majetschak and others 2003; Pisitkun and others 2004). Higher ubiquitin concentrations in systemic circulation have been reported in multiple diseases such as; allergic, autoimmune, infections, and kidney failure (Asseman and others 1994; Okada and others 1995; Akarsu and others 2001b; Majetschak and others 2003). Extracellular ubiquitin is proposed to have pleiotropic effects on hematopoiesis, leukocytes, and platelets and possess antimicrobial activity (Kagan and others 1977; Kagan and others 1979; Pancre and others 1991; Kieffer and others 2003).

The G protein-coupled receptor CXC chemokine receptor (CXCR)4 is abundantly expressed in immune cells and tissues, including cardiac fibroblast (Hu and others 2007). CXCR4 plays an important role in a variety of biological processes and is suggested to be involved in the pathophysiology of various disease processes such as cancer, HIV, ischemic myocardial injury, and angiogenesis (Tripathi and others 2013). Stromal derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) is the cognate ligand for CXCR4 that is implicated in homing of hematopoietic stem cells during wound repair (Chu and others 2010). SDF-1 $\alpha$  is shown to have cardioprotective effects against ischemia/reperfusion injury by promoting antiapoptotic program (Hu and others 2007). Interestingly, CXCR4 is identified as a receptor for UB in THP1 leukemia cell line (Saini and

others 2010). Using C-terminal truncated ubiquitin and ubiquitin mutants, Saini et al. provide evidence that the ubiquitin-CXCR4 interaction follows a 2-site binding mechanism in which the hydrophobic surfaces surrounding Phe-4 and Val-70 are important for receptor binding, whereas the flexible C terminus facilitates receptor activation (Saini and others 2011).

#### Extracellular UB and $\beta$ -AR-Stimulated Myocyte Apoptosis

As discussed above,  $\beta$ -AR stimulation induces apoptosis in adult cardiac myocytes. However,  $\beta$ -AR stimulation induces apoptosis only in a fraction ( $\sim 15$ – $20\%$ ) of myocytes, although all the cells are exposed to the same stimulus. Previously, a search to identify survival factor(s) secreted or released by myocytes that protect 80–85% of cells from apoptosis (in our lab) using 2-dimensional gel electrophoresis followed by MALDI TOF and MS/MS, led to identification of UB in the conditioned media of myocytes treated with  $\beta$ -AR agonist (isoproterenol) (Singh and others 2010). Inhibition of  $\beta_1$ -AR and  $\beta_2$ -AR subtypes inhibited  $\beta$ -AR-stimulated increases in extracellular levels of UB, whereas activation of adenylyl cyclase using forskolin mimicked the effects of  $\beta$ -AR stimulation. Pretreatment with UB inhibited  $\beta$ -AR-stimulated increases in apoptosis. Inhibition of phosphoinositide 3-kinase using wortmannin and LY-294002 prevented antiapoptotic effects of extracellular UB. UB pretreatment inhibited  $\beta$ -AR-stimulated activation of GSK-3 $\beta$  and JNK and increases in the levels of cytosolic cytochrome *c*. The use of methylated UB suggested that the antiapoptotic effects of extracellular UB are mediated via monoubiquitination. Together, these studies suggested that  $\beta$ -AR stimulation increases levels of UB in the conditioned media. Extracellular UB plays a protective role in  $\beta$ -AR-stimulated apoptosis, possibly via the inactivation of GSK-3 $\beta$ /JNK and mitochondrial pathways (Singh and others 2010).



### Specific Aims

The overall goal of the present study was to further understand the role extracellular ubiquitin plays in cardiac myocyte apoptosis, the signaling mechanisms involved, and the implications in the processes of cardiac remodeling. We hypothesized that extracellular ubiquitin modulates cardiac myocyte apoptosis and fibroblast phenotype and function, thereby modulating chronic  $\beta$ -AR receptor-stimulated and I/R-induced myocardial remodeling. The specific aims of the study were as follows: 1) investigate the in vivo role of exogenous ubiquitin in  $\beta$ -AR-stimulated myocardial remodeling with effects on LV function, fibrosis, and myocyte apoptosis; 2) study the role of extracellular ubiquitin in modulation of cardiac fibroblast phenotype and function; and 3) determine the effect of extracellular ubiquitin on the process of myocardial I/R injury at the cellular and tissue levels.

## CHAPTER 2

### EXOGENOUS UBIQUITIN MODULATES CHRONIC $\beta$ -ADRENERGIC RECEPTOR-STIMULATED MYOCARDIAL REMODELING: ROLE IN AKT ACTIVITY AND MATRIX METALLOPROTEINASE EXPRESSION

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## Abstract

$\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation increases extracellular ubiquitin (UB) levels, and extracellular UB inhibits  $\beta$ -AR-stimulated apoptosis in adult cardiac myocytes. This study investigates the role of exogenous UB in chronic  $\beta$ -AR-stimulated myocardial remodeling. L-isoproterenol (ISO; 400 $\mu$ g/kg/h) was infused in mice in the presence or absence of UB (1 $\mu$ g/g/h). Left ventricular (LV) structural and functional remodeling was studied 7 days after infusion. UB infusion enhanced serum UB levels. In most parts, UB alone had no effect on morphometric or functional parameters. Heart weight-to-body weight ratios were increased to a similar extent in ISO and UB+ISO groups. Echocardiographic analyses showed increased percent fractional shortening, ejection fraction and LV circumferential stress and fiber shortening velocity in ISO group. These parameters were significantly lower in UB+ISO vs ISO. Isovolumic contraction and relaxation times, and ejection time were significantly lower in ISO vs UB+ISO. The increase in the number of TUNEL-positive myocytes and fibrosis was significantly higher in ISO vs UB+ISO. Activation of Akt was higher, while activation of GSK-3 $\beta$  and JNKs was lower in UB+ISO vs ISO. Expression of MMP-2, MMP-9 and TIMP-2 was higher in UB+ISO vs ISO. In isolated cardiac fibroblasts, UB enhanced expression of MMP-2 and TIMP-2 in the presence of ISO. Neutralizing UB antibodies negated the effects of UB on MMP2 expression, while recombinant UB enhanced MMP-2 expression. UB activated Akt, and inhibition of Akt inhibited UB+ISO-mediated increases in MMP-2 expression. Thus, exogenous UB plays an important role in  $\beta$ -AR-stimulated myocardial remodeling with effects on LV function, fibrosis and myocyte apoptosis.

## Introduction

Ubiquitin (UB), a highly conserved protein of ~8.5 kDa, is found in all eukaryotic cells. The most important intracellular function of UB is to regulate protein turnover by the ubiquitin-proteasome pathway (15). The ubiquitin-proteasome pathway may regulate receptor internalization, hypertrophic response, apoptosis, and tolerance to ischemia and reperfusion in cardiac myocytes (56). UB is a normal constituent of plasma. Elevated levels of UB are described in the serum or plasma of patients with parasitic and allergic diseases (4), alcoholic liver disease (48), type 2 diabetes (1),  $\beta$ 2-Microglobulin amyloidosis (34) and chronic hemodialysis patients (2). Patients with traumatic brain injury are shown to have increased UB levels in the cerebrospinal fluid (28). Extracellular UB is proposed to have pleiotropic functions including regulation of immune response, anti-inflammatory and neuroprotective activities (27; 29; 36), as well as growth regulation and apoptosis control in hematopoietic cells (9). The biological functions of extracellular UB in the heart remain largely unexplored. Specifically, the role of exogenous UB in myocardial remodeling has not yet been investigated.

Sympathetic nerve activity increases in the heart during cardiac failure. Prolonged stimulation of the  $\beta$ -adrenergic neurohormonal axis contributes to the progression of heart failure and mortality in animal models and human patients (13; 43). Stimulation of  $\beta$ -adrenergic receptor ( $\beta$ -AR) increases expression and activity of matrix metalloproteinase (MMP-2 and MMP-9) in cardiac myocytes *in vitro* and *in vivo* (23; 31). It induces apoptosis in cardiac myocytes *in vitro* and *in vivo* (19; 42; 43; 55).  $\beta$ -AR-stimulated apoptosis in adult rat ventricular myocytes (ARVMs) is demonstrated to occur via the GSK-3 $\beta$ -/JNK-dependent mitochondrial death pathway (30; 39). Recently, we provided evidence that stimulation of  $\beta$ -AR increases

extracellular levels of UB in ARVMs, and extracellular UB plays a protective role in  $\beta$ -AR-stimulated apoptosis via the inactivation of GSK-3 $\beta$  and JNKs pathways (44).

Here, we investigated the *in vivo* role of exogenous UB in cardiac myocyte apoptosis and myocardial remodeling following chronic  $\beta$ -AR stimulation in mice. We report that exogenous UB plays an important role in  $\beta$ -AR-stimulated myocardial remodeling with effects on left ventricular function, fibrosis and myocyte apoptosis. It may inhibit myocyte apoptosis via the activation of Akt and inactivation of GSK-3 $\beta$  and JNKs, while inhibits fibrosis by modulating expression and activity of MMPs and TIMPs.

## Methods and Material

### Experimental Animals

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal protocol was approved by the University Committee on Animal Care. Animals were anesthetized using a mixture of isoflurane (2.5%) and oxygen (0.5 l/min) and the heart was excised following a bilateral cut in the diaphragm. Mice were euthanized by exsanguination. The studies were performed using male ICR mice (25-30 g; purchased from Harlan Lab.).

### Mice Treatment

Mice were randomly assigned to 4 groups (sham, ISO, UB+ISO and UB). The mice in UB+ISO and UB groups received intraperitoneal injection of UB (1  $\mu$ g/g; U6253; Sigma) 1h prior to pump implantation. The mice were infused with ISO (isoproterenol; 400  $\mu$ g/kg/h; ISO group), UB+ISO (1  $\mu$ g/g/h+400  $\mu$ g/kg/h; UB+ISO group) and UB (1 $\mu$ g/g/h; UB group) at the

rate of 1.0 µl/h for 7 days using mini-osmotic pumps (Alzet Corp.). L-isoproterenol and UB were dissolved in acidified isotonic saline (0.001 N HCl). Sham animals were infused with acidified isotonic saline solution. The dose of ubiquitin and ISO were selected based on previously published reports (14; 23; 42).

### Echocardiography

Transthoracic two-dimensional M-mode echocardiogram and pulsed wave Doppler spectral tracings were obtained using a Toshiba Aplio 80 Imaging System (Tochigi, Japan) equipped with a 12 MHz linear transducer (12; 23). Echocardiographic procedures were performed prior to and 7 days after implantation of pumps. The animals were anesthetized during procedure using a mixture of isoflurane (1.5%) and oxygen (0.5 l/min), and their body temperature maintained at ~37°C using a heating pad. M-mode tracings were used to measure LV end-systolic diameter (LVESD) and end-diastolic diameter (LVEDD). Percent fractional shortening (%FS) and ejection fraction (EF%) were calculated as described (12; 23). Doppler tracings of mitral and aortic flow were used to measure isovolumic relaxation time (IVRT), isovolumic contraction time (IVCT) and ejection time (ET). LV circumferential stress and fiber shortening velocity (Vcf) was calculated as  $Vcf = (LVEDD - LVESD) / LVEDD \times LVET$ , where LVEDD and LVESD are LV diastolic and systolic diameters, respectively, and LVET is LV ejection time (38). All echocardiographic assessments were performed by the same investigator blinded to the treatments. A second person also performed measurements on a separate occasion using the same recordings with no significant differences in interobserver variability.

### Morphometric Analyses

Animals were euthanized and the hearts were arrested in diastole using KCl (30 mM) followed by perfusion fixation with 10% buffered formalin. Cross sections (4  $\mu\text{m}$  thick) were stained with Masson's trichrome for the measurement of fibrosis using Bioquant image analysis software (Nashville, TN).

### Measurement of Serum UB Levels

Serum UB levels, 7 days after ISO-infusion, were measured using ELISA kit (Novatein Biosciences).

### Myocyte Cross-Sectional Area

Heart sections (4  $\mu\text{m}$  thick) were deparaffinized and stained with TRITC-labeled wheat germ agglutinin (WGA). The sections were visualized using fluorescent microscopy (20X; Nikon) and images were recorded using Retiga 1300 color-cooled camera. Suitable area of the sections was defined as the one with nearly circular capillary profiles.

### TUNEL Staining

To measure apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining followed by Hoechst 33258 staining was carried out on 4  $\mu\text{m}$ -thick paraffin-embedded sections as per manufacturer's instructions (Cell death detection assay kit, Roche). To identify apoptosis associated with cardiac myocytes, the sections were immunostained using  $\alpha$ -sarcomeric actin antibodies (1:50; 5C5 clone, Sigma, St Louis, MO). Hoechst 33258 (10  $\mu\text{M}$ ; Sigma, St Louis, MO) staining was used to count the total number of

nuclei. TUNEL-positive nuclei that were clearly seen within cardiac myocytes were counted. The number of apoptotic cardiac myocyte nuclei was counted and index of apoptosis was calculated as the percentage of apoptotic myocyte nuclei/total number of nuclei.

### Fibroblast Isolation and Treatment

Adult rat cardiac fibroblasts were isolated as described (53). The cells were grown to confluence and serum-starved for 48 h before use. First and second passage cells were used for all the experiments. The cells were pretreated with UB (10 µg/ml; Sigma) for 30 min followed by treatment with ISO for indicated time points. To neutralize effects of UB, cells were pretreated with anti-UB antibodies (5 µg/ml; U5379; Sigma) for 30 min. To verify effects of UB, cells were pretreated with recombinant UB (rUB; 10 or 100 µg/ml; Boston Biochem) for 30 min followed by treatment with ISO for 48 h. To inhibit Akt, cells were pretreated with MK-2206 (1 µM; Chemie Tek) for 30 min, then with UB (10 µg/ml) for 30 min followed by treatment with ISO.

### Western Analysis

LV tissue lysates were prepared in RIPA buffer [10 mM Tris-HCl (pH 7.2), 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride] using a tissue homogenizer. Cell lysates were prepared using lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride]. Equal amounts of total proteins (70 µg from tissue lysates or 20 µg from cell lysates) were resolved on 10% SDS-polyacrylamide gels. The proteins were transferred onto PVDF membrane. The blots were then probed with primary antibodies directed



against p-Akt (1:1000; Cell Signaling Tech.), p-GSK-3 $\beta$  (1:1000; Cell Signaling Tech.), p-JNKs (1:1000; Millipore), p-ERK1/2 (1:2000; Cell Signaling Tech.), MMP-2 (1:5000; Millipore), MMP-9 (1:5000; Millipore), or TIMP-2 (1:1000; Santa Cruz) and appropriate secondary antibodies. Membranes were then stripped and probed with Akt, GSK-3 $\beta$ , JNKs, ERK1/2, actin (Chemicon) or GAPDH (Santa Cruz) antibodies to normalize protein loading. Band intensities were quantified using Kodak photo documentation system (Eastman Kodak Co.).

### In-gel Zymography

Gelatin in-gel zymography in the concentrated conditioned media of fibroblasts (2  $\mu$ g) or LV (50  $\mu$ g) lysates was performed as described (53). Clear and digested regions representing MMP-2 activity were quantified using a Kodak documentation system.

### Statistical Analysis

Data are expressed as the mean  $\pm$  SEM. Data was analyzed using Student's *t* test or a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Probability (P) values of <0.05 were considered to be significant.

## Results

### Morphometric Studies

None of the animals died during the course of the experiment. No significant change in body weight (BW) in any group was observed during the experiment. ISO-infusion increased heart weight (HW) as well as the HW/body weight (HW/BW) ratios in ISO and UB+ISO groups

as compared to sham ( $p<0.05$ ; Table 2.1) with no significant difference between the two ISO groups. UB alone had no effect on HW or HW/BW ratio.

Table 2.1. Morphometric Measurements

Parameters	SHAM (n=6)	ISO (n=6)	UB+ISO (n=6)	UB (n=6)	P value
BW (g)	30.27±1.00	31.95±0.86	31.60±0.64	31.00±0.76	NS
HW (mg)	129.7±6.74	167.4±8.98*	178.6±7.46*	144.5±10.8	*<0.01
HW/BW (mg/g)	4.29±0.21	5.22±0.17*	5.65±0.21*	4.65±0.29	*<0.01

HW, heart weight; BW, body weight. Values are mean±SEM; \*comparison versus SHAM group; NS, not significant.

### Serum UB Levels

Measurement of serum UB levels using ELISA demonstrated presence of 16.2±1.3ng/ml UB in sham animals. ISO had no effect on serum UB levels. Serum UB levels were increased in UB-infused groups when compared to sham or ISO groups ( $P<0.05$ ; Fig 2.1). However, the increase in serum UB levels was higher in UB alone when compared to UB+ISO group.

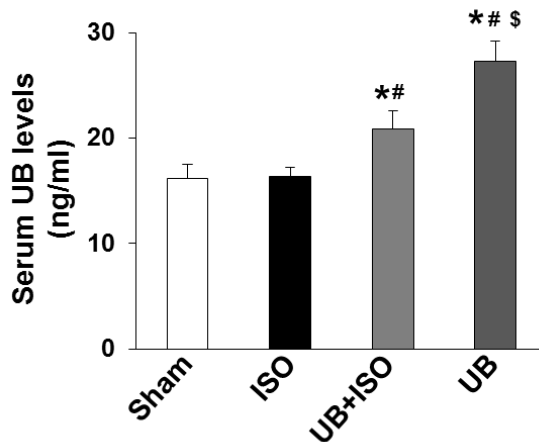


Figure 2.1. Serum UB Levels. UB infusion increases serum UB levels. \* $P<0.05$  versus sham; # $P<0.05$  versus ISO; \$ $P<0.05$  vs UB+ISO; n=5-6.

## Echocardiographic Measurements

M-mode echocardiography showed no significant difference in LVESD, LVEDD, %FS and EF% between sham and UB groups. ISO-infusion decreased LVESD in ISO, not in UB+ISO, group. LVEDD remained unchanged between the two groups. %FS, EF% and Vcf were increased in ISO, not in UB+ISO, group when compared to sham. In fact, %FS, %EF and mean Vcf were significantly lower in UB+ISO when compared to ISO group (Table 2.2).

Table 2.2. M-mode Echocardiographic Measurements

Parameters	SHAM (n=6)	ISO (n=6)	UB+ISO (n=6)	UB (n=5)	P value
LVESD (mm)	2.73±0.04	2.36±0.06*	2.78±0.06 <sup>#</sup>	2.74±0.16	*<0.05; <sup>#</sup> <0.05
LVEDD (mm)	3.90±0.08	3.89±0.02	4.11±0.06	3.97±0.16	NS
%FS	30.01±0.99	39.26±1.55*	32.50±1.03 <sup>#</sup>	31.31±1.88	*<0.01; <sup>#</sup> <0.05
EF%	57.73±1.39	70.07±2.02*	61.14±1.45 <sup>#</sup>	59.51±2.84	*<0.01; <sup>#</sup> <0.05
Mean Vcf (circ/sec)	5.99±0.23	10.10±0.62*	6.97±0.28 <sup>#</sup>	6.09±0.39	*<0.001; <sup>#</sup> <0.001

Values are mean±SEM; LVESD, LV end-systolic diameter; LVEDD, LV end-diastolic diameter; %FS, percent fractional shortening; EF%, percent ejection fraction; Vcf, mean velocity of circumferential shortening; \*comparison versus SHAM group; <sup>#</sup>comparison versus ISO group; NS, not significant.

Doppler tracings revealed no difference in any parameters between sham and UB groups (Table 2.3). ISO-infusion decreased IVRT, IVCT, and ET in both groups when compared to sham. However, decrease in these parameters was significantly lower in ISO when compared to UB+ISO group (p<0.05; Table 2.3). ISO-infusion increased heart rates in both groups with no significant difference between ISO and UB+ISO groups.

Table 2.3. Doppler Echocardiographic Measurements

Parameters	SHAM (n=6)	ISO (n=6)	UB+ISO (n=6)	UB (n=5)	P value
IVRT (ms)	12.41±0.01	5.07±0.01*	7.26±0.01* <sup>#</sup>	12.98±0.01	*<0.05; #<0.01
IVCT (ms)	11.98±0.01	5.19±0.01*	7.67±0.01* <sup>#</sup>	13.11±0.01	*<0.01; #<0.001
ET (ms)	50.22±0.01	39.07±0.01*	46.70±0.01* <sup>#</sup>	51.47±0.01	*<0.05; #<0.001
HR (bpm)	428.37±19.36	572.87±23.7*	506.98±17.98*	388.64±18.31	*<0.05

Values are mean±SEM; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; ET, ejection time ; HR, heart rate ; \*comparison versus SHAM group; <sup>#</sup>comparison versus ISO group; NS, not significant.

### Fibrosis, Apoptosis and Hypertrophy

ISO-infusion increased fibrosis in both groups (Fig 2.2A). Quantitative analysis of trichrome-stained sections showed that the increase in fibrosis was significantly lower in the UB+ISO group when compared to ISO (percent fibrosis, WT-sham,  $0.17 \pm 0.05$ ; ISO,  $1.78 \pm 0.15^*$ ; UB+ISO,  $1.09 \pm 0.16^{*#}$ ; UB,  $0.29 \pm 0.13$ ; \*P<0.01 vs Sham; <sup>#</sup>P<0.05 vs ISO; n = 5-6; Fig 2.2B).

ISO-infusion increased the number of TUNEL-positive myocytes in both groups. However, the percentage of apoptotic myocytes was significantly lower in UB+ISO group when compared to ISO (percentage of apoptotic myocyte nuclei/total number of nuclei; Sham,  $0.07 \pm 0.03$ ; ISO,  $0.35 \pm 0.07^*$ ; UB+ISO,  $0.15 \pm 0.05^{\#}$ ; UB,  $0.02 \pm 0.01$ ; \*P<0.01 vs Sham; <sup>#</sup>P<0.05 vs ISO; n = 3-5; Fig 2.2C).

ISO-infusion increased myocyte cross-sectional area to a similar extent in both groups when compared to sham ( $\mu\text{m}^2$ ; Sham,  $137.5 \pm 8.5$ ; ISO,  $297.4 \pm 9.2^*$ ; UB+ISO,  $227.3 \pm 28.5^*$ ; UB,  $149 \pm 4.8$ ; \*P<0.01 vs Sham; n = 3-4; Fig 2.2D).

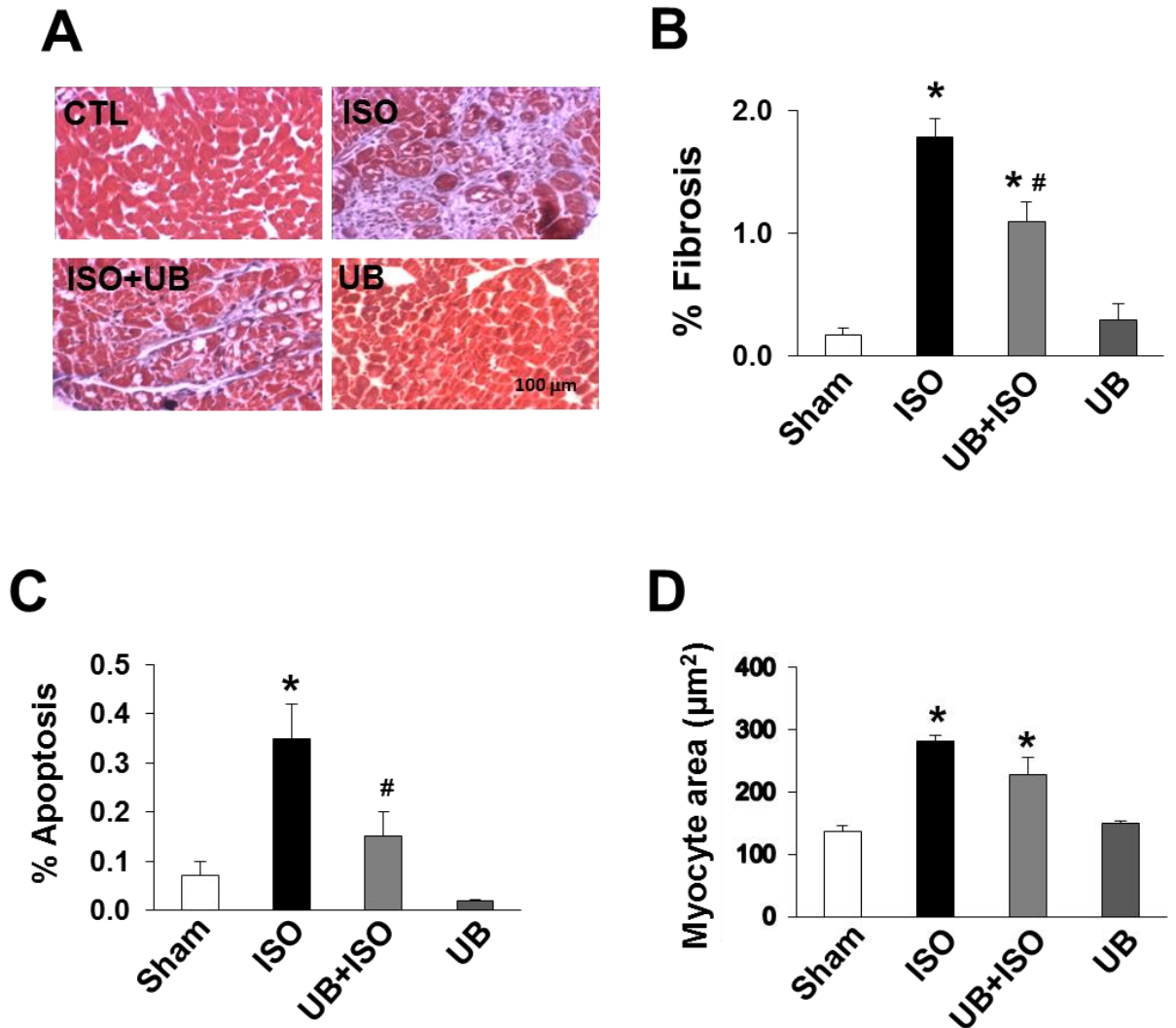


Figure 2.2. Myocardial Remodeling 7 Days after ISO-Infusion. A & B) Analysis of fibrosis. Panel A exhibits Masson's trichrome-stained sections 7 days after ISO-infusion. Panel B shows quantitative analysis of fibrosis. \* $P < 0.01$  versus sham; # $P < 0.05$  versus ISO;  $n = 5-6$ . C) Quantitative analysis of TUNEL-stained myocytes 7 days after ISO-infusion. \* $P < 0.01$  versus sham; # $P < 0.05$  versus ISO;  $n = 3-5$ . D) Quantitative analysis of myocyte cross-sectional area. \* $P < 0.01$  versus sham;  $n = 3-4$ .

## Activation of Akt and GSK-3 $\beta$

Previously, we have provided evidence that inhibition of PI3-kinase inhibits the protective effects of UB in  $\beta$ -AR-stimulated apoptosis in ARVMs (44). PI3-kinase activates Akt and activation of Akt plays an anti-apoptotic role (22). Western blot analysis of LV lysates using phospho-specific anti-Akt antibodies showed a significant increase in Akt phosphorylation (activity) in both ISO and UB+ISO groups when compared to sham. However, the increase in Akt activity was significantly higher in UB+ISO group when compared to ISO ( $p < 0.05$ ; Fig 2.3A). UB alone had no effect on Akt activity.

Activation of GSK-3 $\beta$  plays a pro-apoptotic role in  $\beta$ -AR-stimulated apoptosis (30). Phosphorylation of an N-terminal serine residue (serine-9) inactivates GSK-3 $\beta$ . Akt is one of the upstream kinases involved in phosphorylation (serine-9) and inactivation of GSK-3 $\beta$  (16). Western blot analysis of LV lysates using phospho-specific anti-GSK-3 $\beta$  antibodies showed decreased phosphorylation (activation) of GSK-3 $\beta$  in ISO, not in UB+ISO, group. GSK-3 $\beta$  phosphorylation was significantly higher in UB+ISO when compared to ISO group (WT-sham,  $0.76 \pm 0.1$ ; ISO,  $0.46 \pm 0.04^*$ ; UB+ISO,  $0.64 \pm 0.04^\#$ ; UB,  $0.57 \pm 0.1$ ;  $*P < 0.05$  vs Sham;  $^\#P < 0.05$  vs ISO;  $n = 4-7$ ; Fig 2.3B). UB alone had no effect on GSK-3 $\beta$  phosphorylation.

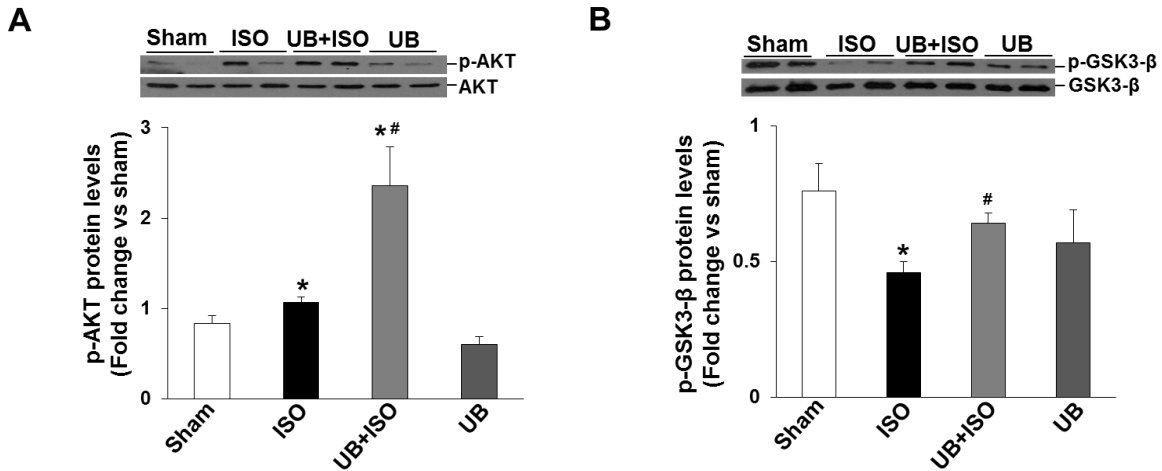


Figure 2.3. Activation of Akt and GSK-3 $\beta$  in the Heart. Total LV lysates (70  $\mu$ g), were analyzed by western blot using phospho-specific Akt (serine-473), total Akt, phospho-specific GSK-3 $\beta$  (serine-9) and total GSK-3 $\beta$  antibodies. The lower panels exhibit mean data normalized to total Akt or GSK-3 $\beta$ . A) Phosphorylation (activation) of Akt. \*P<0.05 versus sham; #P<0.05 versus ISO; n = 6. B) Phosphorylation (inactivation) of GSK-3 $\beta$ . \*P<0.05 versus sham; #P<0.01 versus ISO; n = 4-7.

#### Activation of JNKs and ERK1/2

$\beta$ -AR-stimulated activation of c-Jun NH<sub>2</sub>-terminal kinase (JNK) pathway is demonstrated to play a pro-apoptotic role in  $\beta$ -AR-stimulated apoptosis (39). Previously, we have provided evidence that active GSK-3 $\beta$  may act upstream in the activation of JNKs (44). Western blot analysis of LV lysates using phospho-specific anti-JNKs antibodies showed increased phosphorylation (activation) of JNKs in ISO, not in UB+ISO, group. Activation of JNKs was significantly lower in UB+ISO group when compared to ISO (WT-sham,  $0.68 \pm 0.16$ ; ISO,  $4.32 \pm 0.35$ ; UB+ISO,  $1.88 \pm 0.59^{\#}$ ; UB,  $0.99 \pm 0.43$ ; \*P<0.05 vs Sham;  $^{\#}$ P<0.05 vs ISO; n = 4-7; Fig 2.4A). UB alone had no effect on JNKs phosphorylation. ISO in the presence or absence of UB had no effect on the activation of ERK1/2 (Fig 2.4B).

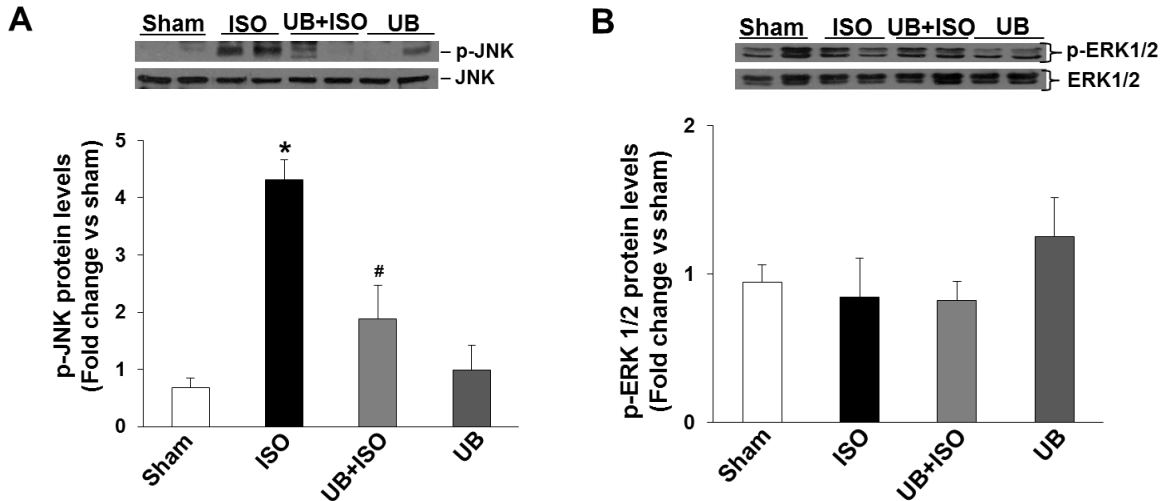


Figure 2.4. Activation of JNKs and ERK1/2 in the Heart. Total LV lysates (70  $\mu$ g), were analyzed by western blot using phospho-specific JNKs, total JNKs, phospho-specific ERK1/2 and total ERK1/2. The lower panels exhibit mean data normalized to total JNKs or ERK1/2. A) Phosphorylation (activation) of JNKs. \* $P < 0.05$  versus sham; # $P < 0.05$  versus ISO;  $n = 6$ . B) Phosphorylation (activation) of ERK1/2;  $n = 5$ .

#### Expression and Activation of MMP-2 and MMP-9

MMPs (MMP-2 and MMP-9) play a significant role in myocardial fibrosis and remodeling (46). Western blot analysis of LV lysates demonstrated increased expression (protein levels) of MMP-2 in both ISO groups (Fig 2.5A). However, the increase in MMP-2 expression was significantly higher in UB+ISO group when compared to ISO. In gel zymography showed increased MMP-2 activity in both ISO groups. However, the increase in MMP-2 activity was significantly higher in UB+ISO group when compared to ISO (Fig 2.5B). Increased MMP-9 expression was only observed in UB+ISO group (WT-sham,  $0.79 \pm 0.1$ ; ISO,  $0.73 \pm 0.1$ ; UB+ISO,  $1.16 \pm 0.1^{* \#}$ ; UB,  $0.66 \pm 0.2$ ; \* $P < 0.05$  vs Sham; # $P < 0.05$  vs ISO;  $n = 6$ ; Fig 2.6A). In gel zymography failed to show active MMP-9 bands in the LV lysates from these groups (data not shown).



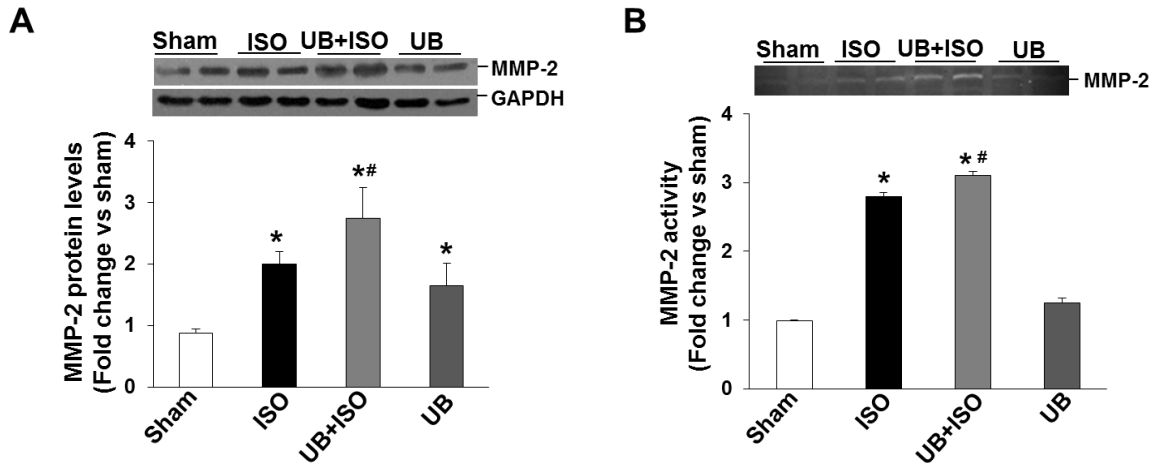


Figure 2.5. Expression and Activation of MMP-2 in the Heart. Total LV lysates (70µg) were analyzed by western blot (using anti-MMP-2 antibodies) or gelatin in-gel zymography. A) Expression of MMP-2. GAPDH immunostaining indicates protein loading. \*P<0.05 versus sham; #P<0.05 versus ISO; n = 6. B) MMP-2 activity. \*P<0.01 versus sham; #P<0.05 versus ISO; n = 4.

#### Expression of TIMP-2 and TIMP-4

Tissue inhibitors of MMPs (TIMPs) play an important role in regulation of MMPs activity (32; 51). Western blot analysis of LV lysates demonstrated that ISO alone has no effect on TIMP-2 protein levels. However, UB alone or in the presence of ISO significantly increased TIMP-2 protein levels (Fig 2.6B). ISO in the presence or absence of UB had no effect on protein levels of TIMP-4. UB alone increased TIMP-4 protein levels when compared to ISO or UB+ISO groups (data not shown).

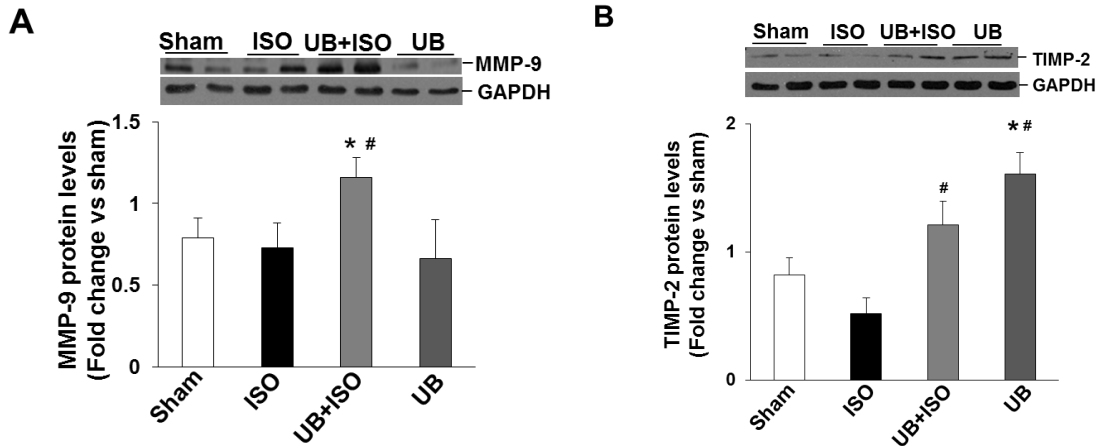


Figure 2.6. Expression of MMP-9 and TIMP-2 in the Heart. Total LV lysates (70µg) were analyzed by western blot using anti-MMP-9 or anti-TIMP-2 antibodies. GAPDH immunostaining indicates protein loading. A) Expression of MMP-9. \*P<0.05 versus sham; #P<0.05 versus ISO; n = 6. B) Expression of TIMP-2. \*P<0.01 versus sham; #P<0.05 versus ISO; n = 4.

### UB Modulates Expression of MMPs and TIMP-2 in Cardiac Fibroblasts

ISO treatment increased MMP-2 expression in adult cardiac fibroblasts as analyzed by western blots. However, the increase in MMP-2 expression was significantly higher in UB+ISO group when compared to ISO (Fig 2.7A). In-gel zymography showed a trend towards increased MMP-2 activity in response to ISO. However, MMP-2 activity was significantly higher in UB+ISO group as compared to control and ISO-treated samples (Fig 2.7B). These effects of UB on MMP-2 expression were negated by pretreatment with a neutralizing anti-UB antibodies (Fig 2.7C). Treatment with rUB also enhanced ISO-mediated increases in MMP-2 expression (Fig 2.7D). However, the effects were observed at a higher concentration (100 µg/ml). MMP-9 expression tended to be higher in UB+ISO group when compared to sham (P=0.06; n=6; Fig 2.8A). ISO treatment had no effect on TIMP-2 protein levels. However, TIMP-2 protein levels were significantly higher in UB+ISO samples when compared to control or ISO-treated samples (Fig 2.8B).

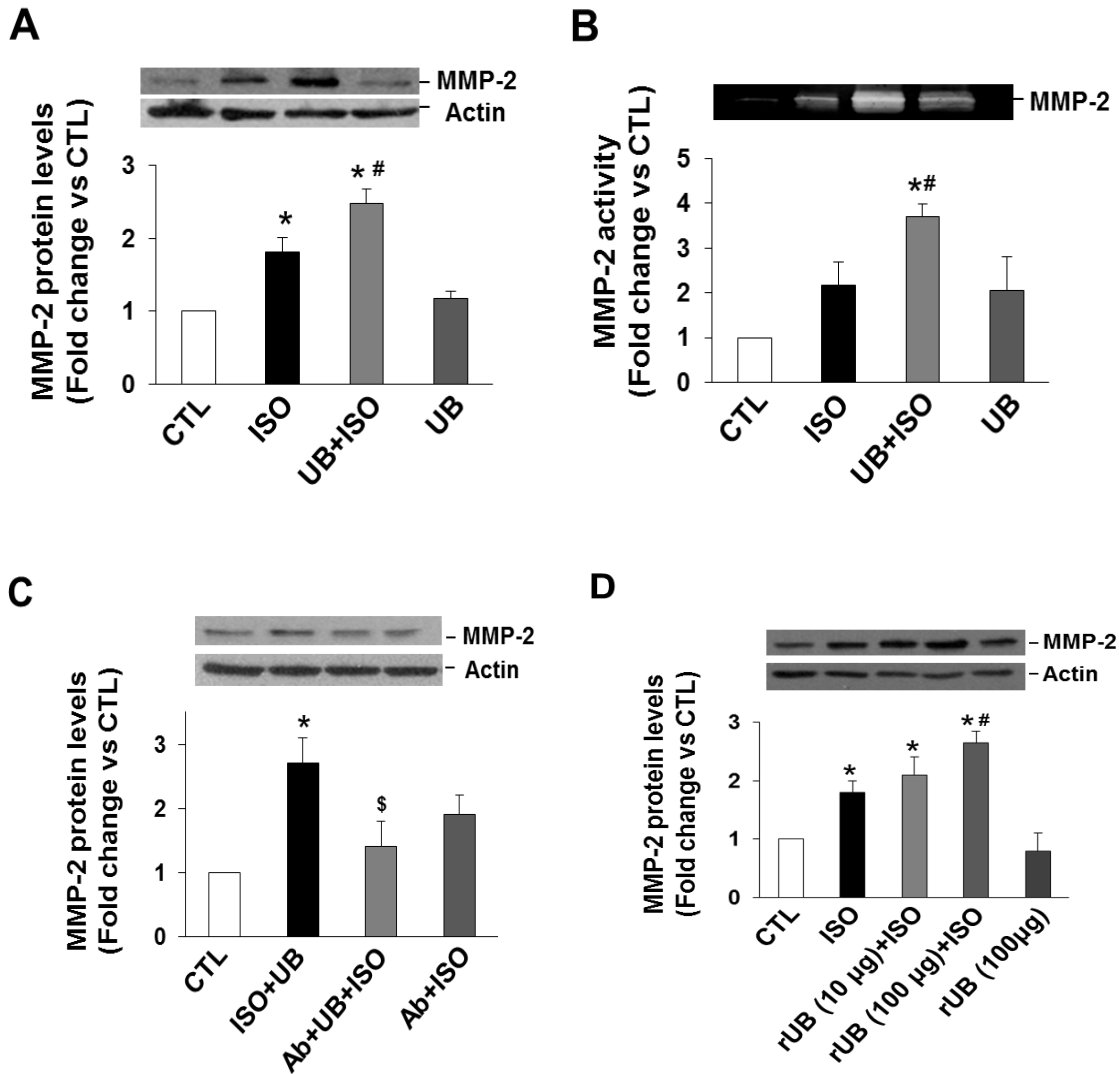


Figure 2.7. Expression and Activity of MMP-2 in Cardiac Fibroblasts. A & B) Cardiac fibroblasts were pretreated with UB (10 µg/ml) for 30 min followed by treatment with ISO (10 µM) for 48 h. A) Cell lysates (20 µg) were analyzed by western using anti-MMP-2 antibodies. \*P<0.05 versus control (CTL); #P<0.05 versus ISO; n = 11. B) Concentrated conditioned media (2 µg) were analyzed by gelatin in-gel zymography. \*<0.01 versus CTL; #P<0.05 versus ISO; n = 3. C) Cells were pretreated with neutralizing anti-UB antibody (Ab) for 30 min followed by treatment with UB for 30 min. The cells were then treated with ISO for 48 h. Cell lysates (20 µg) were analyzed by western using anti-MMP-2 antibodies. \*P<0.05 versus CTL; \$P<0.05 versus UB+ISO; n=3. D) Cells were pretreated with rUB (10 or 100 µg/ml) for 30 min followed by treatment with ISO for 48 h. Cell lysates (20 µg) were analyzed by western using anti-MMP-2 antibodies. \*P<0.05 versus CTL; #P<0.05 versus ISO; n=3. Actin immunostaining indicates protein loading.

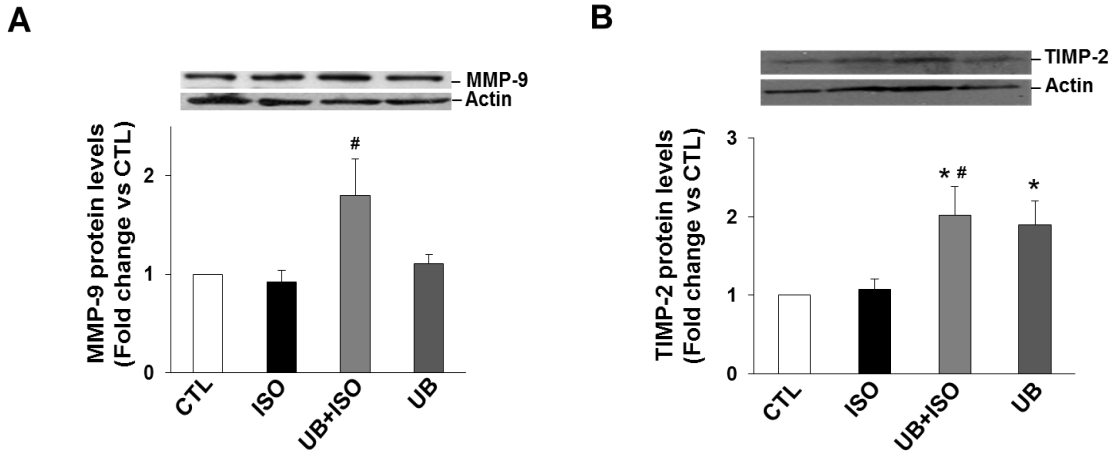


Figure 2.8. Expression of MMP-9 and TIMP-2. Cardiac fibroblasts were pretreated with UB (10  $\mu\text{g/ml}$ ) for 30 min followed by treatment with ISO (10  $\mu\text{M}$ ) for 48 h. Cell lysates (20  $\mu\text{g}$ ) were analyzed by western blot using anti-MMP-9 or anti-TIMP-2 antibodies. Actin immunostaining indicates protein loading. A) Expression of MMP-9. <sup>#</sup> $P=0.06$  versus ISO;  $n = 6$ . B) Expression of TIMP-2. <sup>\*</sup> $P<0.05$  versus CTL; <sup>#</sup> $P<0.05$  versus ISO;  $n = 3-4$ .

#### UB Activates Akt, and Inhibition of Akt Inhibits MMP-2 Expression in Cardiac Fibroblast

To investigate if UB alone or in combination with ISO activates Akt, cardiac fibroblasts were pretreated with UB followed by treatment with ISO for 15 min. Analysis of cell lysates using phospho-specific antibodies showed that UB or ISO alone had no effect on Akt activation. However, UB in the presence of ISO significantly increased Akt activity as compared to control or ISO-treated samples (Fig 2.9A). MMP-2 activation is shown to be dependent on activation of Akt in the heart (45). Inhibition of Akt using MK-2206 (18; 37) significantly inhibited UB+ISO-mediated increases in MMP-2 expression (fold change vs CTL; UB+ISO,  $1.71 \pm 0.1^*$ ; MK+UB+ISO,  $1.23 \pm 0.2^{\#}$ ; MK,  $0.59 \pm 0.3^*$ ; <sup>\*</sup> $P<0.05$  vs Sham; <sup>#</sup> $P<0.05$  vs UB+ISO;  $n = 5$ ; Fig 2.9B).

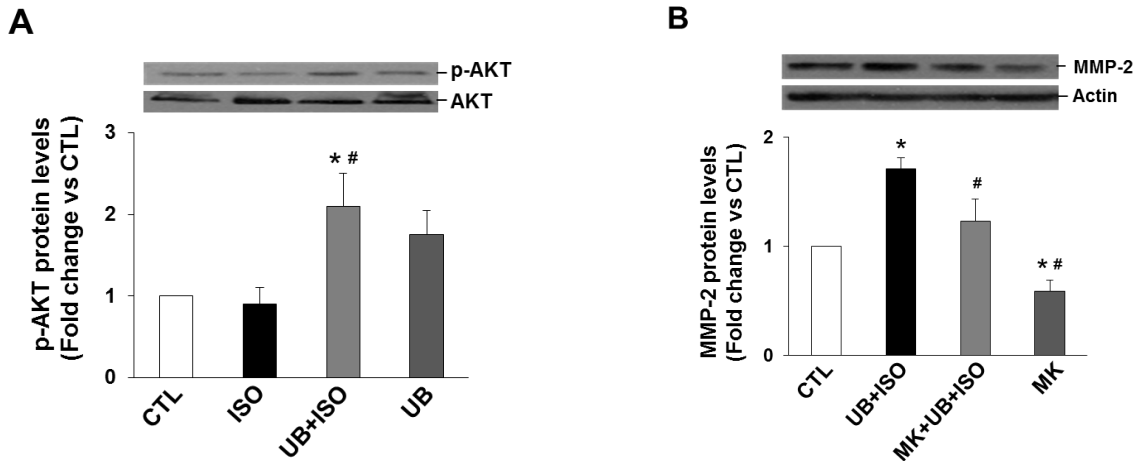


Figure 2.9. Activation of Akt and its Role in MMP-2 Expression. Cardiac fibroblasts were pretreated with UB (10  $\mu\text{g/ml}$ ) for 30 min followed by treatment with ISO (10  $\mu\text{M}$ ) for 15 min to measure Akt activation (A). To measure MMP-2 expression, cardiac fibroblasts were pretreated with MK-2206 (MK; 1  $\mu\text{M}$ ) for 30 min, then with UB (10  $\mu\text{g/ml}$ ) for 30 min followed by treatment with ISO (10  $\mu\text{M}$ ) for 48 h (B). Cell lysates (20  $\mu\text{g}$ ), were analyzed by western blot using phospho-specific anti-Akt (serine-473) or anti-MMP-2 antibodies. The lower panels exhibit mean data normalized to total Akt or actin. A) Phosphorylation (activation) of Akt. \* $P < 0.05$  versus CTL; # $P < 0.05$  versus ISO;  $n = 5$ . B) Expression of MMP-2. \* $P < 0.05$  versus CTL; # $P < 0.05$  versus UB+ISO;  $n = 5$ .

### Discussion

Previously, we have provided evidence that  $\beta$ -AR stimulation increases levels of extracellular UB and treatment with UB plays a protective role in  $\beta$ -AR-stimulated apoptosis in ARVMs (44). This is the first study investigating the role of exogenous UB in the heart, specifically in response to chronic  $\beta$ -AR stimulation. Here we confirm our previous finding of an anti-apoptotic function for UB *in vivo*, and show that UB plays an important role in  $\beta$ -AR-stimulated myocardial remodeling with effects on left ventricular function, fibrosis, and myocyte apoptosis. UB infusion enhanced serum UB levels. Exogenous UB depressed  $\beta$ -AR-stimulated increases in systolic and diastolic functional parameters of the heart. Increase in cardiac myocyte apoptosis and myocardial fibrosis was significantly lower in the presence of exogenous UB.

Exogenous UB enhanced activation of anti-apoptotic kinase Akt, while decreasing the activation of pro-apoptotic kinases, GSK-3 $\beta$  and JNKs. It increased protein levels of MMP-2, MMP-9 and TIMP-2 in the presence of ISO. In isolated cardiac fibroblast, UB enhanced expression of MMP-2 and TIMP-2. It activated Akt and inhibition of Akt decreased MMP-2 expression.

Sympathetic nerve activity increases in the heart during cardiac failure. Prolonged stimulation of the  $\beta$ -adrenergic neurohormonal axis contributes to the progression of heart failure and mortality in animal models and human patients (3; 13). Catecholamines, released during heightened adrenergic drive, accumulate in the interstitial space of the heart (6; 8). This accumulation of catecholamines may contribute to left ventricular dysfunction (10). UB is a normal constituent of plasma or serum (11; 29; 47). Levels of UB increase in plasma or serum of patients under a variety of pathological conditions (26). However, the role of plasma UB in the heart has not yet been investigated. Previously, we have shown that  $\beta$ -AR stimulation increases extracellular levels of UB in ARVMs (44). Here we observed basal presence of UB in the serum of ICR mice. Infusion of UB enhanced serum UB levels in the presence or absence of  $\beta$ -AR stimulation. It was interesting to note that serum UB levels were lower in the presence of  $\beta$ -AR stimulation, and  $\beta$ -AR stimulation alone had no effect on serum UB levels. These observations suggest the possibility that  $\beta$ -AR stimulation may enhance UB absorption *in vivo*. Isoproterenol is shown to increase cardiac output without affecting renal blood flow in dog, human, and lamb (5; 40). However, the possibility of increased renal blood flow and renal clearance of UB in the presence of isoproterenol cannot be ruled out. A significant finding of this study is that exogenous UB has the potential to play an important role in the remodeling process of the heart by affecting  $\beta$ -AR-stimulated increases in myocyte apoptosis and myocardial fibrosis.

Ventricular hypertrophy is considered as an important compensatory mechanism that allows the heart to maintain its output. Chronic  $\beta$ -AR-stimulation is shown to induce hypertrophy, and increase heart rate and LV systolic function (20; 49; 50). Indicators of hypertrophy such as HW/BW ratio, and myocyte cross-sectional area were increased to a similar extent in both ISO-groups. Interesting finding of this study is that exogenous UB in the presence of ISO restored systolic function to normal levels as indicated by decreased %FS, %EF and Vcf. It partially restored ISO-mediated decrease in IVRT, IVCT, and ET. Changes in heart rate are suggested to affect echocardiographic parameters in mice (52). As observed previously (23), ISO-infusion increased heart rate in ICR mice. UB alone or in the presence of ISO had no effect on basal or ISO-mediated increases in heart rates. Therefore, the observed changes in echocardiographic parameters between ISO and UB+ISO groups may not be due to the changes in heart rate. Extracellular UB is shown to promote intracellular  $Ca^{++}$  flux and reduce cAMP levels through a G protein-coupled receptor in THP1 cells (41). Therefore, UB may affect systolic and diastolic parameters of the heart by modulating levels of intracellular  $Ca^{++}$  and/or cAMP. However, further investigations are needed to understand the mechanism by which exogenous UB modulates heart function in the presence of  $\beta$ -AR agonist.

Cardiac myocyte apoptosis plays a crucial role in the pathogenesis of heart failure (17; 21; 33).  $\beta$ -AR-stimulated activation of JNKs and GSK-3 $\beta$  plays a pro-apoptotic role via the involvement of mitochondrial death pathway (30; 39). Previously, UB is shown to activate PI3-kinase and inhibit  $\beta$ -AR-stimulated activation of JNKs and GSK-3 $\beta$ , and mitochondrial death pathway of apoptosis (44). PI3-kinase is an upstream activator of Akt (35), while Akt acts upstream in the inactivation of GSK-3 $\beta$  (16). Active GSK-3 $\beta$  may act upstream in the activation of JNKs (44). Here we show exogenous UB enhances  $\beta$ -AR stimulated activation of Akt, while inhibiting

activation of GSK-3 $\beta$  and JNKs. These *in vivo* data confirm our previous *in vitro* findings, and suggest that activation of PI3-Kinase/Akt pathway may be a mechanism involved in anti-apoptotic effects of exogenous UB.

Chronic sympathetic stimulation is shown to induce growth of interstitial tissue in the heart leading to fibrosis (7). MMPs and TIMPs play an important role in the remodeling of extracellular matrix (46). Previously, we have shown that chronic  $\beta$ -AR stimulation increases myocardial fibrosis (23). Consistent with these observations, we observed increased fibrosis following chronic  $\beta$ -AR stimulation in ICR mice. The new finding of this study is that exogenous UB inhibits chronic  $\beta$ -AR-stimulated increases in myocardial fibrosis. It enhanced chronic  $\beta$ -AR-stimulated increases in the expression and activity of MMP-2. It also increased expression of MMP-9 and TIMP-2 in the heart. Use of isolated adult cardiac fibroblasts confirmed our *in vivo* findings with respect to the expression of MMPs and TIMP-2 in response to UB. Of note, a higher concentration of rUB (as compared to bovine UB) was required to observe increased expression of MMP-2 in the presence of isoproterenol in fibroblasts (Fig 2.7D). The reasons may include differential modification of UB from different sources. UB is shown to be modified by acetylation of lysines, oxidation of methionine, and nitration of tyrosine (25; 54). These modifications may influence interaction of extracellular UB with its receptor and/or other proteins. The concentration of TIMP-2 is suggested to determine the role of TIMP-2 in activation of MMP-2. At low concentrations, TIMP-2 may activate MMP-2 on the cell surface with the MT1-MMP-TIMP-2 complex serving as a receptor for proMMP-2, whereas at higher concentrations, TIMP-2 may neutralize MT1-MMP and prevent activation of MMP-2 (24). The data presented here suggest that exogenous UB decreases chronic  $\beta$ -AR-stimulated myocardial fibrosis by modulating expression of MMPs and TIMP-2. Previously, we have reported that



chronic  $\beta$ -AR stimulation increases MMP-9 expression in the heart (23). Here we did not observe increased expression of MMP-9 in response to chronic  $\beta$ -AR stimulation. The observed discrepant findings may relate to the use of different mouse strains. Previous study used 129xblack Swiss mice, while the current study uses ICR mice.

Increased MMP-2 expression is suggested to be dependent on Akt activation in the rat heart during ischemia/reperfusion (45). In the current study, UB enhanced Akt activation in the heart and in isolated cardiac fibroblasts in the presence of  $\beta$ -AR stimulation. Inhibition of Akt decreased UB+ISO-mediated increases in MMP-2 expression. These data suggest a potential relationship between Akt activation in response to UB treatment and MMP-2 expression in the heart.

#### Perspective

The data presented here are novel and of significant interest since exogenous UB modulates  $\beta$ -AR-stimulated myocardial function, and inhibits myocardial fibrosis and cardiac myocyte apoptosis. The structural changes related to cardiac myocyte apoptosis and extracellular matrix play a significant role in modulation of myocardial function and in the progression to heart failure. Therefore, elucidation of processes that can shift the balance from myocyte apoptosis to survival may have clinical implications. In addition, analysis of components of extracellular matrix, including collagen type I and IV, laminin, fibronectin etc, may provide insight into the modulation of heart function in the presence of exogenous UB. It should be emphasized that our data on heart function and signaling are obtained seven days after ISO-infusion. The experimental time-point should be extended beyond seven days to investigate long-term effects of exogenous UB.

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### Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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## CHAPTER 3

### EXTRACELLULAR UBIQUITIN MODULATES CARDIAC FIBROBLAST PHENOTYPE AND FUNCTION: ROLE OF CXCR-4 RECEPTOR

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## Abstract

Stimulation of  $\beta$ -adrenergic receptor ( $\beta$ -AR) increases extracellular levels of ubiquitin (UB), and exogenous UB plays an important role in  $\beta$ -AR-stimulated myocardial remodeling with effects on LV function, fibrosis and myocyte apoptosis. Cardiac fibroblasts play a critical role in maintaining the normal function of the heart, and in the reparative and fibrosis processes during pathological cardiac remodeling. This study investigated the role of extracellular UB on cardiac fibroblast function and phenotype, and involvement of CXCR-4 receptor (a potential receptor for extracellular UB) in UB-mediated modulation of fibroblast function and phenotype. Using primary cultures of adult rat cardiac fibroblasts, we provide evidence that extracellular UB interacts with the cell surface. Extracellular UB induced rearrangement of fibrillar actin into the formation of stress fibers as analyzed by phalloidin-FITC and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, a marker for transdifferentiation of fibroblasts into myofibroblasts) staining. However, it inhibited the formation of lamellopodia and filopodia. Western blot analysis revealed increased expression of  $\alpha$ -SMA in response to extracellular UB. Extracellular UB co-immunoprecipitated with CXCR-4 receptor. Proximity ligation assay confirmed interaction of extracellular UB with CXCR-4. Extracellular UB inhibited the migration of cells into the wound. It activated ERK1/2, while it had no effect on the activation of Akt. It increased the expression of vascular endothelial growth factor A (VEGF-A), while decreasing the expression of  $\beta$ 3 integrins. Most of the effects of extracellular UB were negated by pretreatment with AMD-3100. Two UB mutants (mUB V70 and mUB F4), unable to interact with CXCR-4, had no effect on the expression of VEGF-A and  $\beta$ 3 integrins. Thus, extracellular UB, at least in part acting via the CXCR-4 receptor, plays an important role in modulation of fibroblast function and phenotype.

## Introduction

Ubiquitin (UB), a highly conserved protein of ~8.5 kDa, is found in all eukaryotic cells. The most important intracellular function of UB is to regulate protein turnover by the ubiquitin-proteasome pathway (16). The ubiquitin-proteasome pathway may regulate receptor internalization, hypertrophic response, apoptosis, and tolerance to ischemia and reperfusion in cardiac myocytes (56). UB is a normal constituent of plasma. Elevated levels of UB are described in the serum or plasma of patients with parasitic and allergic diseases (3), alcoholic liver disease (47), type 2 diabetes (1),  $\beta$ 2-Microglobulin amyloidosis (35) and chronic hemodialysis patients (2). Patients with traumatic brain injury are shown to have increased UB levels in the cerebrospinal fluid (27). Extracellular UB is proposed to have pleiotropic functions including regulation of immune response, anti-inflammatory and neuroprotective activities (26; 28; 36), as well as regulation of growth and apoptosis in hematopoietic cells (10). Previously our lab has shown that  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation increases extracellular levels of UB, and treatment of adult rat ventricular myocytes with UB inhibits  $\beta$ -AR-stimulated apoptosis (41). *In vivo*, exogenous UB decreased  $\beta$ -AR-stimulated increases in cardiac myocyte apoptosis and myocardial fibrosis (11).

The G protein-coupled receptor CXC chemokine receptor (CXCR)-4 is abundantly expressed in immune cells and tissues, including cardiac fibroblast (21). CXCR-4 plays an important role in a variety of biological processes, and is suggested to be involved in the pathophysiology of various disease processes, such as cancer, HIV, ischemic myocardial injury, and angiogenesis (49). Stromal derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) is the cognate ligand for CXCR-4 which is implicated in homing of hematopoietic stem cells during wound repair (7). SDF-1 $\alpha$  is shown to have cardioprotective effects against ischemia/reperfusion injury by promoting anti-

apoptotic program (21). Interestingly, CXCR-4 has been identified as a receptor for UB in THP1 leukemia cell line (38). Using C-terminal truncated ubiquitin and ubiquitin mutants, Saini et al., provide evidence that the ubiquitin-CXCR-4 interaction follows a two-site binding mechanism in which the hydrophobic surfaces surrounding Phe-4 and Val-70 are important for receptor binding, whereas the flexible C terminus facilitates receptor activation (39). Recently, we provided evidence that extracellular UB, acting via CXCR-4 receptor, increases expression of angiogenic molecules and stimulates angiogenesis in cardiac microvascular endothelial cells (45).

Cardiac fibroblast play a critical role in maintaining the normal function of the heart, and in the remodeling process of the heart that occurs in response to myocardial infarction, hypertension and heart failure (42). The objective of this study was to investigate the role of extracellular UB on cardiac fibroblast phenotype and function, and to define the role of CXCR-4 receptor in modulation of fibroblast phenotype and function in response to extracellular UB. The data presented here suggest that extracellular UB interacts with fibroblasts and CXCR-4 receptor, induces cytoskeletal reorganization, induces intracellular signaling, and inhibits migration of cells into the wound. Most of these effects of UB are inhibited by CXCR-4 receptor antagonist. The data presented here also suggest that extracellular UB and SDF-1 $\alpha$  may elicit different signaling mechanisms.

## Materials and Methods

### Vertebrate Animals

All experiments and procedures were reviewed and approved by the East Tennessee State University Institutional Committee on Animal Care and conform to the *Guide for the Care and*

*Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats (average wt: 200-225g; Harlan, Indianapolis, IN) were used for isolation of resident cardiac fibroblast cells. Rats were anesthetized using a mixture of isoflurane (2.5%) and oxygen (0.5 l/min), and the heart was excised following a bilateral cut in the diaphragm. Animals were euthanized by exsanguinations.

### Fibroblast Isolation and Treatment

Adult rat cardiac fibroblasts were isolated as described (54). The cells were grown to confluence and serum-starved for 48 h before use. Experiments were performed using first and second passage cells. The cells were pretreated with AMD 3100 (10  $\mu$ M; Sigma) or PD98059 (15 $\mu$ M; Calbiochem) for 30 min followed by treatment with UB (10  $\mu$ g/ml; Sigma), biotin-UB (500 ng/ml; Boston Biochem), mutated UB (mUB V70 and mUB F4; 10 $\mu$ g/ml; Boston Biochem) or SDF-1 $\alpha$  (1nM; Peprotech) for indicated time points. To investigate cellular interaction of UB, cells were treated with FITC-UB (500 ng/ml; Boston Biochem) for 30 min.

### Immunocytochemical Analysis

Immunocytochemical analysis was carried out as described (31). Briefly, isolated cardiac fibroblast were plated on coverslips, fixed in 3.7% formaldehyde and permeabilized using 0.2% Triton X-100. Coverslips were incubated in anti- $\alpha$ -SMA (1:500; Sigma) antibodies in 1% BSA followed by Alexa Fluor-conjugated (1:500; Invitrogen) secondary antibodies. The coverslips were then counterstained with Hoechst 33258, mounted and visualized using a fluorescent microscope.



### Actin Polymerization Assay

Serum starved confluent cultures of cardiac fibroblast were treated with UB for 24 h. Cells were washed twice with PBS, fixed in 3.7% formaldehyde solution, and permeabilized with 0.2% Triton X-100. Nonspecific binding was blocked by incubating slides for 20 min at room temperature in blocking solution (1% BSA in PBS). Cells were stained with Phalloidin-FITC (1U/slide in blocking solution; Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature in the dark. After washing, the slides were mounted with SlowFade (Invitrogen Corp., Carlsbad, CA) and visualized under fluorescent microscope using a rhodamine filter. Images were acquired using a Nikon TE-2000 microscope with a Retiga-1300 color cooled camera.

### Co-immunoprecipitation Assay

Total cell lysates (300 µg proteins) were incubated overnight at 4°C with 3 µg of anti-CXCR-4 (Abcam) antibody in 500 µl of TNT buffer (20 mM Tris·HCl, pH 7.5, 137 mM NaCl, 20 mM NaF, 5 mM EDTA, 1 mM PMSF, 10 mM sodium pyrophosphate, 0.2 M sodium orthovanadate, 8 µg/ml aprotinin and 2 µg/ml leupeptin, digitonin 0.05%, and 1% Triton X-100). Protein A/G beads (60 µl, Thermo Scientific, Rockford, IL) were added to the mixture and incubated for an additional hour with rocking. The immunocomplexes were then washed six times with cold PBS. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were then probed with anti-UB (Santa Cruz) antibody as described for western blot analyses.

### DuoLink In Situ Proximity Ligation Assay

To detect the interaction between CXCR-4 and UB, in situ proximity ligation assay (PLA) was performed using Duolink kit according to the manufacturer's protocol (Olink Bioscience, Uppsala, Sweden). After fixing and permeabilization, the cells were incubated overnight with anti-UB antibodies (monoclonal; Santa Cruz) and anti-CXCR-4 antibodies (polyclonal; Abcam). The cells were washed with PBS and incubated with appropriate secondary antibodies. After a ligation reaction at 37°C of the DNA chains directly coupled to the PLA probes, the cells were mounted and detection of the amplified probe was performed using fluorescent microscope.

### Migration Assay

Movement of cells through a wound introduced in a cell monolayer was determined as described (19). Briefly, cardiac fibroblasts were grown as a confluent monolayer. After cells were made quiescent in serum free medium for 48 h, a wound was created in the center of the cell monolayer by gentle removal of attached cells using a sterile plastic pipette tip. Cell debris was removed by a PBS wash and images of the wound were acquired using a Nikon TE-2000 microscope with a Retiga-1300 color cooled camera. The cells were then incubated in serum-free DMEM containing UB for 24 and 48 h. Images were again acquired using as described above. The wound area was measured using Bioquant Image Analysis software (Bioquant Image Analysis Corp., Nashville, TN). The ability of cells to migrate into the wound area was assessed by comparing micrographs at time zero, 24 and 48 h along the wounded area. The percentage of wound area recovered was calculated by dividing the wound area after 24 h or 48 h by the initial wound area at time zero, multiplied by 100.

### Western Blot Analysis

Cell lysates were prepared using lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, 1% Triton X-100, and 1 mM PMSF]. Equal amounts of total proteins (20 µg from cell lysates) were resolved on 10% SDS-polyacrylamide gels. The proteins were transferred onto PVDF membrane. The blots were then probed with primary antibodies directed against p-Akt (1:1000; Cell Signaling Tech.), p-ERK1/2 (1:2000; Cell Signaling Tech.), β3-integrin (1:1000; Santa Cruz), VEGF-A (1:200; Santa Cruz), or α-SMA (1:20000; Sigma) and appropriate secondary antibodies. Membranes were then stripped and probed with Akt, ERK1/2, actin (Chemicon) or GAPDH (Santa Cruz) antibodies to normalize protein loading. Band intensities were quantified using Kodak photo documentation system (Eastman Kodak Co.). To detect biotin-labelled UB, the membranes were incubated overnight in extra-avidin peroxidase (Sigma; 1:2000 dilution in TBST). The biotin-labelled UB was visualized using chemiluminescence reagents.

### Statistical Analysis

Data are expressed as the mean ± SEM. Data was analyzed using Student's *t* test or a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Probability (P) values of <0.05 were considered to be significant.

## Results

### Extracellular UB Interacts with Fibroblasts and Induces Cytoskeletal Reorganization

To investigate if extracellular UB interacts with cells, cells were treated with FITC-UB for 30 min. As a negative control, cells were incubated with FITC-labeled secondary antibodies

(1:500) for 30 min. Visualization of cells using fluorescent microscope showed clear staining of cells with FITC-UB. No staining was observed in control cells or cells incubated with FITC-labeled secondary antibodies (Fig 3.1).

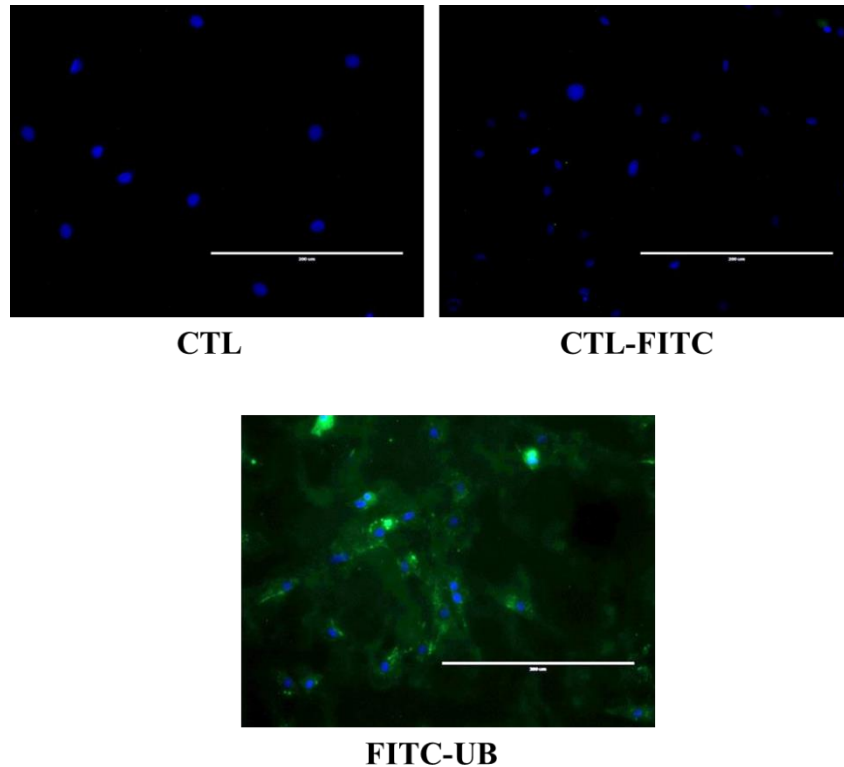


Figure 3.1. Cellular Interaction of Extracellular UB. Cardiac fibroblasts were incubated with FITC-UB (500 ng/ml) or FITC-labelled secondary antibodies (CTL-FITC) for 30 min. The cells were visualized by fluorescent microscope and photographed. CTL, control.

The rearrangement of actin in fibroblast is one of the driving forces of migration into the wound (23; 53). In anchorage dependent cells, like fibroblast, there are two distinct cytoskeletal arrangements: the geodom (polygonal net) and stress fibers (13). To analyze the actin cytoskeleton, fibroblasts were treated with UB for 24 h followed by Phalloidin-FITC staining. This analysis showed the presence of geodom in untreated cells (Fig 3.2A). UB treated cells mostly showed absence of geodom. UB treatment enhanced polymerization of actin into stress fibers (yellow arrows). However, there was no presence of filamentous membrane projections

called filopodia or lamellipodia in the UB-treated cells (Fig 3.2B). Similar observations were made when fibroblasts were immunostained for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; Fig 3.2 C&D). Western blot analysis of cell lysates using anti- $\alpha$ -SMA antibodies showed increased expression of  $\alpha$ -SMA 48 h after UB treatment (Fig 3.2E).

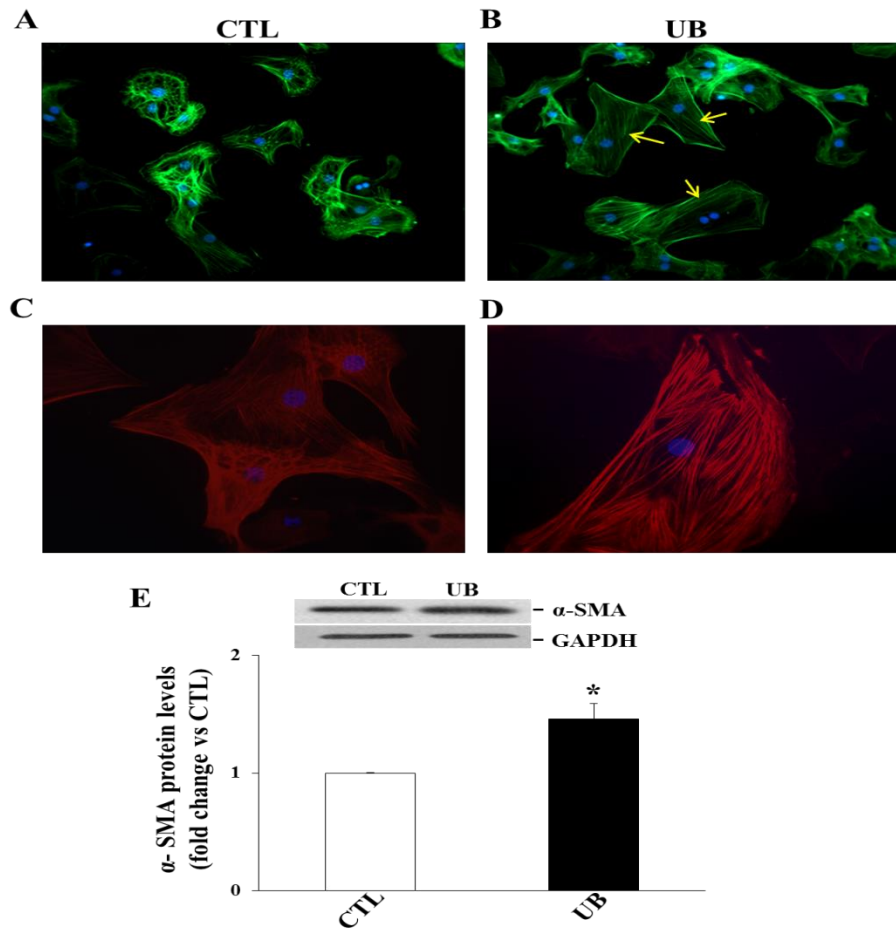


Figure 3.2. Extracellular UB Induces Cytoskeleton Rearrangement, and Increases Expression of  $\alpha$ -Smooth Muscle Actin ( $\alpha$ -SMA): A-D. Semi-confluent cultures of cardiac fibroblast were treated with UB for 24 h. The cells were stained with Phalloidin-FITC or anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibodies and visualized using fluorescent microscope (20 or 40X). Panels A & B depict representative images from CTL and UB-treated cells following Phalloidin-FITC staining. The yellow arrows indicate bundles of actin filaments called stress fibers that are required for the traction of the rear of the cells toward the leading edge during migration (B). Panels C & D depict representative images from CTL and UB-treated cells following immunocytochemical staining using anti- $\alpha$ -SMA antibodies. E. Western blot analysis of  $\alpha$ -SMA. Cells were treated with UB for 48 h. Cell lysates were analyzed by western using anti- $\alpha$ -SMA

antibodies. The lower panel exhibits the mean data normalized with GAPDH. \*P<0.05 vs control (CTL); n=5.

### UB Interacts with CXCR-4 Receptors

CXCR-4 is identified as a receptor for extracellular UB in THP-1 cells (38). Co-immunoprecipitation is considered a standard method to determine if two proteins of interest form a complex. To investigate the interaction of UB with CXCR-4 receptor, fibroblasts were treated with Biotin-UB for 30 min. Cell lysates were immunoprecipitated with anti-CXCR-4 antibody. The resultant immunoprecipitates were analyzed by western blots using extra-avidin peroxidase to detect Biotin-UB. This analysis showed the presence of Biotin-UB in cell lysates immunoprecipitated with anti-CXCR-4 antibodies (Fig 3.3A). Binding specificity of UB with CXCR-4 receptor was next analyzed by pretreatment of cells with AMD-3100 (CXCR-4 antagonist) or SDF-1 $\alpha$  (a known ligand for CXCR-4 receptor) for 30 min followed by treatment of cells with Biotin-UB for different time points (15 min, 3 h and 24 h). Western blot analysis of cell lysates using extra-avidin peroxidase showed that pretreatment with AMD-3100 almost completely inhibits binding of Biotin-UB to the cells at all different time-points. SDF-1 $\alpha$  also decreased binding of Biotin-UB at all different time points. However, this inhibition was more pronounced at 3 and 24 h (Fig 3.3B). Pretreatment with AMD3100 also inhibited binding of FITC-UB to the cells (Fig 3.3C). To confirm interaction of UB with CXCR-4, we used a recently developed commercial assay (Duolink) called proximity ligation assay (PLA). The PLA identifies interactions between two proteins in their native form. This assay results in a fluorescent signal in the form of a spot when the two proteins of interest are closer than 40nm. The specificity of the assay was tested using single antibodies directed against UB and CXCR-4. This assay showed a few positive spots for interaction of UB with CXCR-4 receptor in untreated

cells. This may be due to the fact that endogenous UB can bind to CXCR-4 receptors on the cytoplasmic side of the transmembrane protein (29). However, clear increase in the positive signal for the interaction of UB with CXCR-4 was observed in cells treated with UB as indicated by an increase in bright red fluorescent spots when compared to CTL (Fig 3.3D).

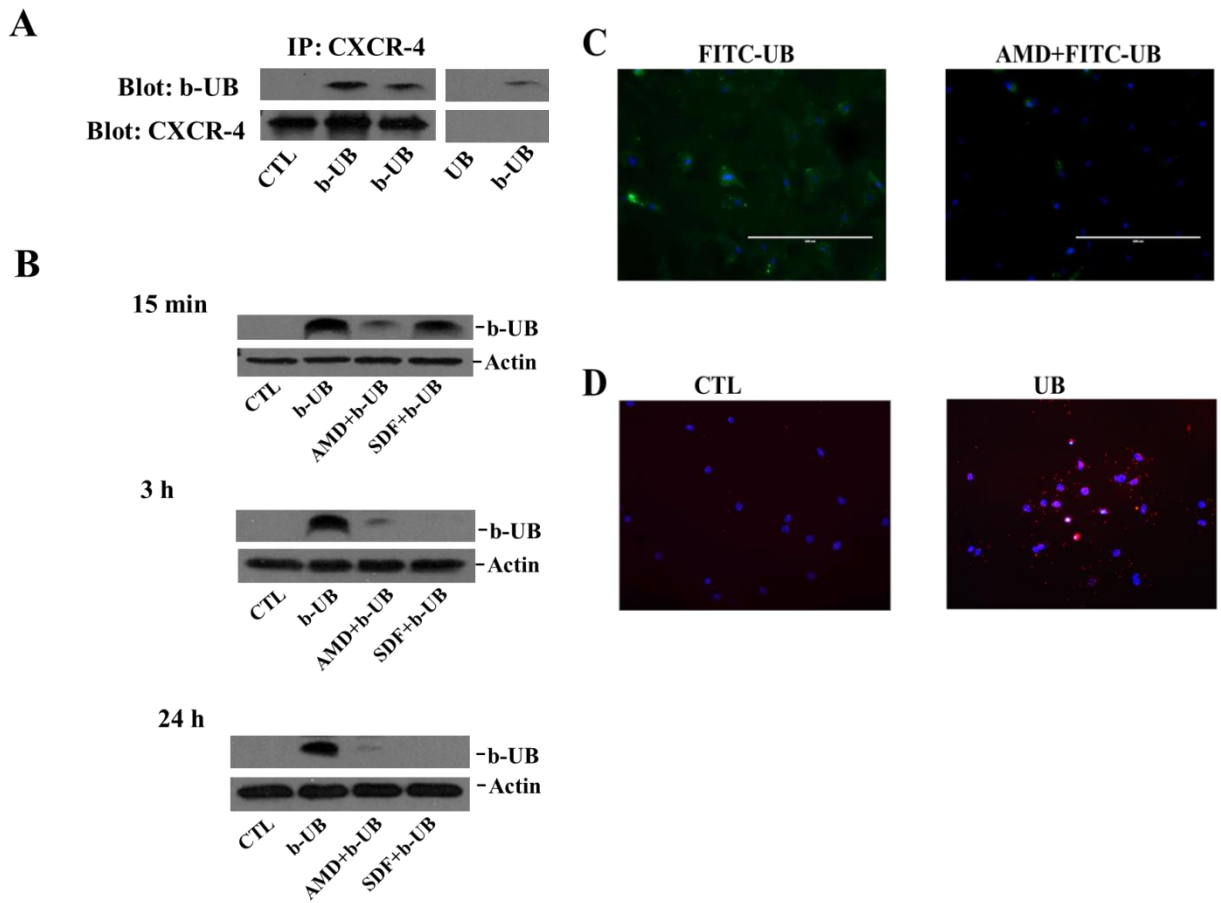


Figure 3.3. Interaction of UB with CXCR-4 Receptor: A. Fibroblast were treated with biotin-labeled UB (b-UB; 10  $\mu$ g/ml) for 30 min. Cell lysates were immunoprecipitated with anti-CXCR-4 antibodies. Immunoprecipitates were analyzed by western blot using extra-avidin peroxidase. Unlabeled UB and b-UB served as negative and positive controls, respectively. B. Cells were pretreated with AMD 3100 (AMD; 10  $\mu$ m) or SDF-1 $\alpha$  (SDF; 1 nM) for 30 min followed by treatment with b-UB for 15 min, 3 h or 24 h. Cell lysates were analyzed by western blot using extra-avidin peroxidase. C. Cells were pretreated with AMD for 30 min followed by treatment with FITC-UB for 30 min. Cells were visualized using fluorescent microscope. D. Fibroblast were treated with UB for 24 h. The cells were then used for PLA using anti-UB and anti-CXCR-4 antibodies. Increased fluorescent red staining in UB-treated sample indicates interaction of UB and CXCR-4.

### Extracellular UB Inhibits Migration of Fibroblast into the Wound

Fibroblasts play a key role in the normal healing process after injury (30). They migrate into the wound site and proliferate, and play a significant role in the reconstitution and deposition of fibrotic scar (14). They also play a significant role in the repair processes of the heart following myocardial infarction (43; 55). To examine the functional effects of extracellular UB on fibroblast cell migration, we used an *in vitro* migration (scratch) assay as previously described (19). Migratory potential of fibroblasts into the wound was clearly present in the untreated cells as observed by the decrease in the percent wound area recovered following 24 and 48 h after incubation (Fig 3.4A). Interestingly, UB treatment significantly inhibited the percent wound area recovered at both time points (24 h; CTL, 45 % vs UB, 14 %; 48 h; CTL, 64 % vs UB, 35 %; n=5; Fig 3.3B&C). Pretreatment with AMD3100 significantly enhanced the migration of cells into the wound at both time points (CTL, 45±3; UB, 14±4\*; AMD+UB, 34±6<sup>§</sup>; AMD, 47±3; 24 h; CTL, 64±2; UB, 35±6\*; AMD+UB, 59±5<sup>§</sup>; AMD, 63±5; 48 h; \*P<0.05 vs CTL; <sup>§</sup>P<0.05 vs UB; n=5; Fig 3.4B&C). AMD 3100 alone had no effect on the migration of cells into the wound.



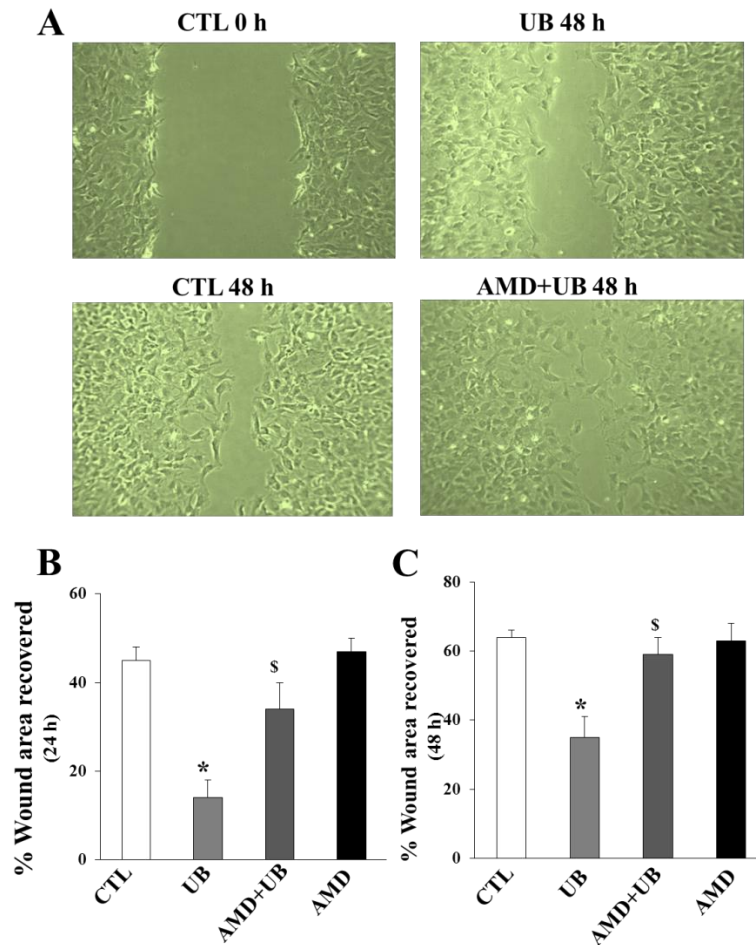


Figure 3.4. Extracellular UB Inhibits Migration of Cardiac Fibroblast: Confluent fibroblast monolayers were wounded using a sterile pipette tip and pretreated with AMD followed by treatment with UB for 24 or 48 h. The ability of cells to migrate into the wound was assessed following 24 or 48 h after UB treatment. Panel A demonstrates the wound area at 0 and 48 h after treatment. Wound healing was assessed by calculating percent of recovered wound area. Panel B and C represents the mean data of percent wound area recovered 24 and 48 h after UB treatment; \*P<0.05 versus CTL; \$P<0.05 versus UB; n = 5.

### Activation of Intracellular Signals by Extracellular UB, and Involvement of CXCR-4 Receptor

A variety of growth factors activate ERK1/2 (52). UB treatment is also shown to activate ERK1/2 in THP-1 cells (40). To analyze the activation ERK1/2 in cardiac fibroblasts and involvement of CXCR-4 receptors, cells were treated with AMD3100 or PD98059 (a selective inhibitor of ERK1/2 pathway) followed by treatment with UB for 15 min. Western blot analysis

of cell lysates using phospho-specific anti-ERK1/2 antibodies showed increased phosphorylation (activation) of ERK1/2 in response to UB. Pretreatment with PD98059 completely inhibited activation of ERK1/2, while inhibition of ERK1/2 was only partial in the presence of AMD3100 (CTL,  $1\pm 0.01$ ; UB,  $4.73\pm 0.8^*$ ; AMD+UB,  $2.4\pm 0.8^{*\$}$ ; PD+UB<sup>\$</sup>,  $0.05\pm 0.02$ ; AMD,  $0.93\pm 0.38$ ; \*P<0.05 vs CTL; <sup>\$</sup>P<0.05 vs UB; n = 5; Fig 3.5A). Similar observations were made when cells were treated with SDF-1 $\alpha$  in the presence of AMD3100 or PD98059 (CTL,  $1\pm 0.01$ ; SDF,  $3.95\pm 0.94^*$ ; AMD+SDF,  $2.52\pm 0.62^{*\$}$ ; PD+SDF,  $0.74\pm 0.68^{\$}$ ; AMD,  $0.93\pm 0.38$ ; \*P<0.05 vs CTL; <sup>\$</sup>P<0.05 vs SDF; n = 5; Fig 3.5B). AMD 3100 alone had no effect on the activation of ERK1/2.

Previously, we have provided evidence that inhibition of PI3-kinase inhibits the protective effects of UB in  $\beta$ -AR-stimulated apoptosis in ARVMs (41). PI3-kinase acts upstream in the activation of Akt, and activation of Akt is generally considered as an anti-apoptotic signal (22). Western blot analysis of cell lysates using phospho-specific anti-Akt antibodies showed no significant increase in Akt phosphorylation in response to UB treatment. On the other hand, SDF-1 $\alpha$  increased Akt phosphorylation within 15 min. Pretreatment with AMD3100 inhibited SDF-1 $\alpha$ -mediated activation of Akt (CTL,  $1.00\pm 0.01$ ; UB,  $1.24\pm 0.19$ ; AMD+UB,  $1.36\pm 0.33$ ; SDF,  $1.74\pm 0.23^*$ ; AMD+SDF,  $0.81\pm 0.26^{\$}$ ; AMD,  $1.25\pm 0.24$ ; \*P<0.05 vs CTL; <sup>\$</sup>P<0.05 vs SDF; n = 5, Fig 3.5C).

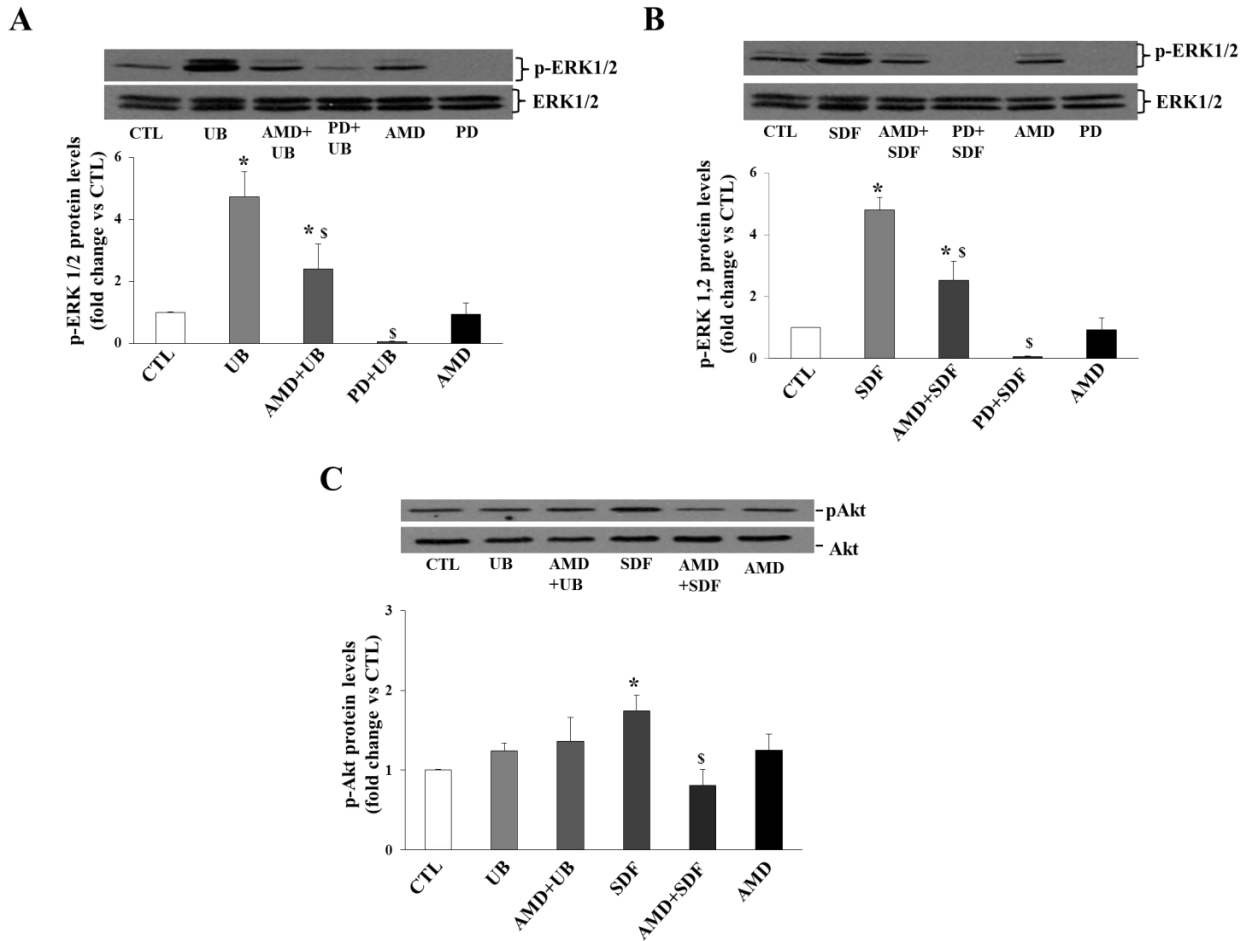


Figure 3.5. UB Activates ERK1/2, not Akt: Serum starved confluent cultures of fibroblasts were pretreated with AMD, or PD98059 (PD; 15  $\mu$ M) for 30 min followed by treatment with UB or SDF-1a (SDF) for 15 min. Cell lysates were analyzed by western blot using phospho-specific ERK1/2 or Akt (serine-473) antibodies. The lower panels exhibit mean data normalized to total ERK1/2 or Akt. Both UB and SDF increased activation of ERK1/2; \* $P < 0.05$  versus CTL, <sup>s</sup> $P < 0.05$  versus UB,  $n = 6$  (A); \* $P < 0.05$  versus CTL; <sup>#</sup> $P < 0.01$  versus SDF,  $n = 4-7$  (B); \* $P < 0.05$  versus CTL, <sup>s</sup> $P < 0.05$  versus SDF,  $n = 4-6$  (C).

### Extracellular UB Affects Expression of $\beta 3$ -integrin and VEGF-A

Integrins provide linkages between ECM proteins and the actin cytoskeleton, thereby providing adhesion of cells to the substratum and playing a crucial role in cell migration (20; 50). Cardiac fibroblasts predominantly express  $\beta 3$  integrins (5). Western blot analysis of cell lysates using anti- $\beta 3$ -integrin antibodies showed a significant decrease in the expression of  $\beta 3$  integrin

in UB-treated (24 h) cells compared to CTL (Fig 3.6A). AMD3100 alone had no significant effect on  $\beta 3$  integrin expression. However, pretreatment with AMD3100 almost completely inhibited UB-mediated decrease in the expression of  $\beta 3$  integrin (CTL,  $1.00 \pm 0.01$ ; UB,  $0.45 \pm 0.09^*$ ; AMD+UB,  $0.99 \pm 0.22$ ; AMD,  $0.97 \pm 0.18$ ;  $*P < 0.05$  vs CTL;  $n = 5$ ; Fig 3.6A). The UB-CXCR-4 interaction is suggested to involve the hydrophobic surfaces surrounding UB residues Phe-4 and Val-70 (39). To further investigate the role CXCR-4 in  $\beta 3$  integrin expression, cells were treated with two different mutated UBs; F4A and V70A. Western blot analyses of cell lysates showed that these two mutations in the UB have no effect on  $\beta 3$  integrin expression (Fig 3.6B).

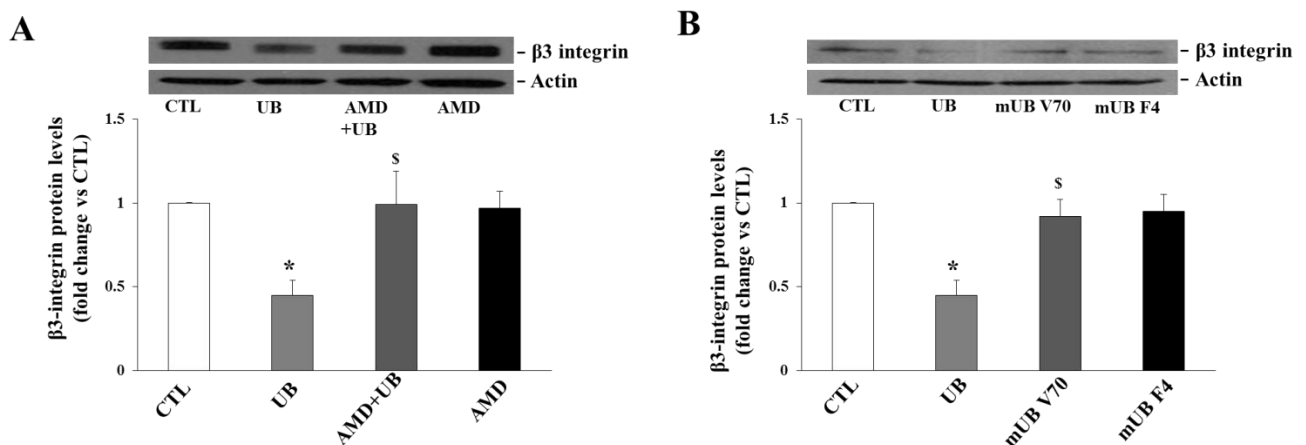


Figure 3.6. UB Decreases  $\beta 3$ -Integrin Expression, and Mutated UBs (UBV70A and UBF4A) have no Effect: Serum starved confluent cultures of fibroblasts were pretreated with AMD for 30 min followed by treatment with UB (10  $\mu\text{g}/\text{ml}$ ) for 24 h (A). The cells were also treated with mutated UBs (UBV70A and UBF4A) for 24 h (B). Cell lysates were analyzed by western blot using anti- $\beta 3$ -integrin antibodies. The lower panels exhibit the mean data normalized to actin.  $*P < 0.05$  versus CTL;  $^sP < 0.05$  versus UB;  $n = 6-7$ .

VEGF-A is suggested to play a role in tissue repair (12). Recently, we have shown that extracellular UB increases VEGF-A expression in microvascular endothelial cells (45). Western blot analysis of cell lysates using anti-VEGF-A antibodies showed a significant increase in the

expression of VEGF-A in UB-treated (24 h) cells compared to CTL (Fig 3.7A). AMD3100 alone had no significant effect on VEGF-A expression. However, it almost completely inhibited UB-mediated increases in VEGF-A expression (CTL,  $1.00\pm 0.01$ ; UB,  $3.22\pm 0.6$ ; AMD+UB,  $1.49\pm 0.3$ , AMD,  $1.15\pm 0.1$ ; \* $P < 0.05$  vs CTL;  $n = 5$ ; 7A). Treatment of cells with mutated UBs had no effect on VEGF-A expression (Fig 3.7B).

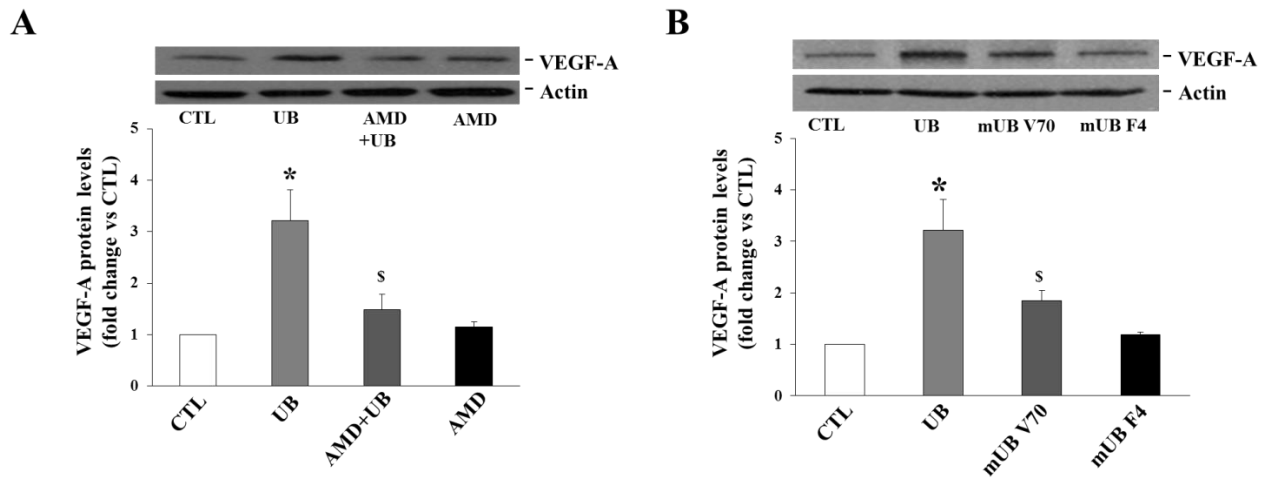


Figure 3.7. UB Increases VEGF-A Expression, while Mutated UBs (UBV70A and UBF4A) have no Effect: Serum starved confluent cultures of fibroblasts were pretreated with AMD for 30 min followed by treatment with UB (10  $\mu\text{g/ml}$ ) for 24 h (A). The cells were also treated with mutated UBs (UBV70A and UBF4A) for 24 h (B). Cell lysates were analyzed by western blot using anti-VEGF-A antibodies. The lower panels exhibit the mean data normalized to actin. \* $P < 0.05$  versus CTL; § $P < 0.05$  versus UB;  $n = 6$ .

### Discussion

Cardiac fibroblasts play a critical role in maintaining normal function of the heart, and in cardiac remodeling during pathological conditions, including hypertension and following myocardial infarction (42). A major finding of this study is that extracellular UB modulates fibroblasts phenotype and function. The data presented here shows that extracellular UB 1) interacts with the cell surface; 2) induces cytoskeletal rearrangement; 3) inhibits migration of fibroblasts into the wound and expression of  $\beta 3$  integrins; 4) activates intracellular signaling

involving ERK1/2 pathway; 5) increases VEGF-A expression. Most of the effects of UB are inhibited by the inhibition of CXCR-4 receptor, suggesting that involvement of CXCR-4 in modulation of fibroblast function and phenotype in response to extracellular UB.

UB is a normal constituent in the plasma (47). Extracellular UB is proposed to have pleiotropic functions including regulation of immune response, anti-inflammatory and neuroprotective activities (26; 28; 36). Interaction and internalization of extracellular UB is shown in human peripheral blood mononuclear cells and monocytic leukemia cells. Inflammatory stimuli increase uptake of labelled UB suggesting the possibility that uptake of extracellular UB could be directed towards restoring intracellular UB equilibrium (37). Previously, using N-terminal biotin-labelled UB, we provided evidence for the cellular interaction/uptake of extracellular UB in adult rat cardiac myocytes.  $\beta$ -AR stimulation enhanced this interaction/uptake by  $\sim$ 1.9-fold (41). This is the first study indicating interaction of extracellular UB by cardiac fibroblasts. In THP-1 cells, extracellular UB is shown to induce intracellular signaling, including phosphorylation of ERK1/2 and Akt (40). UB-mediated phosphorylation of these kinases occurred transiently which declined within 30 min. However, SDF-1 $\alpha$  phosphorylation was sustained for 30 min. Furthermore, UB showed weaker chemotactic activity in cell migration assay when compared to SDF-1 $\alpha$  (40). In adult rat cardiac myocytes, extracellular UB alone had no effect on the activation of JNKs and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). However, it inhibited  $\beta$ -AR-stimulated activation of JNKs and GSK-3 $\beta$  (41). In cardiac fibroblasts, extracellular UB and SDF-1 $\alpha$  activated ERK1/2. However, Akt activation was only observed with SDF-1 $\alpha$ . SDF-1 $\alpha$  also binds to CXCR-7 (4). However, overexpression of CXCR-7 in HEK293 failed to increase UB receptor binding, suggesting that CXCR-7 may not serve as a receptor for extracellular UB (40). Collectively, these data suggest

that the response to UB may be cell-type specific, and differential responses between UB and SDF-1 $\alpha$  could be due to the involvement of receptor/s other than CXCR-4.

Activated fibroblasts play a critical role in both the reparative and fibrotic processes. In normal tissues, resident fibroblasts are quiescent, producing limited amounts of extracellular matrix proteins and exhibiting few actin-associated cell-matrix and cell-cell contacts (48). Tissue injury induces alterations in the microenvironment, resulting in differentiation of fibroblasts into myofibroblasts (18). Myofibroblasts are characterized by the presence of microfilamentous contractile apparatus enriched in  $\alpha$ -SMA and its assembly into stress fibers (18). Formation of stress fibers is important for fibroblast migration (8). Immunocytochemical analysis of cells using phalloidin-FITC and  $\alpha$ -SMA staining showed that intracellular UB induces structural changes in the cardiac fibroblasts. UB treatment enhanced polymerization of actin into the stress fibers. Extracellular UB also increased expression of  $\alpha$ -SMA, a hallmark of differentiated fibroblasts into myofibroblasts. However, it was interesting to note the absence of filamentous membrane projections called filopodia or lamellipodia in UB-treated cells. UB-treated samples also exhibited decreased migration of fibroblasts into the wound. This finding is in contrast to cardiac microvascular endothelial cells where extracellular UB promotes the formation of stress fibers, lamellopodia and filopodia (45). Previously, we have shown that exogenous UB decreases  $\beta$ -AR-stimulated increases in myocardial fibrosis (11). UB-mediated decrease in the fibroblast migration into the wound may help explain decreased fibrosis in the heart during  $\beta$ -AR stimulated cardiac remodeling. However, further investigations are needed to prove this thesis.

Integrins are cell surface receptors that play a role in cell adhesion and migration, as well as in growth and survival (20; 25). Fibroblasts are capable of modulating their microenvironment through autocrine and paracrine signaling, thereby playing a role in tissue remodeling following

tissue injury. Fibroblasts are also shown to play a role in the angiogenic process (32; 51). Fibroblasts secrete fibroblast growth factor and VEGF-A (33). VEGF-A is considered as a potent angiogenic inducer. In this study, we found that UB treatment increases VEGF-A expression. Increased expression of VEGF-A is consistent with our previous finding in microvascular endothelial cells where UB treatment increased VEGF-A expression (45). Cardiac fibroblasts express  $\beta 3$  integrin (17).  $\beta 3$  integrin is shown to play a critical role in cardiac fibroblasts adhesion, migration, proliferation and extracellular matrix production since  $\beta 3^{-/-}$  cardiac fibroblast exhibit a significant reduction in these processes (5). The data presented here demonstrates significant reduction in  $\beta 3$  integrin expression following UB treatment. The absence of filopodia and lamellopodia, and decreased expression of  $\beta 3$  integrins can help explain the reduced migration of fibroblasts into the wound in UB-treated samples. Increased expression of VEGF-A may act as a paracrine factor for cardiac microvascular endothelial cells to induce angiogenesis.

Recently, CXCR-4 has been identified as a receptor for extracellular UB in human myeloid cells (38). SDF-1 $\alpha$  (also called CXCL-12) is considered a natural ligand for CXCR-4 (6). SDF-1 $\alpha$ /CXCR-4 axis is suggested to play a pivotal role in the regulation of bone marrow-derived stem cell homing, repopulation and mobilization of stem cells into the peripheral blood (24). Mice lacking SDF-1 $\alpha$  or CXCR-4 confirm the essential role of SDF-1 $\alpha$ /CXCR-4 axis in hematopoiesis, cardiogenesis and vasculogenesis during embryonic development (15; 34; 46; 57). Cardiac myocytes and non-myocyte cell populations of the heart express CXCR-4 receptors (9). Previously, we provided evidence for CXCR-4 as a receptor for extracellular UB in microvascular endothelial cells (44). Here, we provide evidence for CXCR-4 as a receptor for extracellular UB in cardiac fibroblasts. We show that extracellular UB co-immunoprecipitates



with CXCR-4. Interaction of extracellular UB with CXCR-4 was confirmed using the PLA technique. AMD 3100 and SDF-1 $\alpha$  time-dependently decreased interaction of extracellular UB with CXCR-4 as analyzed by western blot. AMD3100 also decreased binding of FITC-labeled UB. It enhanced the migration of cells in the wound area, decreased activation of ERK1/2 and expression of VEGF-A and restored expression of  $\beta$ 3 integrin. Use of two different mutated UB proteins (F4A and V70A) on the expression of  $\beta$ 3-integrin and VEGF-A further supports the involvement of CXCR-4 receptor in extracellular UB signaling. It is interesting to note that AMD 3100 almost completely inhibits UB-mediated increases in  $\beta$ 3 integrins and VEGF-A expression. However, inhibition of ERK1/2 was partial. The reasons for this differential response may include differences in binding affinities for AMD 3100 and UB and/or incubation durations.

#### Perspectives

Cardiac fibroblasts are important in maintaining the normal function of the heart as well as in wound repair following injury to the heart. UB is found in normal plasma. A variety of disease states increase levels of UB in the plasma. Here we provide evidence that extracellular UB modulates cardiac fibroblast function and phenotype via the involvement of CXCR-4 receptor. Further investigation of signaling mechanisms leading to UB-mediated regulation of cardiac fibroblast function and phenotype may help uncover novel strategies to improve cardiac remodeling and function.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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## CHAPTER 4

### EXOGENOUS UBIQUITIN MODULATES ISCHEMIA/REPERFUSION INJURY IN ISOLATED HEARTS AND CARDIAC MYOCYTES

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## Abstract

Exogenous ubiquitin (UB) plays an important role in  $\beta$ -AR-stimulated myocardial remodeling with effects on LV function, fibrosis and myocyte apoptosis. Extracellular UB modulates cardiac fibroblast function and phenotype, and increases expression of angiogenic molecules in cardiac microvascular endothelial cells. This study tested the hypothesis that exogenous UB plays a protective role in ischemia/reperfusion (I/R) injury in the heart and cardiac myocytes. Isolated mouse hearts underwent 15 min of baseline perfusion, 25 min of global ischemia and 40 min of reperfusion. UB (10  $\mu$ g/ml) was infused within 5 min before ischemia. Isolated adult rat ventricular myocytes (ARVMs) were exposed to simulated ischemia/reperfusion by placing the cells in a hypoxic chamber for 2.5 h followed by reoxygenation (H/R) for indicated time points. Measurement of infarct size using TTC-staining showed that UB infusion significantly decreases infarct size. UB I/R hearts showed a significant improvement in functional recovery as measured by increased % left ventricular developed pressure. Activation of proapoptotic proteins, p-STAT-1 and caspase-9, was significantly lower in UB I/R hearts when compared to I/R. In ARVMs, apoptosis was lower in UB H/R group when compared to H/R. Activation of Akt was higher, activation of GSK-3 $\beta$  was lower in UB H/R cells versus H/R. The number of reactive oxygen species and superoxide positive ARVMs was lower in UB-treated samples. TMRM staining showed that UB treatment increases the number of ARVMs with polarized mitochondria. CXCR-4 antagonist (AMD3100) significantly negated the protective effects of UB on H/R-induced apoptosis and activation of Akt and GSK-3 $\beta$ . UB mutants, unable to bind to CXCR-4, had no effect on H/R-induced apoptosis, activation of Akt and GSK-3 $\beta$  and oxidative stress. Thus, exogenous UB, most likely acting via CXCR-4, plays a protective role in I/R injury with

effects on infarct size, heart function, cardiac myocyte apoptosis, ROS and intracellular signaling.

### Introduction

Myocardial ischemia/reperfusion (I/R) injury occurs from reperfusion of blood and oxygen in acute myocardial infarction. I/R injury increases reactive oxygen species (ROS) production and induces cardiac myocyte apoptosis (25; 27; 55). The apoptotic pathway involves mitochondrial dysfunction, which is activated in myocytes by hypoxia, ischemia/reperfusion, and oxidative stress (9). Mitochondria are considered as a major source of ROS and an increase in ROS causes mitochondrial dysfunction including a loss in mitochondrial membrane potential (24; 28). Mitochondrial dysfunction is a major contributor to loss of myocytes during myocardial ischemia and subsequent reperfusion (42). Despite advances in understanding the molecular and cellular mechanisms that regulate the damage caused by I/R injury, the treatment for I/R injury remains a challenge.

Ubiquitin (UB), a highly conserved protein of ~8.5 kDa, is found in all eukaryotic cells. The most important intracellular function of UB is to regulate protein turnover by the ubiquitin-proteasome pathway (17). The ubiquitin-proteasome pathway may regulate receptor internalization, hypertrophic response, apoptosis, and tolerance to ischemia and reperfusion in cardiac myocytes (61). UB is a normal constituent of plasma. Elevated levels of UB are described in the serum or plasma of patients with parasitic and allergic diseases (4), alcoholic liver disease (54), type 2 diabetes (2),  $\beta$ 2-Microglobulin amyloidosis (41) and chronic hemodialysis patients (3). Patients with traumatic brain injury are shown to have increased UB levels in the cerebrospinal fluid (37). Extracellular UB is proposed to have pleiotropic functions including regulation of immune response, anti-inflammatory and neuroprotective activities (36;

38; 43), as well as regulation of growth and apoptosis in hematopoietic cells (11). C-X-C chemokine receptor (CXCR-4) plays an important role in a variety of biological processes, and is suggested to be involved in the pathophysiology of various disease processes, such as cancer, HIV, ischemic myocardial injury, and angiogenesis (56). Stromal derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) is the cognate ligand for CXCR-4 which is implicated in homing of hematopoietic stem cells during wound repair (8). Interestingly, CXCR-4 has been identified as a receptor for UB in THP1 leukemia cell line (46). Using C-terminal truncated ubiquitin and ubiquitin mutants, Saini et al., provide evidence that the ubiquitin-CXCR-4 interaction follows a two-site binding mechanism in which the hydrophobic surfaces surrounding Phe-4 and Val-70 are important for receptor binding, whereas the flexible C terminus facilitates receptor activation (47).

Previously our lab has shown that  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation increases extracellular levels of UB, and treatment of adult rat ventricular myocytes with UB inhibits  $\beta$ -AR-stimulated apoptosis (49). *In vivo*, exogenous UB decreased  $\beta$ -AR-stimulated increases in cardiac myocyte apoptosis and myocardial fibrosis (12). Our recent work suggest that extracellular UB modulates cardiac fibroblast function and phenotype (unpublished work), and increases expression of angiogenic molecules in cardiac microvascular endothelial cells (51). The objective of this study was to investigate the role extracellular UB plays in I/R injury in isolated hearts and cardiac myocytes. The data presented here suggest that extracellular UB decreases infarct size, improves functional recovery of the heart, and decreases activation of STAT-1 and caspase-9. In ARVMs, UB treatment decreased H/R-induced myocyte apoptosis, activated Akt while inactivating GSK-3 $\beta$ , decreased oxidative stress and improved the number of myocytes with polarized mitochondria. Most of these effects of UB are negated by CXCR-4



antagonist AMD 3100. Mutated UB, unable to bind CXCR-4 had no effect on H/R-induced apoptosis, intracellular signaling or oxidative stress.

## Methods and Materials

### Vertebrate Animals

All experiments and procedures were reviewed and approved by the East Tennessee State University Institutional Committee on Animal Care and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male C57BL/6 mice (8 to 10 week-old; Jackson Laboratories, Bar Harbor, ME) were used. Mice were anesthetized using a mixture of isoflurane (2.5%) and oxygen (0.5 l/min) and the heart was excised following a bilateral cut in the diaphragm. Animals were euthanized by exsanguinations.

### Isolated Heart Perfusion System (Langendorff)

Langendorff global I/R experiments were performed in isolated mouse hearts as previously described (29). The aorta was quickly cannulated and the heart was perfused using Krebs buffer containing (in mM): 118.5 NaCl, 4.7 KCl, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 24.8 NaCHO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, and 10.6 glucose. The buffer was equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and maintained at 37°C. A fluid-filled silicon balloon was inserted in the left ventricle through the mitral valve for left ventricular developed pressure (LVDP) measurement with a pressure transducer (AD Instruments, Dunedin, New Zealand). Hearts were allowed to stabilize during a 15 min baseline period followed by 25 min ischemia and 40 min reperfusion. UB (10 µg/ml in Krebs buffer) treatment was started within 5 min before ischemia. I/R hearts received Krebs

buffer throughout the procedure. Immediately at the end of reperfusion, hearts were snap frozen with liquid nitrogen and stored at -80°C.

### Infarct Size

Infarct size was measured using tetrazolium chloride (TTC; Sigma) staining. Each heart was sliced transversely to yield five slices. The slices were incubated in 1% TTC for 15 min at 37°C. Infarct size was calculated, using Bioquant image analysis software, as the percentage of total white area divided by the total tissue area.

### Cardiac Myocyte Isolation, Treatment and Hypoxia/Reoxygenation

Calcium-tolerant ARVMs were isolated from the hearts of adult male Sprague-Dawley rats (150-200 g) as described previously (39). ARVMs were plated in Dulbecco's modified Eagle's medium (DMEM; Mediatech) supplemented with 25 mM HEPES, 0.2% albumin, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, and 0.1% penicillin-streptomycin at a density of 30–50 cells/mm<sup>2</sup> on 100-mm tissue culture dishes or coverslips (Fisher Scientific) precoated with laminin (1 µg/cm<sup>2</sup>). ARVMs were cultured overnight in the above medium. After changing the medium, the ARVMs were treated with UB (10 µg/ml; Sigma) or mutated UB (mUB V70 and mUB F4; 10µg/ml; Boston Biochem) for 30 min at 37°C with 5% CO<sub>2</sub>. To simulate ischemia/reperfusion, ARVMs were incubated at 37°C with 5% CO<sub>2</sub> and 0.1% O<sub>2</sub> in a hypoxia chamber (Pro-Ox Model C21, BioSpherix Ltd, Redfield NY) for 2.5 h. After changing the media containing the corresponding treatments, ARVMs were then incubated at 37°C with 5% CO<sub>2</sub> (reoxygenation) for indicated time points. To investigate the role of CXCR-4, cells were pretreated with AMD 3100 for 30 min prior to UB treatment.

### Apoptosis

To detect apoptosis, myocytes were placed in a hypoxic chamber for 2.5 h, followed by 18 h of reoxygenation. TUNEL-staining assay was performed using *in situ* death detection kit according to the manufacturer's instructions (Roche Molecular Biochemicals). Hoechst 33258 (10  $\mu$ M; Sigma, St Louis, MO) staining was used to count the total number of nuclei. The percentage of TUNEL-positive cells (relative to total ARVMs) was determined by counting ~200 cells in 10 randomly chosen fields per coverslip for each experiment.

### Measurement of Mitochondrial Membrane Polarization

ARVMs were plated on 50-mm MatTek dishes (MatTek, Ashland, MA) and incubated for 30 min in 3 ml of culture medium containing 200 nM TMRM at 37°C and 5% CO<sub>2</sub> (58). After 30 min of incubation, the culture media was substituted with PBS plus 5 mM glucose and 10 mM succinate. Before imaging, a cover slip was placed over the cells, and 15  $\mu$ l sterile oxyrase was added. Oxyrase selectively removes oxygen creating depletion of oxygen in the myocyte's immediate surroundings. Images of cells were captured using the Zeiss AxioCam MRm monochrome digital camera. The number of polarized cells (i.e., those exhibiting TMRM fluorescence) was counted at each time point of oxyrase incubation and was expressed as a percent of total cells before anoxia (basal) imaged using fluorescent microscopy.

### ROS Detection

ROS was detected using the Total ROS detection kit from Enzo Life Sciences and used according to manufacturer's instructions. This kit is designed to directly monitor real time ROS production in live cells using fluorescent microscopy. For this, UB or mutated UB treated cells

were exposed to hypoxia 2.5 h followed by reoxygenation for 1 h. Cells were loaded with ROS-responsive fluorescence probe for 1 h during reoxygenation. After washing with phosphate-buffered saline (PBS), cells were visualized using a fluorescent microscope. Superoxide staining appears as red dots, while ROS-positive cells exhibit green fluorescence.

### Western Analysis

LV tissue lysates were prepared in RIPA buffer [10 mM Tris-HCl (pH 7.2), 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride] using a tissue homogenizer. Cell lysates were prepared using lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride]. Equal amounts of total proteins (60 µg from tissue or cell lysates) were resolved on 10% SDS-polyacrylamide gels. The proteins were transferred onto PVDF membrane. The blots were then probed with primary antibodies directed against p-Akt (1:1000; Cell Signaling Tech.), p-GSK-3β (1:1000; Cell Signaling Tech.), caspase-9 (1:1000; Millipore), p-STAT-1 (1:1000; Millipore), and appropriate secondary antibodies. Membranes were then stripped and probed with Akt, GSK-3β, STAT-1, or actin (Chemicon) antibodies to normalize protein loading. Band intensities were quantified using Kodak photo documentation system (Eastman Kodak Co.).

## Statistical Analysis

Data are expressed as the mean  $\pm$  SEM. Data was analyzed using Student's *t* test or a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Probability (P) values of  $<0.05$  were considered to be significant.

## Results

### UB Treatment Decreases I/R-induced Infarct Size and Improves Functional Recovery of the Heart

To investigate if extracellular UB treatment modulates global I/R injury, isolated hearts were subjected to 25 min of global ischemia followed by 40 min of reperfusion in the presence or absence of UB. TTC staining showed a significant decrease in % infarct size in UB I/R hearts when compared to I/R alone (I/R,  $39.5 \pm 6.9$ ; UB I/R,  $13.7 \pm 2.8^*$ ;  $*p < 0.05$  vs I/R;  $n = 6$ ; Fig 4.1A). Left ventricular development pressure (LVDP) is a measure of cardiac performance in langendorff perfused hearts. UB I/R hearts showed a greater increase in % LVDP recovery following ischemia when compared to I/R hearts (I/R,  $50.7 \pm 4.4$ ; UB I/R,  $67.3 \pm 4.3^*$ ;  $*p < 0.05$  vs I/R;  $n = 6$ ; Fig 4.1B).

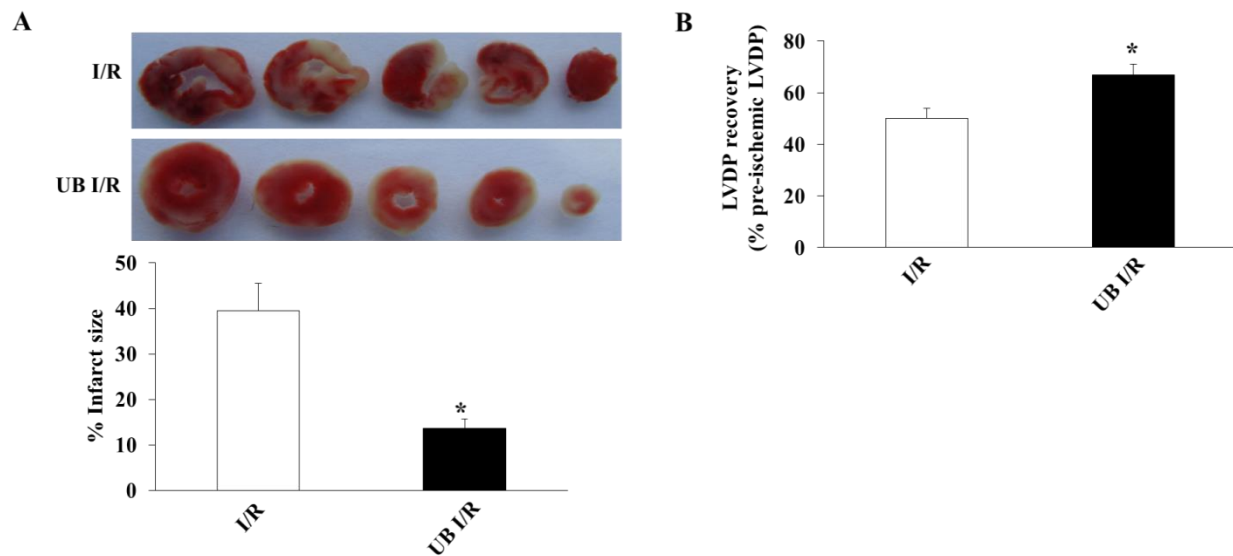


Figure 4.1. UB Decreases Infarct Size and Improves LVDP Recovery. Mouse hearts underwent global ischemia for 25 min, followed by 40 min reperfusion. A. Upper panel depicts representative tetrazolium chloride (TTC) stained hearts showing a decrease in infarcted region (white area) in UB I/R hearts. The lower bar graph exhibits % infarct size. B. Recovery of left ventricular developed pressure. \* $P < 0.05$  vs I/R;  $n = 6$  hearts in each group.

#### UB Decreases Activation of p-STAT-1 and Caspase-9 in I/R Hearts

Proapoptotic proteins, STAT-1 and caspase-9, are activated during I/R injury (35; 52). Western blot analysis of heart homogenate using anti-p-STAT-1 antibodies showed a significant decrease in the phosphorylation of p-STAT in UB I/R group vs I/R (I/R,  $0.90 \pm 0.11$ ; UB I/R,  $0.54 \pm 0.03^*$ ; \* $p < 0.05$  vs I/R;  $n = 4$ ; Fig 4.2A). Presence of ~17 kDa band on western blot using anti-caspase-9 antibodies indicates activation of caspase-9. Western blot analysis of heart homogenate showed decreased levels of active caspase-9 in UB I/R group when compared to I/R (I/R,  $1.63 \pm 0.27$ ; UB I/R,  $0.89 \pm 0.08^*$ ; \* $p < 0.05$  vs I/R;  $n = 4$ ; Fig 4.2B).

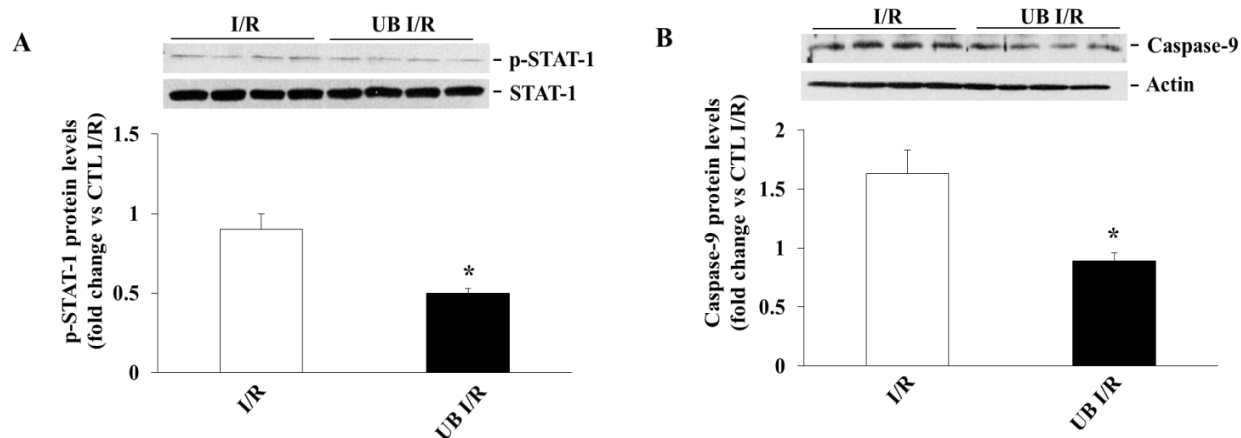


Figure 4.2. UB Decreases I/R-Induced Activation of STAT-1 and Caspase-9: Total heart lysates (60  $\mu$ g) were analyzed by western blot using phospho-specific STAT-1 (A) or anti-caspase-9 antibodies (B). The lower panels exhibit mean data normalized to total STAT-1 or actin. \* $P < 0.05$  versus I/R;  $n = 4$ .

#### UB Treatment Decreases Hypoxia/Reoxygenation-Induced Apoptosis in ARVMs

Hypoxia for 2.5 h followed by reoxygenation for 18 h (H/R) significantly increased apoptosis when compared to control (CTL) cells incubated at 37<sup>0</sup>C with 5% CO<sub>2</sub> (regular incubator). However, UB treated samples showed a significant decreases in TUNEL-positive ARVMs when compared to H/R. Pretreatment with AMD 3100 significantly negated the protective effects of UB (CTL, 3.3 $\pm$ 0.7; H/R, 35.5 $\pm$ 6.3\*; UB H/R, 9.2 $\pm$ 2.2<sup>&</sup>; AMD+UB H/R, 34.1 $\pm$ 4.1\*<sup>\$</sup>; AMD H/R, 24.3 $\pm$ 5.0\*; \* $p < 0.05$  vs CTL; & $p < 0.05$  vs H/R; & $p < 0.05$  vs UB;  $n = 3-4$ ; Fig 4.3A). Mutated UB (mUBV70 or mUBF4) had no effect on H/R-mediated increases in the number of apoptotic ARVMs (CTL, 2.2 $\pm$ 0.2; H/R, 25.7 $\pm$ 2.3\*; mUBV70 H/R, 21.0 $\pm$ 5.4\*; mUBF4 H/R, 21.5 $\pm$ 2.6\*; \* $p < 0.05$  vs CTL;  $n = 3-4$ ; Fig 4.3B).

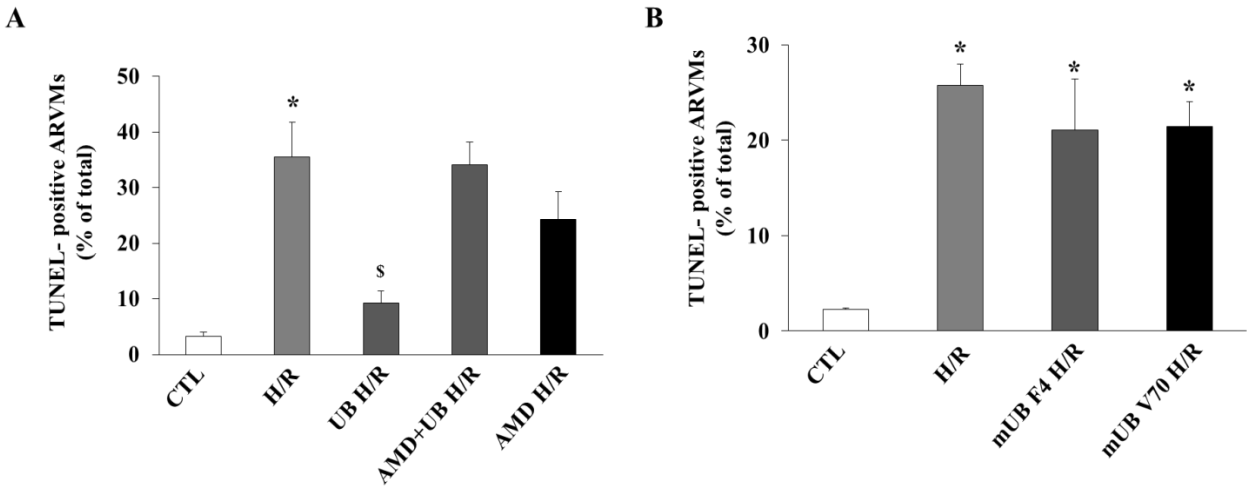


Figure 4.3. UB Inhibits H/R-Induced Apoptosis in ARVMs: A. ARVMs pretreated with AMD 3100 (AMD; 10  $\mu$ M) for 30 min followed by treatment with UB (10  $\mu$ g/ml) for 30 min. The cells then underwent hypoxia for 2.5 h min and reoxygenation for 18 h. \* $p$ <0.05 vs CTL; & $p$ <0.05 vs H/R; & $p$ <0.05 vs UB;  $n$  = 3-5. B. ARVMs were treated with mutated UBs (mUB V70 and mUB F4; 10 $\mu$ g/ml) for 30 min followed by H/R as described. \* $P$ <0.05 versus CTL.

#### Activation of Akt and GSK-3 $\beta$ in ARVMs

PI3-kinase activates Akt, and activation of Akt plays an anti-apoptotic role (26). Western blot analysis of cell lysates using phospho-specific anti-Akt antibodies showed a significant increase in Akt phosphorylation (activation) in UB H/R group when compared to CTL and H/R groups. AMD 3100 pretreatment significantly decreased UB H/R-mediated increase in Akt phosphorylation (CTL,  $1 \pm 0.01$ ; H/R,  $1.11 \pm 0.1$ ; UB H/R,  $2.23 \pm 0.3^*$ ; AMD+UB H/R,  $1.06 \pm 0.2^{\$}$ ; \* $P$ <0.05 vs CTL and H/R;  $^{\$}P$ <0.05 vs UB H/R;  $n$  = 4-6; Fig 4.4A). Treatment with mutated UB (mUBV70) had no effect on the phosphorylation of Akt (CTL,  $1 \pm 0.01$ ; H/R,  $1.11 \pm 0.1$ ; UB H/R,  $2.23 \pm 0.3^*$ ; mUBV70,  $1.09 \pm 0.1^{\$}$ ; \* $P$ <0.05 vs CTL and H/R;  $^{\$}P$ <0.05 vs UB H/R;  $n$  = 4-6; Fig 4.4B).



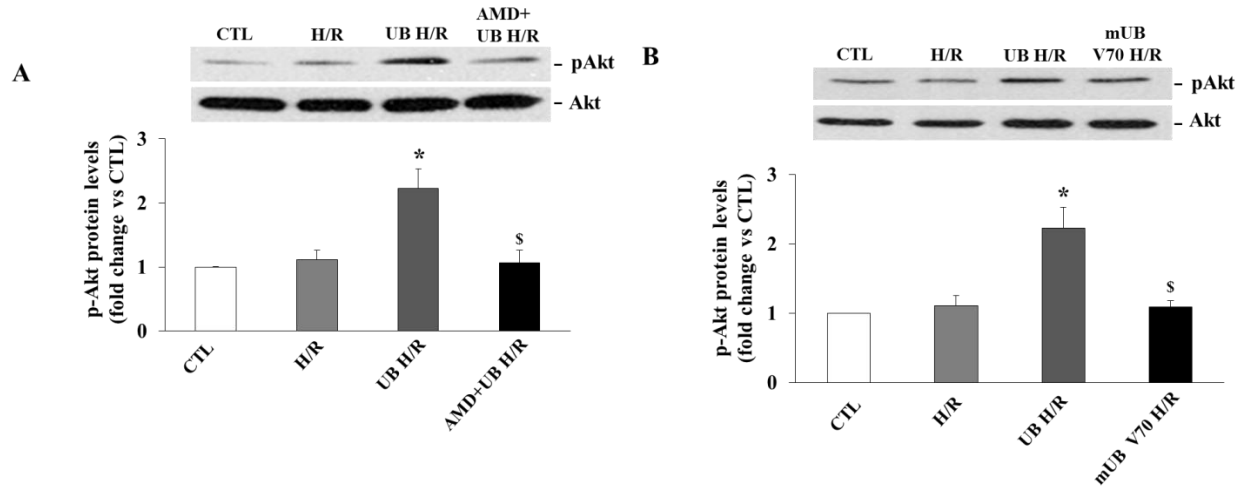


Figure 4.4. UB Activates Akt in ARVMs: A. ARVMs were pretreated with AMD 3100 (AMD; 10  $\mu$ M) for 30 min followed by treatment with UB (10  $\mu$ g/ml) for 30 min. The cells then underwent hypoxia for 2.5 h and reoxygenation for 15 min. B. ARVMs were treated with mutated UBs (mUB V70 and mUB F4; 10 $\mu$ g/ml) for 30 min followed by H/R for as described. Cell lysates (20  $\mu$ g) were analyzed by western using phospho-specific anti-Akt antibodies. The lower panels exhibit mean data normalized to total Akt. \* $P$ <0.05 versus H/R;  $^{\S}$  $P$ <0.05 versus UB H/R;  $n$  = 4-5.

Phosphorylation of an NH<sub>2</sub>-terminal serine residue (Ser<sup>9</sup>) inactivates GSK-3 $\beta$ . Akt is one of the upstream kinases involved in phosphorylation (Ser<sup>9</sup>) and inactivation of GSK-3 $\beta$  (22). Western blot analysis of cell lysates using phospho-specific (Ser<sup>9</sup>) anti-GSK-3 $\beta$  antibodies showed decrease phosphorylation (activation) of GSK-3 $\beta$  in UB H/R group when compared to CTL and H/R groups. AMD 3100 pretreatment significantly decreased UB H/R-mediated increase in Akt phosphorylation (CTL, 1.00 $\pm$ 0.01; H/R, 1.23 $\pm$ 0.2; UB H/R, 2.47 $\pm$ 0.2\*; AMD+UB H/R<sup>§</sup>, 1.25 $\pm$ 0.1; \* $P$ <0.05 vs CTL and H/R,  $^{\S}$  $P$ <0.05 vs UB H/R;  $n$  = 4-6; Fig 4.5A). Treatment with mutated UB (mUBV70) had no effect on the phosphorylation of Akt (CTL, 1 $\pm$ 0.01; H/R, 1.23 $\pm$ 0.2; UB H/R, 2.47 $\pm$ 0.3\*; mUBV70 H/R, 1.06 $\pm$ 0.1<sup>§</sup>; \* $P$ <0.05 vs CTL and H/R;  $^{\S}$  $P$ <0.05 vs UB H/R;  $n$  = 4-6; Fig 4.5B).

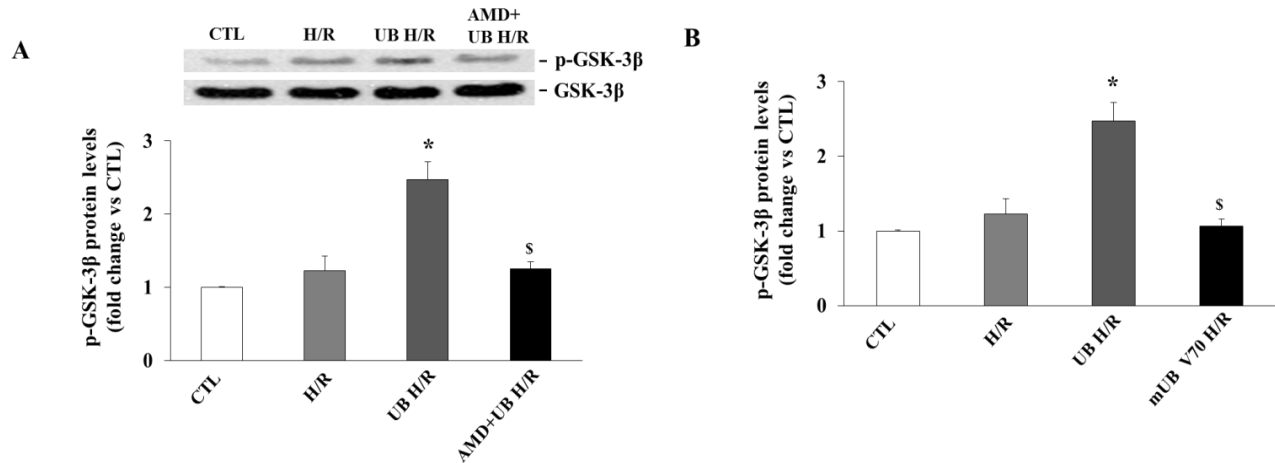


Figure 4.5. UB Inactivates GSK-3 $\beta$  in ARVMs: A. ARVMs were pretreated with AMD 3100 (AMD; 10  $\mu$ M) for 30 min followed by treatment with UB (10  $\mu$ g/ml) for 30 min. The cells then underwent hypoxia for 2.5 h and reoxygenation for 15 min. B. ARVMs were treated with mutated UBs (mUB V70 and mUB F4; 10 $\mu$ g/ml) for 30 min followed by H/R for as described. Cell lysates (20  $\mu$ g) were analyzed by western using phospho-specific anti-GSK-3 $\beta$  antibodies. The lower panels exhibit mean data normalized to total GSK-3 $\beta$ . \*P<0.05 versus H/R; §P<0.05 versus UB H/R; n = 4-5.

#### UB Treatment Decreases the Number of ROS and Superoxide-Positive Myocytes

ROS are implicated in ischemic or hypoxic-toxicity with and without reperfusion (1; 31). To determine the effect UB on oxidative stress, we measured the number ROS and superoxide positive ARVMs following H/R. H/R increased the number of ROS-positive ARVMs when compared to CTL. The UB H/R group showed a significant decrease in total number of ROS-positive ARVMs when compared to H/R. However, mutated UBs showed no significant change in total ROS when compared to H/R (CTL,  $7.2 \pm 2.3$ ; H/R,  $54.2 \pm 7.3^*$ ; UB H/R,  $11.6 \pm 2.6^{\$}$ ; mUBF4 H/R,  $52.0 \pm 5.1^*$ ; mUBV70 H/R,  $57.9 \pm 3.0^*$ ; p<0.05 vs CTL; §P<0.05 vs H/R; n = 4-6; Fig 4.6).

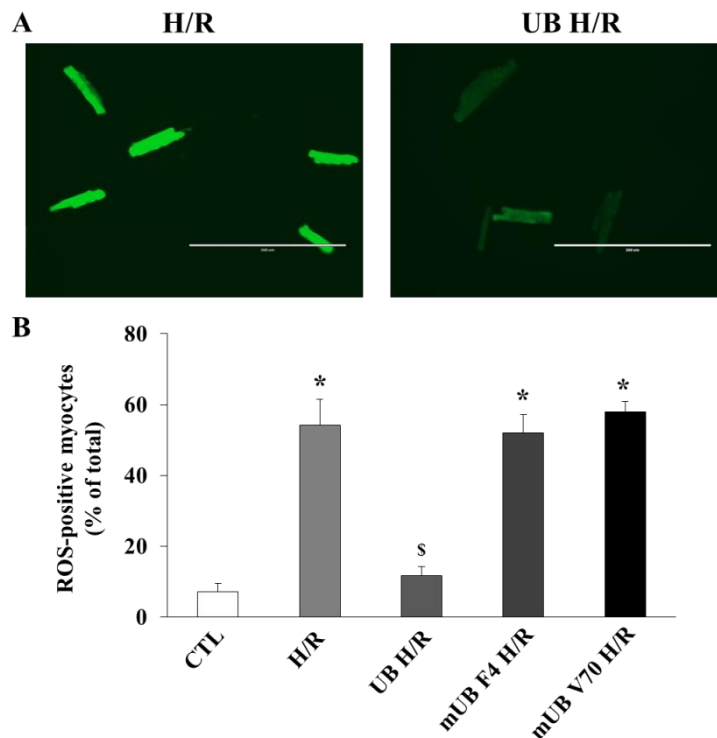


Figure 4.6. UB Decreases the Number of H/R-Induced ROS-Positive ARVMs: ARVMs were pretreated with UB or mutated UBs (mUB V70 and mUB F4; 10 $\mu$ g/ml) for 30 min. The cells then underwent hypoxia for 2.5 h followed by reoxygenation for 1 h. At time of reoxygenation, cells were loaded with total ROS detection stain for 1 h. Green fluorescent staining indicates ROS-positive ARVMs (A). B. Quantitative analysis of ROS-positive ARVMs. \*P<0.05 versus CTL; <sup>s</sup>P<0.05 versus UB H/R; n = 4-6.

Previous reports have shown an increase in superoxide after reperfusion of the isolated ischemic heart (34; 59). Pretreatment with UB showed a significant decrease in superoxide-positive myocytes when compared to H/R myocytes. However, mutated UBs showed no change in superoxide-positive ARVMs when compared to H/R (CTL, 5.6 $\pm$ 1.3; H/R, 20.7 $\pm$ 3.4\*; UB H/R, 7.6 $\pm$ 2.8<sup>s</sup>; mUBF4 H/R, 24.6 $\pm$ 2.8\*; mUBV70 H/R, 22.3 $\pm$ 5.2\*; \*P<0.05 vs CTL; <sup>s</sup>P<0.05 vs H/R; n = 4-6; Fig 4.7).

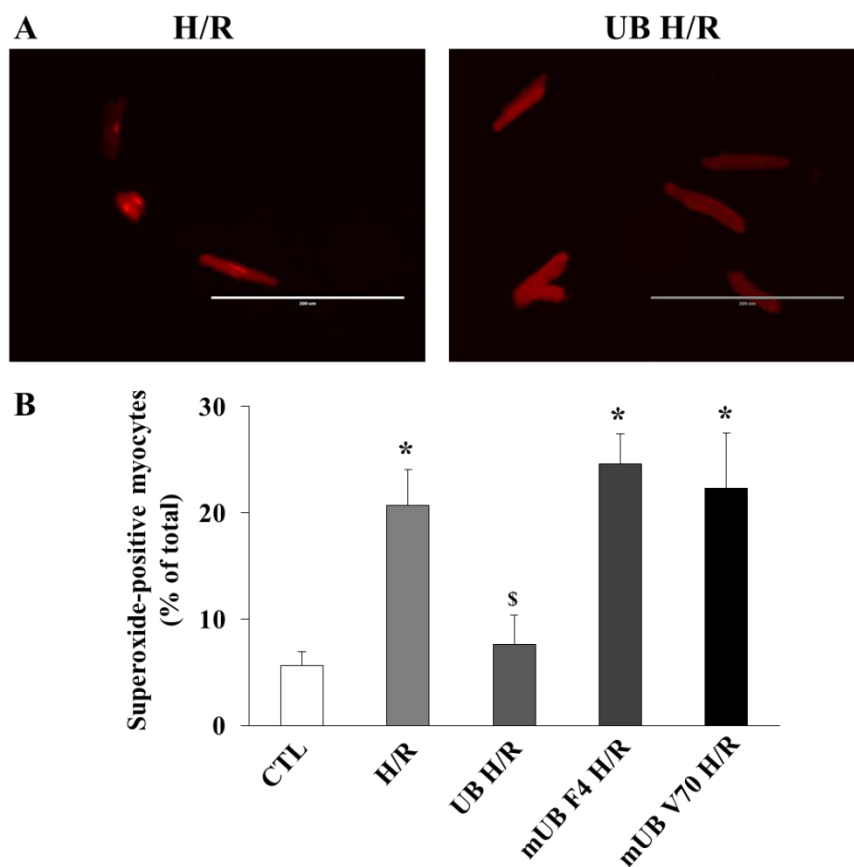


Figure 4.7. UB Decreases the Number of H/R-Induced Superoxide-Positive ARVMs: ARVMs were pretreated with UB or mutated UBs (mUB V70 and mUB F4; 10 $\mu$ g/ml) for 30 min. The cells then underwent hypoxia for 2.5 h followed by reoxygenation for 1 h. At time of reoxygenation, cells were loaded with superoxide detection stain for 1 h. Red fluorescent staining indicates superoxide-positive ARVMs (A). B. Quantitative analysis of superoxide-positive ARVMs. \*P<0.05 versus CTL; <sup>s</sup>P<0.05 versus UB H/R; n = 4-6.

#### UB Treatment Protects Mitochondrial Polarization

H/R induces mitochondrial damage, including loss of membrane potential (13). To investigate if UB treatment protects mitochondrial polarization, ARVMs were subjected to anoxia. Mitochondrial polarization was monitored with TMRM potentiometric dye for 105 min under these conditions. We found a trend towards maintained mitochondrial polarization 60 min after UB treatment. However, the number of ARVMs with polarized mitochondria was significantly greater in UB-treated samples 105 min after UB treatment (Fig 4.8).

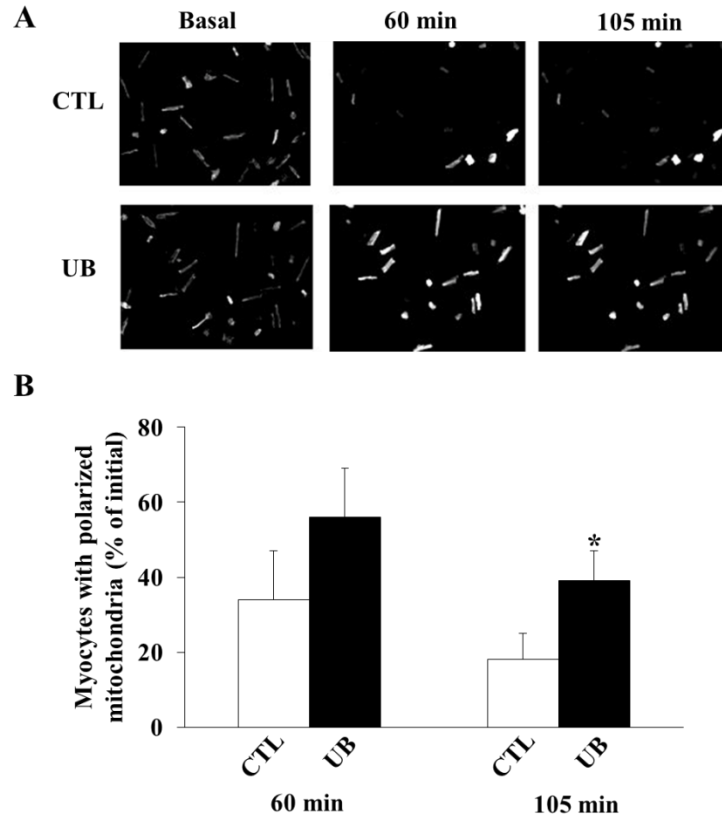


Figure 4.8. UB Treatment Helps Maintain Mitochondrial Membrane Polarization: ARVMs were incubated with oxyrase to create anoxic conditions. Mitochondrial membrane polarization was measured using TMRM loading. Upper panel depicts images of ARVMs obtained using fluorescent microscope before anoxia (basal) and at indicated time points during anoxia. The number of polarized cells (i.e., those exhibiting TMRM fluorescence) was counted at each time point of oxyrase incubation and was expressed as a percent of total cells before anoxia (basal), and are expressed as means  $\pm$  SE (n = 6). \*P<0.05 vs CTL.

### Discussion

Previously, we provided evidence that  $\beta$ -AR stimulation increases levels of extracellular UB and that treatment with UB plays a protective role in  $\beta$ -AR-stimulated apoptosis in ARVMs (49). I/R injury induces cardiac myocyte apoptosis (18). A major finding of this study is that extracellular UB plays a protective role in I/R-induced injury in the heart, and cardiac myocytes apoptosis. The data presented here suggest that UB treatment decreases I/R-induced increases in infarct size while improving functional recovery of the heart following ischemia. These changes

in the heart associate with decreased activation of STAT-1 and caspase-9. In isolated ARVMs, UB decreased H/R-induced apoptosis. It activated Akt and decreased the number of ROS- and superoxide-positive ARVMs. UB treatment also helps maintain mitochondrial membrane polarization under anoxic conditions. Most of the effects of UB were negated by inhibition of CXCR-4 receptor, while mutated UB had no effect.

The primary pathological expression of coronary artery disease is myocardial injury resulting from an I/R insult (45). Cardiac myocytes are highly aerobic cells, and almost all of their energy production comes from oxidative phosphorylation under normal conditions (50). It is clear that production of ROS during both ischemia and reperfusion is a major factor contributing to I/R-induced cardiac injury (14; 15). Global I/R injury is known to cause myocytes apoptosis and create an infarcted region (19). Here we show UB treatment decreased infarct size in isolated hearts when compared to I/R treated hearts. UB treatment also improved LVDP recovery after ischemia.

Previous studies have characterized a proapoptotic effect of STAT-1 by the upregulation of caspase-1 and proapoptotic genes such as FAS, FAS ligand, p21, and p53 (7; 32). Increased STAT-1 activation is reported to impair myocardial performance (23). Mitochondrial damage and leakage of cytochrome c leads to the activation of caspase-9 (53). Caspase-9 is expressed at high levels in the heart and is implicated in mitochondrial-mediated apoptosis (30). The apoptotic cascade is initiated by mitochondrial damage and activation of caspase-9 (48). Caspase-9 is activated during ischemia and remains activated throughout reperfusion in the intact rat heart (48). Activated caspase-9 is detected ~ 17 kDa band on SDS-PAGE. Here, we show that treatment with UB decreases the activation of STAT-1 and caspase-9. These data supports the previous findings of activation of STAT-1 and caspase-9 during I/R injury, and suggest that UB-

mediated decrease in the activation of these molecules may ultimately help preserve heart function.

Apoptosis of cardiac myocytes is identified as an important process in the progression to heart failure (57). Apoptosis contributes, with necrosis, to the cardiac cell loss after I/R injury (48). Using isolated ARVMs, we provide evidence that UB decreases H/R-mediated increases in the number of apoptotic ARVMs. Akt, a central regulator of myocyte survival, is shown to protect myocytes against I/R injury in the mouse heart (16). Several upstream regulators are reported to turn off the activity of GSK-3 $\beta$ , among those Akt is a well-characterized Ser/Thr kinase phosphorylating GSK-3 $\beta$ . Akt phosphorylates GSK3 $\beta$  (Ser-9), and this residue lies in a typical Akt consensus substrate motif (40). Previously, we have shown that activation of GSK-3 $\beta$  plays a pro-apoptotic role in  $\beta$ -AR-stimulated cardiac myocyte apoptosis (39). Exogenous UB inhibited  $\beta$ -AR-stimulated activation of GSK-3 $\beta$  and apoptosis in vitro and in vivo (12; 49). The data presented here demonstrate that UB activates Akt and inhibits activation of GSK-3 $\beta$  within 10 min after reoxygenation. Collectively, these data suggest that activation of Akt signaling pathway may be a key signaling event involved in protecting cardiac myocytes against apoptosis. Inhibition of GSK-3 $\beta$  attenuates I/R injury by inhibiting mitochondrial permeability transition pore (mPTP) opening (60). Therefore, it is possible that extracellular UB may inhibit cardiac myocyte apoptosis by maintaining normal mitochondrial function via the involvement of AKT/GSK-3 $\beta$  pathway/s.

Low to moderate levels of superoxide are produced during ischemia, followed by a burst of ROS production at the onset of reperfusion (5; 33). The cardiac myocyte sarcolemma contains numerous uniport and antiport ion channels along with active transport mechanisms that are required to maintain cellular pH and membrane potential (44; 50). Evidence has been provided

that mitochondria serve as the final arbitrators of life or death during an I/R insult. These organelles are not only required to produce ATP, but they can also trigger both necrosis and apoptosis (20). Free radicals are produced within minutes of reperfusion and continue to be generated for hours after the restoration of blood flow to ischemic tissue (6). Oxidant-mediated cellular injury can lead to damage in the cell membrane, impairment of the cell's ability to regulate ionic homeostasis, and contribute to mitochondrial injury, leading to a decrement in oxidative phosphorylation and opening of the mPTP (33). The influx of hydrogen ions into the mitochondria during hypoxia dissipates the mitochondrial membrane potential ( $\Delta\psi_m$ ) and activates the  $\text{Na}^+-\text{H}^+$  exchanger (NHE) causing an elevation in sodium levels. To reduce the levels of  $\text{Na}^+$  in the mitochondria, the sodium–calcium exchanger ( $\text{Na}^+-\text{Ca}^{2+}$  exchanger) is activated, thus introducing  $\text{Ca}^{2+}$  into the mitochondria. Upon reperfusion, the  $\text{Na}^+-\text{Ca}^{2+}$  exchanger is reversed and starts extruding  $\text{Ca}^{2+}$  from mitochondria (21). It is clear that mitochondrial dysfunction is a major contributor to loss of myocytes during myocardial ischemia and subsequent reperfusion. With a reduced number of myocytes, the heart is no longer able to sustain contractility and heart failure develops (42). Here we show extracellular UB decreases the ROS and superoxide-positive ARVMs during H/R. It also increases the number of ARVMs with polarized mitochondria during anoxic conditions. It should be noted, however, that a significant difference in CTL and UB treatment was only observed 105 min after UB treatment. The treatment time points should be extended beyond 105 min to observe the protective effect of UB in preservation of mitochondrial polarization.

Cardiac myocytes and non-myocyte cell populations of the heart express CXCR-4 receptors (10). In leukemia cell line, CXCR-4 is identified as a receptor for UB (46). UB-CXCR-4 interaction follows a two-site binding mechanism in which the hydrophobic surfaces



surrounding Phe-4 and Val-70 are important for receptor binding (47). Previously, we have shown that the CXCR4 antagonist, AMD3100, inhibits UB-stimulated increases in capillary network formation, suggesting involvement of CXCR4 receptor for intracellular signaling in endothelial cells of the heart in response to UB (51). In this study, we show that AMD 3100 negates the effects of UB treatment with respect to apoptosis and activation of Akt and GSK-3 $\beta$ , suggesting involvement of CXCR-4 in UB signaling. Use of two different UB mutants further supports the involvement of this receptor in anti-apoptotic signaling pathway induced by UB.

### Perspectives

UB is found in normal plasma and is elevated in many diseased states. Previously, we have shown that extracellular UB plays an anti-apoptotic role in  $\beta$ -AR-stimulated cardiac myocyte apoptosis in vitro and in vivo (12; 49). The data presented here suggest a protective role for extracellular UB in I/R-induced cardiac injury and myocyte apoptosis. In the heart, UB treatment improved functional recovery and decreased activation of STAT-1 and caspase-9 during I/R injury. In vitro, UB treatment activated Akt an anti-apoptotic kinase and decreased H/R-induced myocyte apoptosis. The data presented here also suggests an important role for extracellular UB/CXCR4 axis in modulation of oxidative stress and mitochondrial membrane potential. Further identification of signaling mechanisms leading to UB-mediated regulation of I/R injury and cardiac myocyte apoptosis may uncover novel strategies to improve cardiac remodeling and function.

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### Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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## CHAPTER 5

### CONCLUSION

Significant attention is being paid to the development of therapeutics for the treatment and prevention of ischemia/reperfusion (I/R) injury and myocardial infarction (MI). Clinical trials are currently underway using therapeutic agents to reduce the adverse effects of cardiac remodeling caused by I/R injury and MI. Many therapeutic agents and new delivery methods are currently being investigated to reduce cardiac myocyte apoptosis and fibrosis. The future development of therapy directed at pathologic and therapeutic I/R injury and MI is dependent upon a clear understanding of the factors that regulate adverse remodeling of the heart and their mechanism of action.

Cardiovascular disease is the major cause of morbidity and mortality in the United States. I/R injury and MI cause adverse cardiac remodeling leading to a decrease in cardiac function. Cardiac myocytes and resident fibroblast are arguably 2 of the most important cell types of the heart. Due to the limited mitotic capacity of adult cardiac myocytes, it is important to find ways to reduce the loss of myocytes during or after injury to the heart. Fibroblasts play a significant role in wound repair and replacing the loss of myocytes with fibrotic tissue. Collectively, the loss of cardiac myocytes due to apoptosis and replacement with scar tissue causes the heart to become stiff reducing the functioning capacity. One way to repair the myocardium would be to slow or stop the loss of cardiac myocytes and reduce the deposition of fibrotic scar tissue. Therefore, the foundation for successful therapeutic interventions in I/R injury and MI may lie in the field of cardiac myocyte apoptosis and cardiac remodeling.

Cardiac sympathetic nerve activity increases following I/R injury and MI (Jardine and others 2005). Prolonged stimulation of the  $\beta$ -adrenergic neurohormonal axis contributes to the

progression of heart failure and mortality in animal models and human patients (Fowler and others 1986; Singh and others 2001). Stimulation of  $\beta$ -adrenergic receptor ( $\beta$ -AR) increases expression and activity of matrix metalloproteinase (MMP-2 and MMP-9) in cardiac myocytes *in vitro* and *in vivo* (Menon and others 2005; Krishnamurthy and others 2007). It also induces apoptosis in cardiac myocytes *in vitro* and *in vivo* (Shizukuda and others 1998; Iwai-Kanai and others 1999; Zaugg and others 2000; Singh and others 2001).  $\beta$ -AR-stimulated apoptosis in ARVMs is demonstrated to occur via the GSK-3 $\beta$ -/JNK-dependent mitochondrial death pathway (Remondino and others 2003; Menon and others 2007).

Ubiquitin is a highly conserved protein ~ 8.5 kDa and is found in all eukaryotic cells. Since UBs discovery over 3 decades ago, research has focused on UB's role inside the cell. To date, the most important function of UB is the regulation of protein turnover by the ubiquitin-proteasome pathway (Goldberg 2003). However, UB is a normal constituent of plasma and elevated levels of UB are described in several pathological conditions (Asseman and others 1994; Takagi and others 1999; Akarsu and others 2001a; Akarsu and others 2001b). Previously, our lab has provided evidence that stimulation of  $\beta$ -AR receptors increases extracellular levels of UB in adult rat ventricular myocytes (ARVMs), and extracellular UB plays a protective role in  $\beta$ -AR-stimulated apoptosis via the inactivation of GSK-3 $\beta$  and JNK pathways (Singh and others 2010). However, the role extracellular UB plays in hypoxia/reoxygenation-induced cardiac myocyte apoptosis and remodeling has not been elucidated.

The data presented in this study establish a role for extracellular UB in cardiac myocyte apoptosis and myocardial remodeling following chronic  $\beta$ -AR stimulation in the heart. We found that exogenous UB plays an important role in  $\beta$ -AR-stimulated myocardial remodeling with effects on left ventricular function, fibrosis and myocyte apoptosis. The signal transduction

pathways described within for extracellular UB are depicted in Figure 5.1. We confirm our previous finding of an antiapoptotic function for UB *in vivo* and show that UB plays an important role in  $\beta$ -AR-stimulated myocardial remodeling with effects on left ventricular function, fibrosis, and myocyte apoptosis. Exogenous UB enhanced activation of antiapoptotic kinase Akt, while decreasing the activation of proapoptotic kinases, GSK-3 $\beta$  and JNKs.

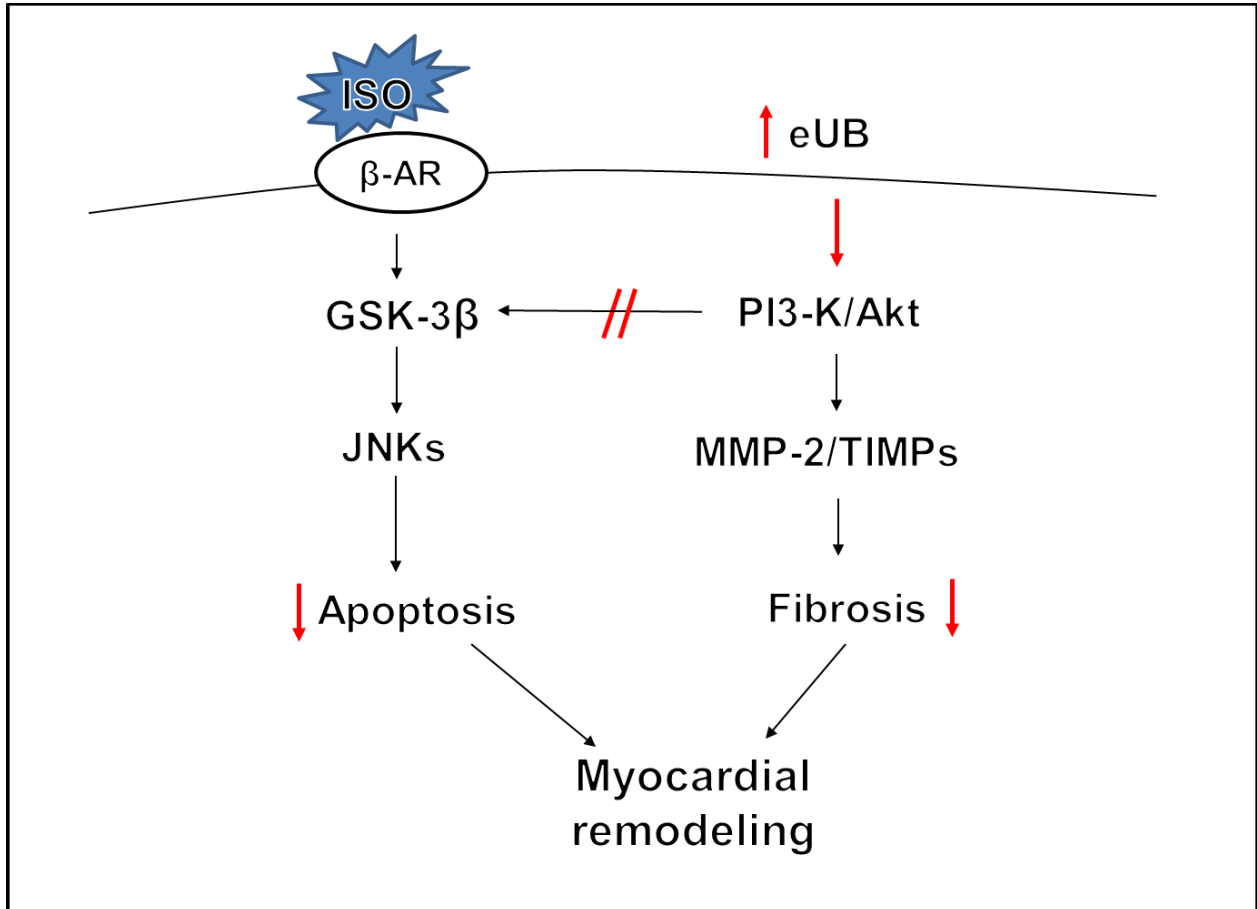


Figure 5.1 Signal Transduction Pathways for Extracellular UB in  $\beta$ -AR-Stimulated Apoptosis and Fibrosis.  $\beta$ -AR,  $\beta$ -adrenergic receptor; eUB, extracellular ubiquitin.

Cardiac fibroblasts play a critical role in maintaining the normal function of the heart, and in the remodeling process of the heart that occurs in response to myocardial infarction (Souders and others 2009). In this study, chronic  $\beta$ -AR-stimulation increased protein levels of MMP-2, MMP-9, and TIMP-2. In isolated cardiac fibroblasts, UB enhanced expression of MMP-2 and

TIMP-2. UB activated Akt and inhibition of Akt decreased MMP-2 expression. We also present evidence that extracellular UB modulates cardiac fibroblast phenotype and function and define a role CXCR4 receptor plays in modulating fibroblast phenotype and function in response to extracellular UB. UB interacts with fibroblast and CXCR4 receptors, induces cytoskeletal reorganization, induces intracellular signaling of ERK1/2, and inhibits migration of fibroblast into the wound. The fibroblast migration may be because UB decreases expression of  $\beta 3$  integrin, a protein involved in fibroblast migration. This may explain the reduction of fibrosis in UB treated  $\beta$ -AR-stimulated hearts.

Apoptosis is programmed cell death and is very organized. It has been reported that after MI or ischemic event, left ventricular (LV) dysfunction occurs, in part, as a result of ongoing cardiac myocyte apoptosis (Sharov and others 1996). Cardiac myocyte apoptosis occurs at an increased rate after injury due to I/R and MI (Olivetti and others 1997). However, the mechanisms of cardiac myocyte apoptosis leading to remodeling are not fully understood. In this study, we present evidence that extracellular UB plays a role in I/R injury in isolated hearts and cardiac myocytes. The data suggest extracellular UB protects the heart by decreasing infarct size, improving functional recovery of the heart, and decreasing activation of STAT-1 and caspase-9. In vitro, UB treatment decreases H/R-induced myocyte apoptosis, activates Akt while inactivating GSK-3 $\beta$ , decreases oxidative stress and improves the number of myocytes with polarized mitochondria. Most of these effects of UB are negated by CXCR-4 antagonist AMD 3100. Mutated UB, unable to bind CXCR-4 has no effect on H/R-induced apoptosis, intracellular signaling, or oxidative stress. Collectively, the data presented in this study shows evidence that extracellular UB modulates chronic  $\beta$ -AR-stimulated myocardial remodeling, fibroblast function, and I/R injury in isolated hearts and myocytes.



Possible future directions for this field of study are numerous. The current study examined the role of exogenous UB 7 days after chronic  $\beta$ -AR stimulation. This time point should be extended beyond 7 days to investigate if UB continues to decrease cardiac myocyte apoptosis, fibrosis, and sustain myocardial function. Analysis of components of extracellular matrix, including collagen type I and IV, laminin, fibronectin, etc. may provide insight into the modulation of heart function in the presence of exogenous UB. In isolated fibroblast, AMD 3100 almost completely inhibits UB-mediated increases in  $\beta$ 3 integrins and VEGF-A expression. However, inhibition of ERK1/2 was partial. Further investigation should be carried out to determine if this differential response is caused by differences in binding affinities for AMD 3100 and UB or incubation durations. The Langendorff global I/R injury is a good model to remove any potential confounding effects derived from systemic actions of the heart. However, the next logical step for I/R injury model would be to move into an animal model of I/R injury. All the current findings have been executed using extracellular UB as a pretreatment before insult. It would be interesting to see if extracellular UB has the same effects postinsult to the heart. Further identification of signaling mechanisms leading to UB-mediated regulation of I/R injury and cardiac myocyte apoptosis may uncover novel strategies to improve cardiac remodeling and function. The structural changes related to cardiac myocyte apoptosis and extracellular matrix play a significant role in modulation of myocardial function and in the progression to heart failure. Therefore, elucidation of processes that can shift the balance from myocyte apoptosis to survival may have clinical implications.

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