The effect of AT_1 or β-adrenergic receptor blockade on cardiotrophin-1 expression in the infarcted rat heart

by

Aran Lindsay Dangerfield

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

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ABSTRACT

Selective blockade of angiotensin type 1 receptors (AT_1) and β-adrenergic (β-AR) signaling pathways improves survival and quality of life in patients with both moderate and severe heart failure. Angiotensin II is a mediator of post-myocardial infarction (MI) wound healing via directly and indirectly triggering increased collagen synthesis by myofibroblasts. Long term AT_1 blockade after MI is associated with improved left ventricular function and decreased cardiac fibrosis. Cardiotrophin-1 (CT-1) is a novel cardioprotective agent which is known to be elevated in various cardiovascular disease states. CT-1 signals through the gp130/LIF receptor complex and is rapidly expressed and significantly elevated immediately following infarction to upwards of 8 weeks post-MI. The beneficial effects of long-term β-blockade in treating heart failure patients may result from its antagonism of the cytotoxic effects of increased circulating catecholamines and the normalization of β-AR signaling however, the relationship between the adrenergic system and CT-1 remains undefined. We sought to investigate the effect of AT_1 (losartan) or β-AR blockade (propranolol) on CT-1 expression *in vivo* in infarcted rat hearts. Following induction of MI, losartan and propranolol were administered in drinking water to separate groups of treatment animals for 24 and 48 hrs as well as 2, 4, and 8 weeks post-MI. Heart tissues were isolated and analyzed via Western blot for expression of CT-1, gp130, phosphorylated STAT3 and total STAT3. AT_1 blockade did not induce an effect on CT-1 expression but β-adrenergic receptor blockade increased CT-1 expression within the infarct scar at 2 and 4 weeks compared to control. Within the propranolol treated rat heart, gp130 expression was significantly downregulated in the scar but not in the viable LV or control tissues. Similarly, the levels of activated phosphorylated STAT3 decreased whereas total STAT3 increased within the infarct scar but not compared to untreated tissue samples. Thus, chronic AT_1-blockade had no effect on CT-1 expression but β-adrenergic blockade is associated with the modulation and regulation of CT-1 expression in the post-MI rat heart.

Keywords: CT-1; cardiotrophin-1; myocardial infarction (MI); AT_1; β-AR; propranolol; losartan; gp130; pSTAT3, Total STAT3, cardiac fibrosis; cardiac myofibroblasts.

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AC – Adenylyl Cyclase

ACE – Angiotensin Converting Enzyme

α-SMA – Alpha-Smooth Muscle Actin

AMI – Acute Myocardial Infarction

AMP – Adenosine Monophosphate

Ang II – Angiotensin II

ARB – Angiotensin Receptor Blocker

ATP – Adenosine Triphosphate

AT_1 – Angiotensin Type 1

AT_2 – Angiotensin Type 2

β-AR – Beta – Adrenergic Receptor

BSA – Bovine Serum Albumin

cAMP – cyclic Adenosine Monophosphate

CAD – Coronary Artery Disease

CLC – Cardiotrophin-Like Cytokine

CT-1 – Cardiotrophin-1

CHF – Congestive Heart Failure

CK-MB – Creatine Kinase Skeletal-Muscle Brain Hybrid Type

CNTF – Ciliary Neurotrophic Factor

COX2 – Cyclo-Oxygenase - 2

CSF-1 – Colony Stimulating Factor

DCM – Dilated Cardiomyopathy

DMEM – Dulbecco's Modified Eagle Medium

ECL – Enhanced Chemiluminescence

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

EGTA – Ethylene Glycol bis (2-aminoethylether) N, N, N', N' – Tetra Acetic Acid

ERK – Extracellular Signal-regulated Kinase

EXP3174 – Losartan Metabolite

FABP – Fatty Acid Binding Protein

FAK – Focal Adhesion Kinase

FBS – Fetal Bovine Serum

FITC – Fluorescein Isothiocyanate

FGF – Fibroblast Growth Factor

gp130 – Glycoprotein 130

GPCR – G-Protein Coupled Receptor

HF – Heart Failure

HRP – Horseradish Peroxidase

IHD – Ischemic Heart Disease

IL – Interleukin

iNOS – inducible Nitric Oxide Synthase

JAB – Jak Binding protein

Jak – Janus kinase

LIF – Leukemia Inhibitory Factor

LOS – Losartan

LV – Left Ventric(le/ular)

MAPK – Mitogen-Activated Protein Kinase

MI – Myocardial Infarction

MMP – Matrix Metalloproteinase

MCP-1 – Monocyte Chemoattractant Protein

mRNA – messenger Ribonucleic Acid

NF-κB – Necrosis Factor-κB

NO – Nitric Oxide

NPN – Neuropoietin

OSM – Oncostatin M

PAGE – Polyacrylimide Gel Electrophoresis

PBS – Phosphate Buffered Saline

PDFG – Platelet Derived Growth Factor

PI3K – Phosphoinositide-3 Kinase

PLC – Phospholipase C

PROP – Propranolol

PVDF – Polyvinyldiene Fluoride

RAS – Renin Angiotensin System

RIPA – Radioimmunoprecipitation Assay

SDS – Sodium Dodecyl Sulfate

SGOT – Serum Glutamic-Oxaloacetic Transaminase

SMEM – S- Minimum Essential Medium (Eagle's)

SOCS – Suppressor Of Cytokine Signaling

SP1.D8 – Procollagen

pSTAT3 – phosphorylated Signal Transducer and Activator of Transcription 3

STAT3 – Signal Transducer and Activator of Transcription 3

TGF- β – Transforming Growth Factor-β

TNF-α – Tumour Necrosis Factor – α

VEGF – Vascular Endothelial Growth Factor

INTRODUCTION

The heart beats about 100,000 times a day or around 3 billion times during the average person's life span (85). As the source providing our tissues, organs and brain with nourishing, nutrient-rich oxygenated blood, the heart is essential in the maintenance of a healthy, functioning individual. Thus, it is not surprising that cardiac dysfunction and disease are the leading cause of death in Canada and the developed world accounting for 37% of mortalities in Canada alone (59).

The heart is composed of four chambers; the left and right atria and the left and right ventricles. The atria collect blood as it returns from the body (right atrium) or lungs (left atrium). During diastole (relaxation), one-way valves open between the atria and ventricles and blood pours into and fills the ventricles. Subsequently during systole, the ventricles contract, closing the valves to the atria and sending blood through a second set of one-way valves to the lungs (right ventricle) or body (left ventricle) (104). Acute myocardial infarction (AMI) is an injurious cardiac event characterized by cardiac myocyte death due to prolonged ischemia (5). The ischemic condition, characterized by a loss in blood flow, is generally the result of the rupture of an atherosclerotic plaque from a blood vessel wall and the subsequent occlusion of a coronary artery (69; 103). In this study, myocardial infarction is the consequence of left coronary artery ligation, leading to subsequent scar formation and eventual progression to heart failure (HF). Heart failure is characterized by the inability of the heart to pump a sufficient amount of blood to meet the metabolic needs of the body (11). The clinical diagnosis of acute heart failure is characterized by symptoms of tachycardia, cutaneous vasoconstriction, diaphoresis, and reduced urinary output (12). After MI, tissue modifications take place

within the infarct zone and the remainder of viable left ventricle (13; 96; 126). The contractile function of the heart is impaired and myocyte necrosis begins within the first 60-90 minutes of ischemia. If severe ischemia persists the myocardial infarction is complete within 3-6 hrs (13). In large, anterior infarcts, expansion, thinning and elongation of the necrotic myocardium occur in the hours and days post-MI (13). Myocyte necrosis results in a reduction in the number of myocytes across the infarct wall (144), and edema and inflammation are localized to the infarcted region (96). Post-MI cardiac preload on the viable, non-ischemic cardiac tissue increases and ventricular emptying is impaired. The left ventricle undergoes sphericalization and dilatation in response to the increased load of blood that is incompletely pumped out of the ventricular cavity (13). The likelihood of aneurysm formation, cardiac rupture and subsequent death are all increased with higher intraventricular systolic pressure, a lack of pre-existing hypertrophy of the ventricular wall, and a large transmural anterior infarction (13).

The positive feedback resulting from the contractile dysfunction and the induction of pathologic hypertrophy and remodeling within the myocardium after infarction likely explain the typically deleterious progressive history and outcome of dilated cardiac conditions. Any attempts to treat congestive HF involve therapies that interrupt this deleterious cycle. Neurohormonal antagonists such as AT_1 inhibitors and β-AR blocking agents are treatments capable of such interruptions, promoting positive post-MI outcomes, though the specific mechanisms by which these drugs induce their beneficial effects are only partially understood (19).

A large MI (defined as < 40% of normal LV function) is generally accompanied by pain and fright and in concert with the ventricular dyskinesis associated with the

occlusion of a major coronary artery result in the activation of the sympathetic nervous system (13). The stimulation of β-adrenergic receptors and the increased contractility of the remnant viable myocardium act to sustain global ventricular function (13). The level of sympathetic nervous drive is reduced by β-adrenergic blockade and has been shown to significantly decrease the risk of morbidity and mortality associated with MI (36; 39). The effect of a given β-blocking agent depends on the way the drug is absorbed, bound to plasma proteins, its metabolism, and also on the extent to which it inhibits the β-receptor (via a lock-and-key fit) (92). Propranolol, the "gold standard" in β-blockade, is a non-specific β-receptor antagonist widely used in the treatment of a variety of conditions from angina to AMI and hypertension among others (92). In heart failure, therapeutic blockade of β-receptors acts to reduce mortality in a manner which is not yet fully understood.

Upregulation of the renin-angiotensin-aldosterone system (RAS) results in increased circulating levels of angiotensin II (Ang II). Ang II is a known hypertrophic agent specific to neonatal cardiac myocytes and is capable of inducing increased matrix production in rat cardiac fibroblasts *in vitro* (9; 66). Ang II has been shown to mediate vasoconstriction and vascular tone, fluid and electrolyte homeostasis and contribute to overall hypertension which can compromise the pumping ability of the failing heart. Blockade of angiotensin converting enzyme (ACE), responsible for the cleavage of angiotensin I to its active form (Ang II) has been shown to improve morbidity and mortality post-MI (106). Unfortunately, ACE inhibition is not well tolerated in all patients and thus interest in alternative therapies like angiotensin type 1 receptor (AT_1R) blockade has increased (106; 116). Though the benefits of AT_1R blockade have been

shown, the precise mechanism of the inhibition of cardiac fibrosis post-MI has yet to be determined.

Cardiotrophin-1 (CT-1) is a novel neuro- and cardioprotective agent, elevated in various cardiovascular disease states (7; 77; 101; 102; 114; 145). A member of the IL-6 superfamily of cytokines, CT-1 signals through the gp130/LIF receptor complex to mediate downstream effects such as regulating the transcription of genes important for a variety of cellular responses. CT-1 has been shown to induce hypertrophy of cardiac myocytes via induction of sarcomeric proteins in series (147). The 5' flanking region of the mouse CT-1 gene contains a norepinephrine response element (NRE) in its promoter (47), though its specific function remains unknown. Previous studies have demonstrated elevated levels of cardiotrophin-1 (45) and decreased levels of gp130 expression in post-MI untreated heart, but to date, the effects of AT_1 or β-AR blockade on regulating CT-1 and gp130 expression have not been published.

The process of post-MI LV remodeling is manifest by changes in wall structure, chamber geometry and pump function (22; 104; 127). Initially, the increased deposition of collagens at the site of injury and within the remnant viable myocardium is beneficial in helping maintain the integrity of the infarct scar. However, upregulation of collagen production eventually becomes deleterious to the heart causing excessive stiffening of the myocardial wall resulting in cardiac rupture and death (126; 126).

In the current investigation, we examine whether either pharmacological β-blockade or AT_1 receptor inhibition are associated with any effect on CT-1 expression in the sham or infarcted rat heart. As the infarct scar is dominated by cardiac myofibroblasts from 3 days to months and years thereafter, and since these cells mediate

wound healing in the infarct zone, our study also addresses CT-1 expression in cardiac myofibroblasts *in vitro*. The first aim of this project was to examine the *in vivo* effect of angiotensin blockade on CT-1 expression using losartan (AT_1 receptor blockade). The second aim was to determine the *in vivo* effect of β-adrenergic receptor blockade using propranolol (non-specific β-blockade) on CT-1 and gp130 expression, and the downstream regulators of transcriptional response (total STAT3 and pSTAT3). The final aim of the project was to establish the *in vitro* effects of β-adrenergic stimulation on CT-1 and gp130 expression.

STATEMENT OF HYPOTHESES

Aim 1: Angiotensin (AT1 receptor) blockade decreases the *in vivo* expression of cardiotrophin in the post-MI heart; particularly, CT-1 expression will be affected in within the infarct scar.

Aim 2: β-adrenergic blockade decreases the *in vivo* expression of cardiotrophin-1, and modifies the expression of gp130, pSTAT3 and total STAT3 after myocardial infarction.

Aim 3: β-adrenergic stimulation increases both CT-1 and gp130 expression *in vitro*.

III. Literature Review

I) Heart Disease and The Induction of MI

Despite advances in the quality of treatments available, coronary artery disease (CAD) remains the leading cause of morbidity and mortality in Canada and the world (69). CAD is a multi-faceted set of conditions including angina pectoris, myocardial infarction (MI) and congestive heart failure (CHF). Most MIs occur as the result of rupture of an atherosclerotic plaque. Thrombolytic occlusion of a coronary artery restricts blood flow to the collateral myocardial tissues causing cell death and tissue necrosis (69; 103). Wound healing post-MI involves the recruitment and proliferation of myofibroblasts and the deposition of extracellular matrix proteins (ECM), most notably collagens I and III, within the infarct scar and viable myocardium. Initially, matrix synthesis provides a beneficial function in maintaining the integrity of the infarct scar; however, over time the excess production of collagens proves detrimental and causes left ventricular stiffening which can lead to cardiac rupture (13).

There are a variety of known stimuli or 'triggers' associated with coronary events that appear to be singularly connected by one uniting factor: stress. Stressors activate the sympathetic nervous system and result in catecholamine release initiating a number of compensatory mechanisms. These mechanisms are believed to have initially evolved to protect humans in the 'fight or flight' response, but may now provoke sclerotic rupture and subsequent cardiac ischemia (69). Heart rate and blood pressure increases in the morning have also been correlated with increased plasma epinephrine and norepinephrine levels (79). Despite our ability to identify stimuli responsible for increasing risk of infarction, and the existence of preventative guidelines to decrease such risks, cardiac

events continue to occur. Apart from survival of the initial cardiac event, the damage occurring as a result of arterial occlusion presents a more serious condition of eventual progression to heart failure. It is therefore essential that we develop a clear-cut understanding of the changes that occur within the post-MI heart to improve both short and long-term survival.

II. Acute Myocardial Infarction

Acute MI develops over the course of several hours and terminates when necrosis of tissues reaches its ultimate extension defined by the boundary of the infarct-associated vascular bed and its potential collateral flow (35). MI is characterized by ischemia when there is less than 40% of normal blood flow to cardiac tissues and the contractile function of the heart becomes impaired (13). Post-MI wound healing is a complex process requiring the activation of multiple parallel systems and takes place over a prolonged period of time after the initial injury.

Infarction results in a series of adaptive cellular and extracellular changes. Cardiac wound healing can be divided into four distinct phases of: i) cell death, ii) the acute inflammatory response, iii) granular tissue formation and iv) repair and remodeling of the infarct scar (4).

Cardiomyocyte death characterizes the first phase of cardiac wound healing. In the adult myocardium, it is generally believed the vast majority of cardiomyocytes do not proliferate. This view is supported in part by clinical observations: functionally significant myocardial regeneration has not been documented in diseases and/or injuries

resulting in cardiomyocyte loss. In addition primary myocardial tumors are rarely observed in adults (121).

Terminally differentiated cardiomyocytes consume the vast majority of the volume of the normal adult mammalian heart (22). These cells constitute the core contractile component of the myocardium and contain several specific contractile proteins which allow them to move in a rhythmic and coordinated fashion (22). Serum markers of myocyte death include increased serum levels of fatty acid binding protein (FABP), troponin T, creatine kinase skeletal-brain hybrid type (CK-MB), and serum glutamic-oxaloacetic transaminase (SGOT). Many such markers can be identified within the first few hours after injury and can be used in general post-MI diagnosis.

Under ischemic conditions myocyte cell death can arise as the result of either apoptosis or necrosis. Apoptosis is characterized by cell swelling and, in rats and humans, peaks within the first 6-8 hrs post-MI (4; 22). Necrosis is believed to take place secondary to apoptosis, characterized by cell shrinkage and occurs 12 hrs to 4 days post-MI (4; 22). It is believed necrosis extends from the inflammatory response at the site of injury and results from the inability of neighbouring cells to phagocytize the majority of the myocytes that have undergone apoptosis (4).

The second phase of wound healing is marked by the inflammatory response stimulated by myocyte cell death. This response initiates a series of events designed to recruit cells to remove and repair damaged tissues and to allow for maintenance of the infarct region via hypertrophy, hyperplasia and ECM production (31; 129). It is believed myocardial cell necrosis results in the release of subcellular membrane constituents rich in mitochondria, and that these components are capable of triggering the complement

cascade within 12-16 hrs after the onset of ischemia (22; 43). The activation of the complement cascade results in the recruitment and trapping of neutrophilic granulocytes and monocytes at the site of injury which act to remove dead cells and other debris. The protein fragment C5a, a component of the complement cascade, initiates the chemotactic recruitment of these mononuclear cells which infiltrate the infarcted myocardium. Transforming growth factor-β (TGF-β) as well as monocyte chemoattractant protein (MCP)-1 secreted by numerous cells of the inflammatory cascade such as neutrophils and platelets also contributes to this cellular influx. Within the infarct site, monocytes differentiate into macrophages likely as a result of localized upregulation of macrophage colony-stimulating factor (M-CSF). These cells serve to regulate the composition of the matrix through synthesis of matrix metalloproteinases and their inhibitors as well as possibly serving as a source of other cytokines and growth factors (43).

Mast cells are granulocytes that originate from CD^{34+} hematopoietic stem cells and circulate as immature precursors in the peripheral blood. At the site of injury, resident cardiac and newly recruited circulating mast cells are an important source of preformed and freshly synthesized cytokines, chemokines and growth factors, most notably TNF-α (4). Mast cells are also important sources of TGF-β, basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), which have been shown to recruit mononuclear cells as well as regulate fibroblast growth, modulate matrix metabolism, and stimulate angiogenesis (43). Mast cell degranulation occurs within the ischemic area and results in rapid release of TNF-α which induces IL-6 expression in infiltrating mononuclear cells. Experimental MI is associated with the coordinated activation of a series of cytokine and adhesion molecules such as NF-κB, which are

activated by cytokines such as TNF-α and by free radicals (43). The genes regulated by NF-κB transcription factors are involved in the inflammatory response, as well as in cell adhesion and growth control. Via the production of a variety of cytokines and growth factors, macrophages, mast cells and lymphocytes create an environment rich in inflammatory cells. Capable of regulating neovessel formation, fibroblast proliferation and matrix metabolism, inflammatory cells begin the healing process within the infarct wound (43).

Activation of matrix production following infarction is a critical step in the acute response to wound healing and scar formation to replace necrotic myocytes and damaged tissues. The third phase of wound healing is distinguished by the formation of granulation tissue in the border zone of the infarct scar two to 3 days post-MI (4). Granulation tissues are characterized by an abundance of small blood vessels. Appearing 3-4 days post-MI, these vessels are either newly formed or derived from pre-existing collateral vessels that retained blood flow during ischemia (4).

Myocardial fibroblasts represent the largest class of cells, in number not size, residing in the LV and serve to regulate matrix turnover and coordinate the fibrotic response to injury (65). Fibroblasts normally function to maintain the integrity of the extracellular matrix and play an integral role in matrix remodeling (45; 123). Within the cardiac wound, fibroblasts are modified via a variety of stimuli including mechanical tension and various cytokines to become a specialized class of hypersecretory myofibroblasts capable of producing an excess of collagens and thus helping maintain the integrity of the scar (29; 133). Myofibroblasts are the main collagen-producing cells in myocardial infarcts which has been shown in many studies to be upregulated in the

infarct scar as well as the remnant viable myocardium (23; 45; 93; 123; 141). These differentiated myofibroblastic cells specifically express α-smooth muscle actin (α-SMA) and embryonic smooth muscle myosin (SMemb) but do not express desmin (a marker of endothelial cells) (41; 45; 115). Myofibroblast differentiation and SMemb expression can be induced by stimulation with TGF-β (41).

Matrix deposition, synthesized by newly formed myofibroblasts, begins with fibrin, followed by fibronectin, tenascin and laminins. These same active myofibroblasts also help regulate the fibrotic process through the synthesis of other matrix proteins and metalloproteinases (42). Composed of collagens and other matrix proteins, proteoglycans, glycosamingoglycans, basement membrane proteins, and bioactive signaling molecules, the cardiac matrix serves an essential function in the heart (3; 78; 140). Granular tissues within the infarct zone are well ordered resulting from the highly organized arrangement of myofibroblasts (4).

The final phase of cardiac wound healing is characterized by the repair and remodeling of the infarct scar. Deleterious remodeling is the leading cause of heart failure and death (43). Even after 2-3 weeks post-MI, the granulation tissue is still rich in myofibroblasts, macrophages and small blood vessels (4). Scar maturation involves a loss of cellularity as a result of apoptosis and leads to a decrease in the cell number within granulation tissues (4). Within the post-MI myocardium, even within the infarct scar, myofibroblasts have been found to persist for more than 20 years (146).

III. Cardiac Wound Healing and the Induction of Heart Failure Post-MI

After a large transmural MI, the whole heart undergoes repair with fibrous tissue that appears both proximally and remotely to the site of injury and contributes to deleterious remodeling in the non-infarcted myocardium as well (128). Under normal conditions, the early LV filling is controlled by relaxation and expansion of the heart muscle and by the heart's ballistic recoil. The final volume of the ventricle is then adjusted by the pressure of the incoming blood. In response to pathological conditions, the heart loses some of its ability to recoil, and suction blood into the ventricle, and thus fills incompletely (104). In heart failure, the heart is unable to deliver an adequate output of blood to supply the demands of the tissues despite adequate LV filling pressure (2; 13). This progressive decline in cardiac output is related to left ventricular remodeling (96). After an infarct, the LV undergoes a series of adaptive changes that result in progressive dilation, hypertrophy, distortion of cavity shape, and deterioration in contractile function (124). The compensatory increase in size of the LV results from the imbalance between the workload required and the inability of the heart to sufficiently pump blood from the ventricles in its weakened state (104). Myocardial hypertrophy, ischemia, and fibrosis cause slow or incomplete LV filling at normal left atrial pressures. Cardiomyocytes, through sarcomeric hypertrophy in series, contribute to the increase in LV chamber size and mass in response to increased loading conditions (55). Left atrial pressure rises to increase LV filling, and results in pulmonary and systemic venous congestion.

Maladaptive left ventricular remodeling primarily occurs as a result of excessive collagen production and the subsequent stiffening of the myocardial wall. In combination, cardiac fibroblasts and myofibroblasts account for ~ 70% of the total cell

population and are exclusively responsible for the synthesis of fibrillar collagens which compose 90% of the total matrix proteins in the heart (139). With normal aging, LV wall thickness, stiffness and systolic blood pressure is increased (2). Post-MI remodeling stimuli such as increased mechanical LV wall stress, neurohormonal activation, cytokines, and oxidative stress lead to hypertrophy of cardiac myocytes, alterations in the interstitial matrix, fetal gene expression, and myocyte death (2). These events stimulate deleterious changes in the structure and function of the LV, which result in further LV dysfunction and increased LV wall stress, and subsequently promote more pathologic remodeling (2). The increased wall tension, increased myocardial oxygen consumption, reduced subendocardial perfusion, and reduced myocyte shortening all affect the pumping ability of the heart. LV remodeling affects survival by reducing hemodynamic function and by increasing the risk of arrhythmias leading to sudden cardiac death.

The major neurohormonal systems activated in heart failure are the sympathetic nervous system, the renin–angiotensin–aldosterone system, natriuretic peptides, endothelin, and TNF-α (2). Activation of neurohormonal systems in chronic heart failure increases LV hypertrophy and later LV dilatation by alterations in afterload, preload, stretch, increased wall stress, interstitial collagen deposits, and by having direct toxic effects.

IV. The Rat Model of Myocardial Infarction

Animal models simulating the human condition of heart disease are an essential component in our understanding of the pathophysiology behind the illness, and in our ability to produce newer and more effective treatment regimens for heart failure (51).

Developed in Canada by Selye *et al.* in the early 1960s, the rat model of MI is one of the few reliable methods of studying scar generation and congestive heart failure *in vivo* (113). In the rat, vascular proliferation and fibrosis of the infarct zone and resorption of necrotic tissue occurs from the onset of infarction, and discrete scar formation is evident at 21 days post-MI (104). The generation of large infarcts, e.g. greater than 30%, is associated with significant myocyte hypertrophy (98). Rats with infarct size greater than 45% have been shown to exhibit overt heart failure which is characterized by elevated filling pressures, reduced cardiac output and low response capacity to preload and afterload stress (137). Previous investigation has verified that the rat model of chronic infarction is an acceptable approximation to the clinical condition. Animals who undergo a left coronary arterial ligation procedure eventually develop heart failure characterized by decreased blood flow to systemic organs in a manner similar to that of the human condition (28; 51). The rat infarct model mimics the human condition, and administration of pharmacologic interventions also proves useful in predicting the response in humans given the same treatment (51). The experimental MI in rat is a well-established model for studying gene expression in pathological conditions in both viable remnant myocardium and within the infarct scar (1; 38; 54; 62; 152). It is for this reason that the rat model of myocardial infarction was chosen as an acceptable and compensatory approximation of heart failure for this study.

V. Post-MI Protein Expression

I. gp130 signaling pathway

Cytokines regulate communication between cells to modulate survival, growth, differentiation and effector functions (56). Rapidly synthesized and secreted by different cells generally after stimulation, cytokines differ from hormones in that they are not stored in glands as preformed molecules. Exhibiting pleiotropism through their effects on many different target cells cytokines can, in nano-to-picomolar concentrations, affect the action of other cytokines in an additive, synergistic or antagonistic manners (56). Cytokines are classified by their a) biological responses as pro or anti-inflammatory cytokines, b) receptor or c) in terms of their three-dimensional structure (56). Glycoprotein 130 (gp130) is a member of the hematopoietic cytokine receptor family and consists of an extracellular region, a single transmembrane segment, and a cytoplasmic domain. The gp130 receptor subunit is considered the most 'promiscuous' of the cytokine receptors as it transduces the signals of a multitude of different ligands including interleukin-6 (IL-6), IL-11, LIF (leukemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), neuropoietin (NPN), cardiotrophin-like cytokine (CLC) and CT-1 (cardiotrophin-1) (87; 112). The physiological responses initiated by gp130 receptor binding are the result of activation of the Janus kinases (Jaks) and transcription factors of the STAT family (Jak-STAT Pathway) (56).

The IL-6 family of cytokines, to which CT-1 belongs, is characterized by a four-α-helix bundle structure (87). After first binding to specific receptors on their target cells, cytokine complexes of IL-6 and IL-11 bind to receptors on their target cells and dimerize with gp130. Receptor-bound CLC, NPN and CNTF form a heterodimer of the gp130 and the LIF receptors (LIF-R). In CT-1 signaling specifically, CT-1 binds directly to the LIF-R, inducing heterodimeric binding with gp130 which then tranduces

downstream signaling efforts. IL-6 cytokines may activate numerous intracellular signaling pathways contributing to disease progression (52). In ventricular restricted gp130 cytokine receptor knockout mice, cardiac structure and function appear to be unaffected under normal conditions. However, after aortic banding the mice display a rapid onset of dilated cardiomyopathy and massive induction of apoptosis whereas control mice developed hypertrophy in response to banding (58). Homozygous gp130 knockout mice were embryonic lethal with a hypoplastic ventricle (151).

In recent years biomarkers have emerged as important tools for diagnosis, risk assessment and therapeutic decision making in cardiovascular disease. One such clinically utilized marker is the B-type (brain) natriuretic peptide (BNP) secreted in response to volume expansion and pressure overload of cardiac ventricles (26; 142). BNP and N-terminal pro-BNP are used for the early diagnosis of HF in patients presenting to the emergency room with shortness of breath (26; 82). Additionally, in patients with chronic HF and acute and chronic coronary artery disease, both BNP and N-terminal pro-BNP are markers of unfavorable prognosis associated with increased mortality (26; 90). Recent studies indicate that in patients with very low ejection fraction but mild to moderate heart failure symptoms, soluble gp130 (sgp130) and brain natriuretic peptide (BNP) were significantly higher in patients whose heart failure worsened or resulted in death. For long-term prognosis, it appears sgp130 serum levels may be superior to BNP or N-BNP in predicting worsening heart failure in patients with advanced left ventricular dysfunction (52). Previous studies from Podewski *et al.* have also shown significant increases in sgp130 in patients with moderate to severe heart failure (99). The authors hypothesize that elevated levels of sgp130 in patients with

congestive heart failure may reflect enhanced IL-6 activity with enhanced gp130 turnover in the myocardium (99). Soluble gp130 may prove to be a valuable independent predictor of progression to heart failure in high risk patients.

II. THE JAK/STAT PATHWAY

The Janus kinases (Jaks) are a family of cytosolic tyrosine kinases that play a critical role in the rapid transduction of signals from the cell surface to the nucleus. Named after Janus, the Roman "god of gates" who was represented as a two-faced divinity, the kinase itself contains two faces; a JH1 (kinase) and a JH2 (pseudokinase) domain (6). The catalytic activity of Jaks is regulated by the JH1 domain. It is suggested the JH2 domain is a potential docking site for signal transducers and activators of transcription (STATs) (6).

The Jak/STAT pathway is capable of transmitting information from extracellular polypeptide signals through transmembrane receptors, directly from the cytoplasm to target gene promoters in the nucleus (56). STATs are ubiquitously expressed and are activated in the cytoplasm, but exert their function in the nucleus. STATs are mainly activated after stimulation of cytokine receptors (6). There is also evidence of STAT activation by other receptor tyrosine kinases such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), colony stimulating factor (CSF-1R), c-kit, insulin receptor and c-met, and by G-protein-coupled receptors (angiotensin II receptors). STAT activity is predominantly regulated by post-translational modifications, specifically the phosphorylation of tyrosine and serine residues.

There are seven different STATs, but the major functional STATs in the heart are STAT3 and STAT1. STAT3 is activated by gp130 and requires tyrosine phosphorylation to allow for dimerization (STAT3, Tyrosine 705). Following tyrosine phosphorylation STAT molecules are transported into the nuclear compartment. STAT dimerization is a prerequisite for DNA binding. After nuclear translocation and dimerization, STATs bind to specific enhancer sequences in DNA stimulating, and in certain cases possibly repressing, the transcription of respective target genes. After growth-hormone stimulation STAT3 is MAPK-dependently serine-phosphorylated; in contrast, after stimulation with IL-6, STAT3 is phosphorylated at the same amino acid independently from MAPK activation (46).

STAT3 has been implicated as a mediator of cardiac hypertrophy and in the induction of endothelial growth factor (46; 73). STAT3 has also been postulated to have a protective role in the heart (72; 88). STAT3 phosphorylation in the heart was examined by Ng *et al.* in 2003 in patients with heart failure resulting from ischemic heart disease (IHD) or idiopathic dilated cardiomyopathy (DCM) (89). Significant STAT3 Y705 phosphorylation was detected in DCM but not IHD(89). Many animal based studies indicate the phosphorylation of the STAT family of transcription factors accompanies cardiac hypertrophy, ischemia/reperfusion and dilated cardiomyopathies (89).

Anti-apoptotic effects of STAT3 have been demonstrated in cultured neonatal cardiac myocytes subject to anoxia, metabolic inhibition and acidosis. In a mouse model of ischemic preconditioning, a role for the Jak-STAT pathway in cardioprotection has been identified (6). *Bolli et al.* identified Jak1, Jak2, STAT1 and STAT3 as the specific

elements involved in the induction of iNOS protein and activity, and delayed protection against infarction.

The Jak-STAT pathway affects cardiac hypertrophy and apoptosis (6). In response to CT-1 stimulation, LIF-β and gp130 become tyrosine phosphorylated by Janus kinases (Jaks), since neither contain any inherent kinase activity (56; 105; 148). Signaling via gp130 and Jak-STAT is controlled by negative-feedback by a family of proteins called the suppressors of cytokine signaling (SOCS). It has been shown that signaling via the gp130-Jak/STAT pathway is profoundly altered in patients with end-stage dilated cardiomyopathies at the level of expression and activated phosphorylation (99). It has also been found that STAT3 protein abundance is significantly reduced in both cardiomyocytes from failing hearts and in non-myocytes (99). The Jak-STAT pathway has been shown to protect cardiomyocytes from apoptosis, induce hypertrophy and promote expression of cardioprotective genes (superoxide dismutase and VEGF) (99).

Jak/STAT pathways have been implicated in the migration of epithelial cells. Through binding to a seven-transmembrane G-protein-coupled receptors, Soriano *et al.* have shown that chemokines activate the JAK/STAT pathway to trigger chemotactic responses (122). The Jak/STAT pathway has been found to be prominently associated with the activation of the autocrine loop of the cardiac rennin-angiotensin system (RAS). Angiotensin II uses a signal pathway in cardiac myocytes in which the promoter of the gene encoding its prohormone, angiotensiongen is a target for the STAT proteins. The Jak/STAT pathway has been shown to be activated in ischemic myocardium (83; 91; 149), but most recently in remote post-MI heart as well (33). This activation is

significant in that El-Adawi *et al.* find that blockade of the Jak/STAT pathway has beneficial functional consequences in early post-MI remodeling equivalent to that seen for AT_1 blockade with losartan (33).

There has been increasing interest in understanding the pathways of signal transduction that mediate the transition of cardiac hypertrophy to overt heart failure because of the benefit of targeting these as potential targets for therapeutic intervention (89).

III. CARDIOTROPHIN-1

Cardiotrophin-1, CT-1, is a member of the IL-6 superfamily of cytokines discovered in 1995 by Pennica *et al.* (95). Other members of the IL-6 family of cytokines include IL-11, LIF, OSM, and CNTF (94). CT-1 is expressed in adult cardiac and skeletal muscle, the ovary, colon, prostate, and testis, and in fetal cardiac, kidney and lung tissues (56; 61). CT-1 is produced and released following myofibroblast cell injury and its expression is augmented in the post-MI heart. Its expression remains elevated into the chronic phase of wound healing, unlike other members of the IL-6 family cytokines (45). CT-1 is a marker of heart disease (80), and has been shown to be cardioprotective (7; 77; 114). Promotion of cardiac hypertrophy via CT-1 stimulation causes in series sarcomeric assembly in myocytes leading to eccentric LV hypertrophy and chamber dilatation (148). Studies indicate CT-1 is capable of altering fibroblast growth (44; 135), and is able to initiate migration and proliferation as well as protein and collagen synthesis (important factors in scar synthesis).

The cardiotrophin-1 signaling cascade requires multiple receptor components and activated factors; glyoprotein130 (gp130) and leukemia inhibitory factor (LIF), Janus

kinases (Jak), and the signal activator and transducers of transcription (STAT3) (56). Cardiotrophin-1 initially binds the LIF receptor and then associates with gp130 forming a dimeric receptor complex which translocates into the nucleus and subsequently transduces the proximal signal (147). CT-1 carries out multiple diverse biological functions (16; 45), CT-1 activates multiple pathways via ERKs (extracellular signal-related kinases), MAPKs (mitogen-activated protein kinases), and PI3-kinases as well as members of the Jak/STAT system. The PI3-kinase enzymes are a group of ubiquitously expressed proteins that have been demonstrated to be essential in a number of biological responses including cell survival, cell proliferation, glucose transport, actin polymerization and membrane ruffling (10).

The mouse gene of CT-1 is 5.4 kb and consists of three exons and two introns. Expression of CT-1 mRNA in cardiac non-myocytes (mostly fibroblasts) was 3.5 times higher than that observed in cardiac myocytes (74). In cardiomyocytes, both *in vivo* and *in vitro* CT-1 mRNA expression was markedly augmented by norepinephrine stimulation (47). The 1.1 kb of the 5' flanking promoter of the mouse CT-1 gene contains a norepinephrine response element (47). Fetal gene induction in the failing human heart is under partial β-adrenergic control (81) thus, β-adrenergic stimulation of CT-1 gene expression is a candidate for a molecular mechanism of adrenergically-driven pathological remodeling of the failing human heart. Angiotensin II has been shown to induce CT-1 expression in cardiac fibroblasts (108).

Elevated serum levels of CT-1 has been observed in human patients with unstable angina, MI and heart failure (130-132). A correlation was made between the level of CT-1 expression and the degree of LV systolic dysfunction. Rats became resistant to

repeated injections of CT-1 via induction of endogenous suppressors of the IL-6 family cytokines (53). Data from Sano *et al.* suggests a substantial amount of the cardiac hypertrophy induced by Angiotensin II and Endothelin-1 is mediated through increased expression of IL-6, LIF and CT-1 in cardiac fibroblasts (108). Thus, CT-1 may be capable of potentiating cross-talk between a number of mediators of fibrosis. CT-1 expression in the myocardium may be a classic example of a cardiac compensatory mechanism that may be both helpful and harmful. Signaling of CT-1 is anti-apoptotic and promotes hypertrophy which may be beneficial in the early settings of hemodynamic overload (108). In circumstances of overt hypertrophy and excessive stiffness associated with the maladaptive and potentially fatal conditions of advanced heart failure, overexpression of such proteins would be deleterious to the health of the heart and patient.

Zolk et al. measured the protein abundance of several key components of the CT-1 signal transduction system. In these experiments, gp130 protein expression was decreased while its mRNA abundance was increased suggesting that gp130. turnover is increased by ligand-dependent internalization and degradation (153). These findings suggest the desensitization of gp130 signaling 'might contribute to deterioration of contractile function' in chronic heart failure by promoting apoptosis and ventricular dilation as was previously noted to have occurred in LV pressure-overloaded cardiac myocyte-restricted gp130 knockout mice (151; 153). However, Zolk et al. did not assess the status of any downstream effectors of gp130, such as tyrosine phosphorylation of STAT-3, and so it is not clear whether the discordant changes in CT-1 and gp130 would produce a net increase or decrease in CT-1 signaling. At present it is unclear whether or

not the changes seen in CT-1 signaling pathways constitute an adaptive or maladaptive response by the hypertrophied, failing human heart.

VI. The Involvement of Angiotensin in the Heart

I. Angiotensin II

The renin-angiotensin-aldosterone system (RAS) plays a key pathophysiologic role in the development of and progression to heart failure (55). The system is one of the most powerful regulators of blood pressure and volume homeostasis in mammals (111). The pathway of angiotensin II production begins with angiotensinogen, produced and released by the liver. Angiotensinogen is cleaved by circulating renin resulting in the formation of the decapeptide angiotensin I which is then cleaved to the octapeptide angiotensin II by angiotensin converting enzyme (ACE) in the endothelial bed (55; 111). Both angiotensin I and II can be generated by pathways independent of renin and angiotensinogen, however, this pathway is responsible for production of the majority of angiotensin II in the circulation (55).

Angiotensin II stimulates the release of vasopressin, luteinizing hormone, oxytocin, and corticotrophin. Angiotensin II is known to regulate vascular tone through effects on vascular smooth muscle via growth stimulation, and aldosterone production and release which leads to increased salt absorption in the kidney and gut and the induction of thirst and sodium appetite in the brain. Vasoconstriction, irregular fluid and electrolyte homeostasis, and increased sympathetic outflow stimulated by angiotensin II all contribute to arterial hypertension and compromise the pumping ability of the failing

heart. Suppression of angiotensin signaling is associated with prevention of ventricular dilatation, improved exercise capacity, attenuation of scar remodeling, and survival in patients and experimental animal models of post-MI heart failure (54; 62-64; 97; 98).

There are two types of angiotensin II receptors, angiotensin type 1 (AT_1) and angiotensin type 2 (AT_2) (55). The clinically important maladaptive cardiovascular effects of angiotensin II are believed to be mediated by the AT_1 receptor which also accounts for its cell growth-promoting effects (55; 111; 136). Extracellular matrix (ECM) production and the secretion of adhesion molecules of fibroblasts is increased in response to angiotensin II (9; 66; 111). Fibroblasts proliferation and enhanced matrix expression also results in excessive, deleterious deposition of matrix material (55). Ang II has been show to increase cardiac fibroblast growth in humans, as well as upregulating proto-oncogene expression, MAPK activity, and mRNA expression of TGF-β_1, fibronectin and laminin (66). Adhesion to collagen and increased focal adhesion kinase (FAK) activity in cardiac fibroblast is also enhanced by angiotensin activation (66). The induction of vagus suppression and β-adrenergic potentiation also increases inotropy and chronotropy in the heart. Angiotensin II promotes rat cardiomyocyte hypertrophy both directly and indirectly by stimulating the release of norepinephrine from cardiac nerve endings and endothelin from endothelial cells (66).

AT_1 receptors mediate the growth-promoting effects that are involved in vascular and left ventricular hypertrophy, as well as mediating the structural remodeling of the heart post-MI (9; 55; 106). As well as producing adverse effects in endothelial functioning and post-ischemic repair, Ang II also appears to participate in inflammatory responses and in the production of free oxygen radicals (27; 106). Alternatively, AT_2

receptor mediated actions may antagonize the adverse effects of the AT_1 receptor though in the adult organism AT_2 receptors are frequently suppressed. Through production of nitric oxide (NO), AT_2 receptors may help restore endothelial function (50) and prevent vascular and cardiac cell growth.

II. AT_1 Receptor Antagonism

The first type of drugs developed to inhibit the renin-angiotensin system specifically targeted the angiotensin converting enzyme (ACE). Normally, ACE acts to reduce the conversion of angiotensin I to angiotensin II, and ACE inhibition reduces angiotensin II concentrations directly by blocking this conversion (55). Despite having beneficial effects in a number of cardiovascular disorders, the use of ACE inhibitors is not well tolerated in all patients (106). Thus, there is increasing interest in alternative and complementary methods of inhibition of the RAS system for cardiovascular patients (106).

In the mid-1980s the first angiotensin receptor type 1 (AT_1) antagonistic drugs were developed (67; 111). Highly specific non-peptidic AT_1-receptor antagonists were created as competitive antagonists and exhibited virtually no agonistic effects at the receptor level. Losartan was the first of these antagonists and the group name was subsequently coined *sartans* (111). Angiotensin receptor blockers (ARBs) provide cardiovascular protection and are both well-tolerated and effective antihypertensive agents (106). In a meta-analysis of hypertensive patients it was demonstrated that ARB treatment when compared with conventional antihypertensive therapy reduces the relative risk of stroke, non-fatal MI and cardiovascular death by 12 % (106).

Angiotensin receptor blockers have certain theoretical advantages over ACE inhibitors given that angiotensin II may be produced independently of ACE (55). ARB therapy has emerged as cardio and renoprotective in HF patients, reducing overall blood pressure in hypertensive patients. A drawback of losartan is that it is a short acting drug which does not produce as significant a change in BP than other longer acting ARBs such as candesartan and irbesartan (117).

It was previously believed that, although other Ang II receptors exist (e.g. AT_2 and AT_4), virtually all the cardiovascular and hemodynamic effects of Ang II were mediated by the AT_1 receptor subtype (111). However, recent developments have indicated the possible involvement of AT_2 receptors in the heart. The AT_2 receptor is normally expressed at high levels in fetal tissues, and decreases rapidly after birth (134). Following vascular and cardiac injury, and during wound healing and renal obstruction, the AT_2 receptor is re-expressed in adults suggesting a role for this receptor subtype in tissue remodeling, growth and development. It is possible, that under physiological conditions, AT_2 receptors may function to antagonize the AT_1-mediated effects by inhibiting cell growth and inducing apoptosis and vasodilation (134).

Losartan has been shown to be greater than 10,000 fold more selective for the AT_1 than the AT_2 receptor (110). I*n vivo*, P450 oxidation of losartan converts a portion of its structure to its metabolites, the most potent of which is called EXP3174 which is 10-40 times more potent than losartan. This metabolite binds noncompetitively and becomes an insurmountable antagonist for the AT_1 receptor (111). Losartan and EXP3174 have been shown to inhibit the thromboxane A2 (TXA2) receptor blocking its vasoconstrictive and platelet aggregative effects (107).

Previous morphological studies indicate the administration of losartan is associated with the inhibition of cardiac fibrosis in the post-MI heart (109; 120). The effect of losartan on cardiac hypertrophy and infarct size in experimental rats at 2 and 4 weeks of treatment has previously been reported by our lab (62). There is also a growing body of evident suggesting that losartan has AT_1 receptor-independent actions related to anti-inflammatory and antiaggregatory mechanisms. These properties are not shared with other ARBs or ACE inhibitors (107). An additional secondary metabolite of losartan (EXP3179) has also been shown to act as an inhibitor of cyclo-oxygenase 2 (COX2) in a similar fashion to that of conventional non-steroidal anti-inflammatories (107). These metabolites also negatively regulate the transcription of proinflammatory genes such as NF-κB.

In patients with hypertension, losartan effectively lowers blood pressure and results in the regression of left ventricular hypertrophy (LVH). In the LIFE (Losartan Intervention for Endpoint Reduction) study, losartan treatment showed a 25% reduction in cardiovascular morbidity and mortality (death, stroke and MI) compared to atenolol (67). In this same study, it was found that losartan was more effective than atenolol in inducing LVH regression and provided superior cardiovascular protection (25).

As multiple profibrotic actions in human cardiac fibroblasts are mediated by angiotensin type-1 (AT_1) receptors (AT_1R), studies have been conducted to determine if AT_1R blockade is useful in attenuating of the development of cardiac fibrosis in humans (66). Studies from Kawano et al. suggest in the human heart, AT_1 receptors are the major functional angiotensin receptor mediating the fibrotic effects of angiotensin II. This underscores the significant role of AT_1R blockade in heart failure and post-MI

remodeling in human patients (66). The AT_1 receptor blocker losartan was shown to improve mortality rates in elderly patients with heart failure compared with the ACE inhibitor captropril (66).

Inhibition of the RAS at different levels has been show to protect vital organs and reduced cardiovascular morbidity and mortality (68; 106). It has also been shown that the beneficial effects of RAS inhibition extend far beyond that of blood pressure control but also the processes of maladaptive vascularization and cardiovascular disease (138). It is for this reason we sought to investigate if the use of AT_1 receptor blockade via losartan would allow cardiotrophin-1 to act unopposed in the post-MI rat heart promoting some of the cytokines' cardioprotective properties.

VII. Adrenergic Signaling in the Heart

I. The Role of β-Adrenergic Receptors

Within the myocardium, the β-adrenergic signaling cascade is an important regulator of normal cardiac function (37). Adrenergic receptors (ARs) belong to the G protein-coupled receptors (GPRC) superfamily that binds the endogenous catecholamines epinephrine and norepinephrine. ARs participate in either the onset or maintenance of several disease states including hypertension, cardiac dysfunction (congestive heart failure, ischemia, arrhythmias), diabetes, glaucoma, depression and impotence (21; 92). The human heart contains two major classes of ARs containing nine receptor subtypes (three α_1, three α_2 and three β subtypes β_1, β_2 and β_3) (92; 118). The pharmacological, structural and molecular data indicate there is significant heterogeneity within this receptor family (92). In human tissues, the primary adrenergic receptors of

norepinephrine are the β_1, β_2 and α_1-receptors (143). The β_1-receptor is predominate in the healthy human heart at a ratio of 70-80 to 30-20 compared to β_2-receptors (14; 75; 143) and the low density α_1-receptors (17; 18). In HF, chronic over stimulation of the sympathetic nervous system results in a downregulation of β_1 receptors in cardiac tissues to a ratio of β_1: β_2: α_1 that is roughly 2:1:1(143).

It was first reported over 40 years ago that plasma norepinephrine concentrations were elevated in patients with heart failure (20). Norepinephrine has a 20 times greater affinity for β_1 than β_2 receptors (15). The binding of norepinephrine results in the activation of adenylate (adenylyl) cyclase which in turn raises intracellular cyclic AMP (cAMP) (92). The membrane bound β-receptor is linked to adenyl cyclase through the G-protein coupled receptor (GPRC) system. In its active stimulatory configuration (G_s) and or its inhibitory form (G_i), the GPCR system acting as either the accelerator or brakes of adrenergic signal transduction (37; 92). When activated, adenyl cyclase produces cAMP from ATP and acts as the intracellular second messenger of the β_1-receptor. β-adrenergic stimulation results in a greater and faster rise in intracellular calcium coupled to increased breakdown of ATP by myosin ATPase. The duration of the interaction of myosin heads and actin tails is reduced by the increased presence of cyclic AMP (cAMP) which increases the phosphorylation of troponin-I (that along with tropomyosin is one of the active regulatory components of binding on the myosin head). The faster rate of turnover enables the cells to relax more quickly and the subsequent increased contractility is linked to an increase in phosphorylation of phospholambin. Taken together, all of these effects increase the overall contractile activity of the heart (92). The increased force of contraction of the heart muscle, termed a positive inotropic effect, results from

the cAMP stimulated "opening" of calcium channels (12; 92). Increased cytosolic calcium re-uptake in the sarcoplasmic reticulum results in a relaxing or lusitropic effect (92). Adrenergic stimulation in the sinus node results in an increase in the pacemaker current (a positive chronotropic effect), and the rate of conduction is accelerated (positive dromotropic effect). Taken in combination, β-adrenergic activation has positive inotropic, chronotropic and dromotropic effects are also *oxygen conserving properties* of great importance in the failing heart(12; 92).

In heart failure, chronic stimulation of β-adrenergic receptors results in receptor internalization and densensitization of β-AR signal transduction (37). A hallmark of heart failure is the selective down-regulation of the β_1-receptor subtype, with a progressive decrease in receptor density with increased severity of HF (40). Such downregulation could be viewed as self-protective, considering the detrimental effects of excess levels of cAMP and calcium ions within the cell signaling system.

The classic model of β-AR signaling in heart failure suggests chronic stimulation of β-ARs leads to chronic G-protein activation, and desensitization or downregulation is a physiologically adaptive mechanism that attempts (but ultimately fails) to stop progressive HF(37).

II. β-AR BLOCKADE

The ability of adrenergic receptors to activate multiple pathways through G proteins and second messengers imparts diversity to adrenergic receptors responsiveness under various conditions. As of 2005, American guidelines recommend that all patients

with AMI are started on β-blockade therapy and continued indefinitely, unless absolutely contraindicated or not tolerated (39).

β-AR blockade is a widely recognized therapeutic tool for heart failure, but the manner in which inhibition of the β-receptor acts to reduce mortality is not yet fully understood. There are several hypotheses to explain the improvements in HF seen with β-blockade which are believed to result from antagonism of the cytotoxic effects of increased circulating catecholamines and the normalization of β-AR signaling. Downregulation of β_1-ARs in myocardial membranes and decreased coupling of remaining receptors to the stimulatory G-protein/adenylyl cyclase system has been shown in heart tissue (100).

Propranolol was the first β-blocker to be introduced and was produced to counteract the cardiac effects of adrenergic stimulation. Propranolol is a non-selective beta blocker, impeding the action of epinephrine on both β_1 and β_2 adrenoreceptors (92). The half-life of propranolol is only 3 hrs, but continued administration saturates the hepatic process that removes propranolol from the circulation; the active metabolite 4-hydroxypropranolol is formed, and the effective half-life becomes longer (92). The biologic half-life of propranolol exceeds the plasma half-life considerably, so that twice-daily dosage of standard propranolol is effective. The higher the dose of any β-blocker, the longer the biologic effects; propranolol is slow release and is highly bound (i.e. it has a good lock and key fit). Propranolol has inhibitory effects on the sinus node, atrioventricular node, and on myocardial contraction as well as chronotropic, dromotropic and inotropic effects (92). It has been the gold standard in treatment after acute myocardial infarction and is also widely used and licensed for multiple other indications

such as angina, hypertension, arrhythmia, migraine prophylaxis, anxiety and essential tremor (92).

Adverse effects of β-blockers include fatigue, insomnia and worsening contraindications such as reactive airway disease, sinus-node dysfunction and abnormalities in the cardiac conduction system (12; 34). The side effects of propranolol include i) smooth muscle spasm (bronchospasm and cold extremities), ii) exaggeration of the cardiac therapeutic actions (bradycardia, heart block, excess negative inotropic effect), and iii) central nervous penetration (insomnia, depression). The central system side effects are likely related to the fact that lipid-soluble β-blockers have high brain penetration. Other side effects include decreased exercise output (-15%) associated with fatigue and impotence. Alternative usage of propranolol in curing stage fright as well as the future incorporation of drug treatment in dealing with post-traumatic stress disorder exemplifies the diverse benefit of the drug in areas other than heart disease (49).

Reduced morbidity and mortality, as well as improvement in the myocardial molecular phenotype, reverse remodeling characterized by decreased ventricular dimensions and improved systolic function have been shown in clinical HF trials using β-blockade (14; 32; 81). Thus far, of the drugs developed to lower morbidity and mortality in HF, antagonism of β-AR has proven most effective (37).

β-AR blockade is now established as a highly effective therapy that reduces morbidity and mortality in patients with heart failure associated with reduced systolic function (34). Newly established guidelines recommend the use of β-AR antagonists in patients with symptomatic LV systolic dysfunction (34). The use of β-antagonist therapy is novel and counterintuitive to many physicians. The implementation of therapy is

viewed as difficult and time consuming because it requires frequent visits for dosage monitoring and regulation of symptoms, physical findings and adverse effects.

Fetal gene induction in the failing human heart is under partial β_1-adrenergic control (81). Therefore, β-adrenergic modulation of CT-1 gene expression is a candidate for the molecular mechanism of adrenergically-driven pathological remodeling in the failing human heart (16) representing another opportunity to explore additional pathways in the management of heart disease.

MATERIALS AND METHODS

I. MYOCARDIAL INFARCTION IN RATS

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following guidelines set forth by the Canadian Council on Animal Care. Myocardial infarction (MI) was produced in 150-200 gram anesthetized (0.01-0.05 mg/kg buprenorphine subcutaneous premedication, 2-2.5% isofluorane inhalation anesthetic) male Sprague-Dawley rats by ligation of the left coronary artery as previously described (30). The resulting scar from the arterial ligation was approximately 40% of the left ventricle, although there was a high mortality rate (upwards of 40% within the first two days post-MI). Sham operated animals received the same procedure as infarcted rats bar arterial ligature and were used as a control for comparative purposes. Post-MI, both sham and infarct groups were treated with either losartan (15 mg/kg/day) or propranolol (30 mg/kg/day) in drinking water vehicle for specific time periods. Animals were sacrificed (at specified stages between 24 hrs and 8 weeks post-MI) and tissues from both the viable region and infarct scar of the left ventricle were subsequently frozen (liquid nitrogen) and placed in the -80°C freezer. Isolated tissue samples were first pulverized in liquid nitrogen, then homogenized and lysed in 2x sodium dodecyl sulfate (SDS) buffer (0.125 M Tris, 2% SDS, 20% glycerol) at 4°C for 60 minutes and then sonicated 3x for 10 seconds. Centrifugation at 14,000g for 10 minutes was used to remove any insoluble materials and the clear supernatant was collected for Western analysis.

II. Isolation and Characterization of Cardiac Myofibroblasts

Ventricular fibroblasts were isolated from the hearts of 150 - 200 g adult male Sprague-Dawley rats as previously described (30). Hearts were then briefly subject to Langendorff perfusion at 37°C with SMEM containing 0.1% collagenase (Worthington Biochemical Corporation, Lakewood, NJ) for 20-25 minutes. Upon completion of the digestion, hearts were removed and placed in diluted collagenase and neutralized by addition of an equal volume of DMEM/F12 medium containing 10% FBS. Liberated cells were collected and centrifuged at 500 x g for 7 minutes. Cells were resuspended in fresh DMEM/F12 containing 10% FBS and plated in 75 cm^2 culture flasks at 37°C with 5% CO_2 for 3 hours. Tissue debris and non adherent cells (myocytes) were removed by changing the culture media and the remaining adherent cells (mainly fibroblasts) were incubated in fresh DMEM/F12 containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μM ascorbate. We (45) and others (84) have previously described the differentiation of myofibroblasts from adult primary fibroblasts when plated at low density as described above. The experiments were conducted on myofibroblasts after the first passage (P1 cells). The cells (myofibroblasts) were observed to express α-smooth-muscle actin and the embryonic form of smooth muscle myosin heavy chain (SMemb) as they do *in vivo* in infarcted rat heart (41). Cells were rendered quiescent by incubation in serum-free medium for 18-24 hours prior to experimentation and then incubated with either or in combinations of losartan (Merck Lot # LLN-056), isoproterenol (I6504 - Sigma-Aldrich, Oakville, ONT), or propranolol (P0884 Sigma-Aldrich, Oakville, ONT). At the specified time-points, cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton

X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris) with 1x protease inhibitor cocktail (Sigma-Aldrich, Oakville, ONT) and 10 mM NaF, 1mM Na_3VO_4 and 1 mM EGTA.

III. Western Analysis

Protein concentrations of tissue and cell lysates were determined using the BCA method as previously described (119). Proteins were separated using 8-12% SDS-PAGE gels and transferred to PVDF membrane (Roche, Indianapolis, IN) for 1 hour at 300 mA. Transferred membranes were blocked with 5% non-fat skim milk in Tris-buffered saline containing 0.2% Tween 20 (TBS-T) or in 5% BSA/TBS-T for phosphorylated antigens for either 1 hour or overnight (O/N). Proteins were visualized using ECL Plus (Amersham) after probing with primary and secondary antibodies. Band intensity was quantified using a CCD camera imaging densitometer (GS670, Bio-Rad Laboratories (Canada) Ltd. Mississauga, Ontario).

IV. Immunofluorescence

Cardiac myofibroblasts were seeded onto coverslips in 6-well dishes and allowed to adhere overnight in DMEM/F12 media containing 10% FBS serum. The cells were rendered quiescent in serum free media for 18-24 hours before 30 minute incubation with either of losartan, propranolol or isoproterenol (in various combinations). After aspiration of media, treated cells were rinsed with 1x PBS and fixed with 4% paraformaldehyde. Permeabilization of cells with 0.1% Triton X-100 was followed by incubation with primary antibodies, biotinylated secondary antibodies, and streptavidin FITC and nuclei were stained with Hoechst 33342. Visualization of cells was

accomplished using epifluorescent microscopy with appropriate filters for various antibody fluorescence (Nikon Canada).

V. Reagents

Recombinant CT-1 and mouse monoclonal CT-1 antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). Angiotensin II was purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario). Rabbit polyclonal antibody against gp130 was bought from Upstate Biotech (Lake Placid, NY). Polyclonal rabbit antibodies against STAT3, STAT3 pY^{705} were from Cell Signaling (New England Biolabs Ltd., Mississauga, Ontario), anti-gp130 antibody was purchased from Upstate (Lake Placid, NY). Secondary anti-mouse and anti-rabbit HRP were acquired from BioRad (Hercules, CA). α-smooth muscle actin antibody was purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, mouse monoclonal antibody against procollagen (SP1.D8) was from the Developmental Studies Hybridoma Bank (Dr. Heinz Furthmayr, Yale University CT). Losartan (i.e. the angiotensin II type I receptor blocker) was obtained from MERCK (Rahway, NJ). Propranolol (i.e. the β-adrenergic receptor blocker) was from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). Culture media (Dulbecco's Modified Eagle Medium, DMEM/F12, and S-Minimum Essential Medium, SMEM), fetal bovine serum (FBS), and antibiotics (penicillin, streptomycin) were purchased from GIBCO BRL (Grand Island, NY). Culture plates and multi-well culture dishes were obtained from Fisher Scientific (Whitby, CA). All other laboratory grade reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario).

VI. STATISTICS

Data are expressed as means ± standard error. Means between control and test conditions were compared using one-way analysis of variance (ANOVA) followed by either Bonferonni's post-hoc analysis, or the Student—Newman—Keuls test for comparing the differences among multiple groups (SigmaStat 3.2). Significant differences were defined via a probability of * $P < 0.05$.

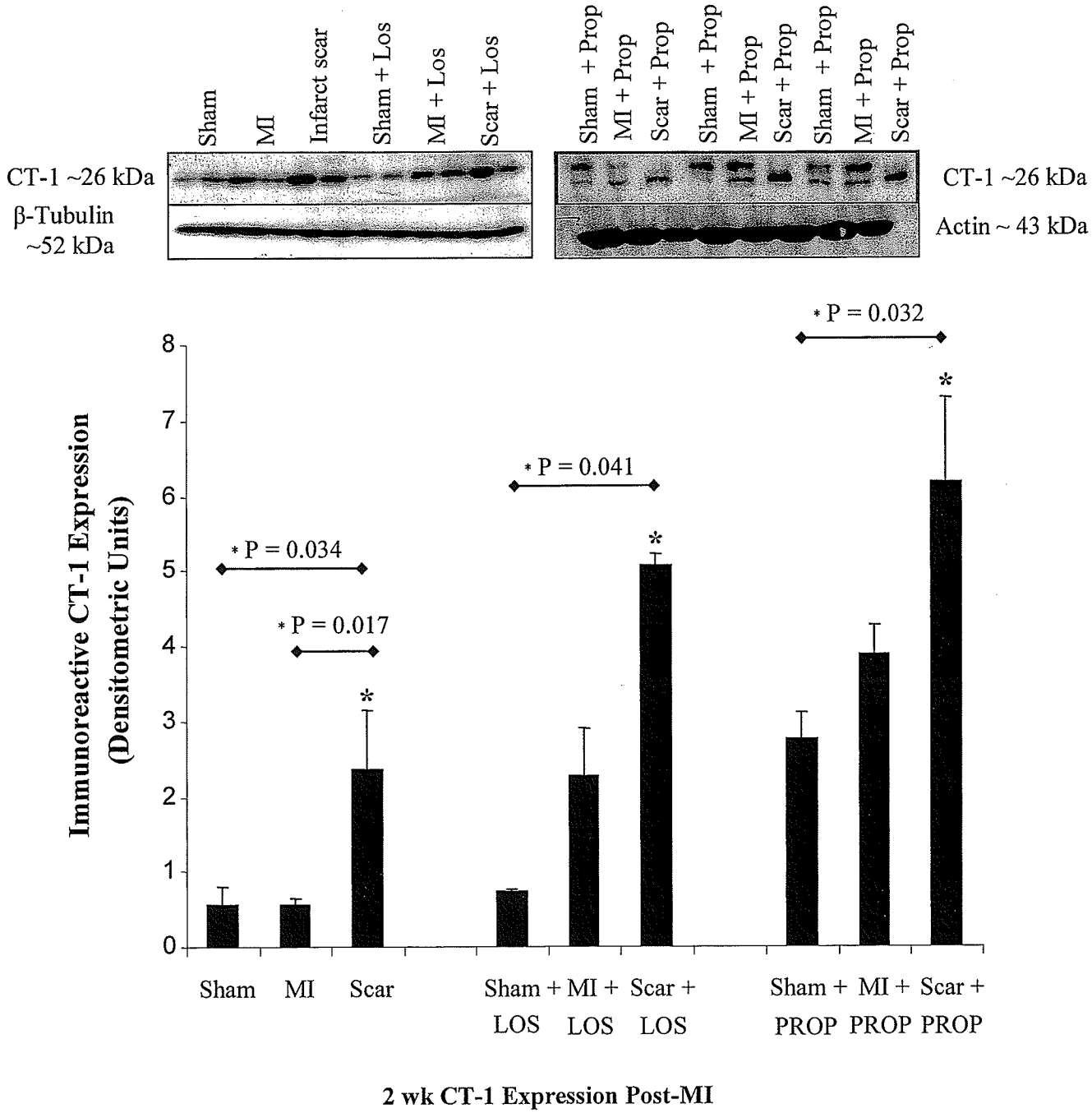


Figure 1. CT-1 protein expression in 2 week control, losartan and propranolol treated tissue samples following MI. A) Representative Western blot of CT-1 expression in control, losartan and propranolol treated tissue samples. Actin and β-Tubulin were used as controls for equal protein loading. B) Protein expression was determined in 50 μg cardiac tissue lysates by Western analysis with mouse monoclonal CT-1 antibody. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM).

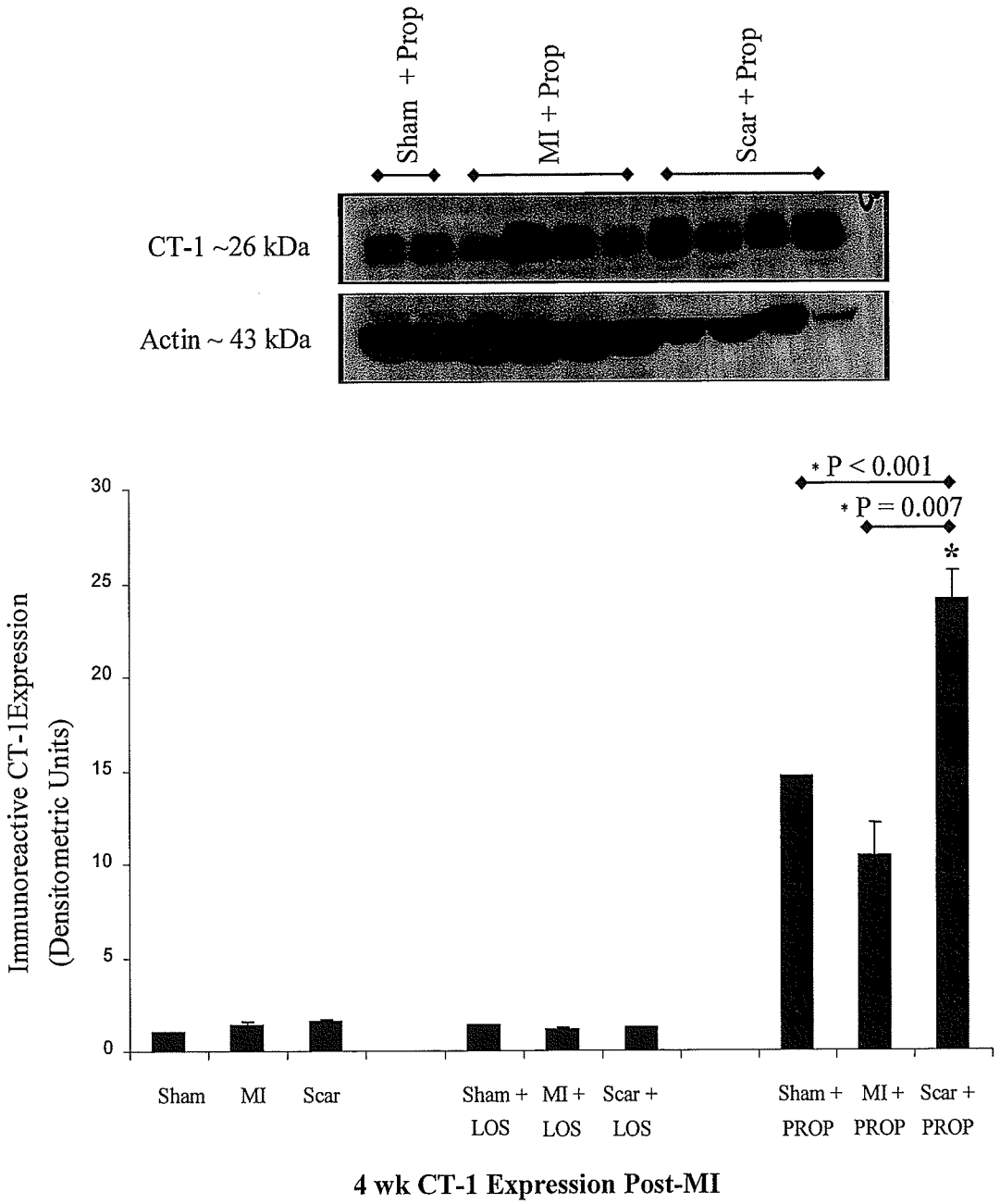


Figure 2. CT-1 protein expression in 4 week control, losartan and propranolol treated tissue samples following MI. A) Protein expression was determined in 50 µg cardiac tissue lysate by Western blot analysis with mouse monoclonal CT-1 antibody. Actin was used as a control for equal protein loading. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM). Note the increased expression of CT-1 in scar tissue samples compared to the relative decrease in protein expression seen in the actin blot (statistical ratios were corrected for actin).

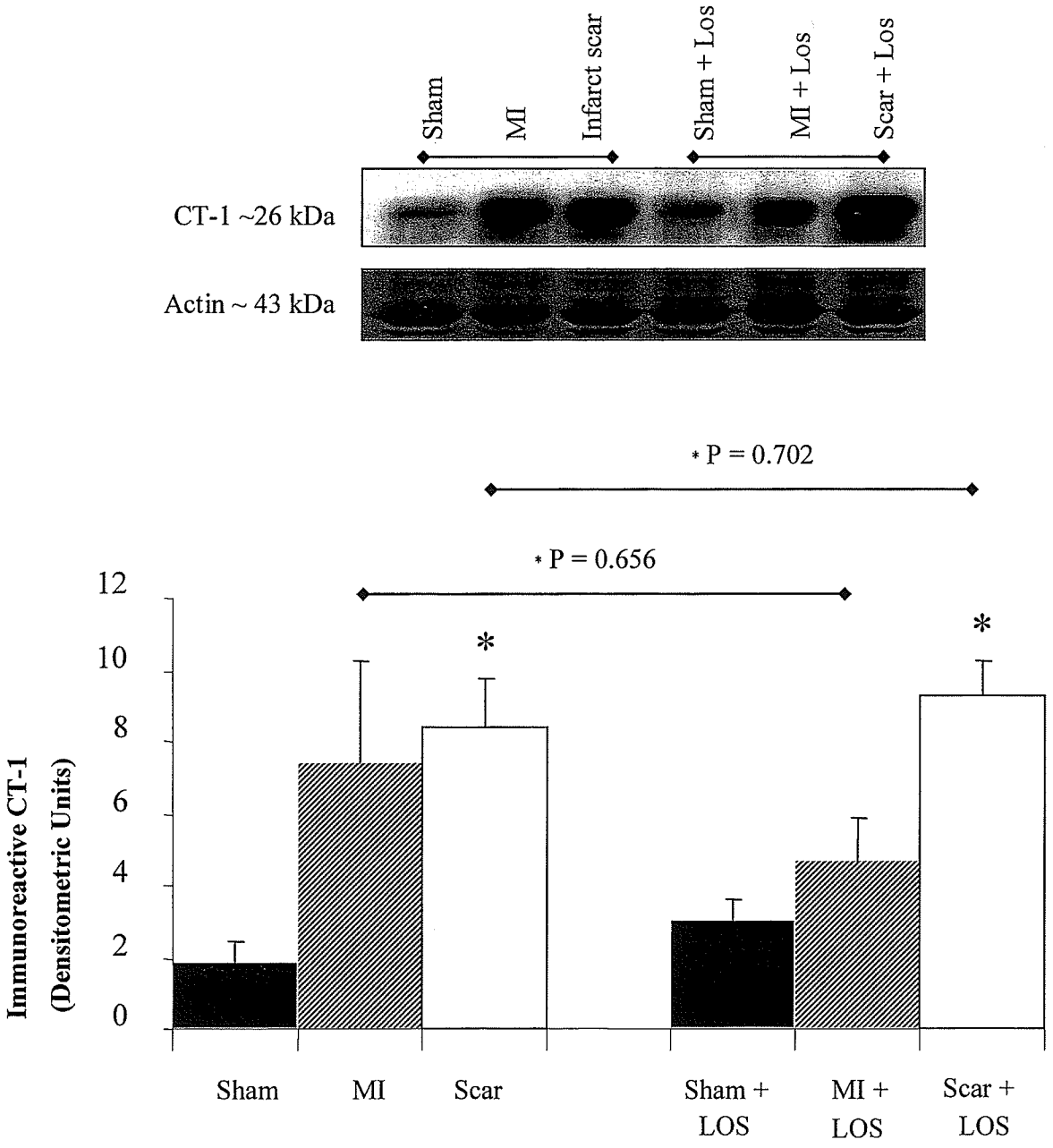


Figure 3. CT-1 protein expression in 8 week control and losartan treated tissue samples following MI. A) Protein expression was determined in 50 µg cardiac tissue lysate by Western blot analysis with mouse monoclonal CT-1 antibody. Actin was used as a control for equal protein loading. B) The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM).

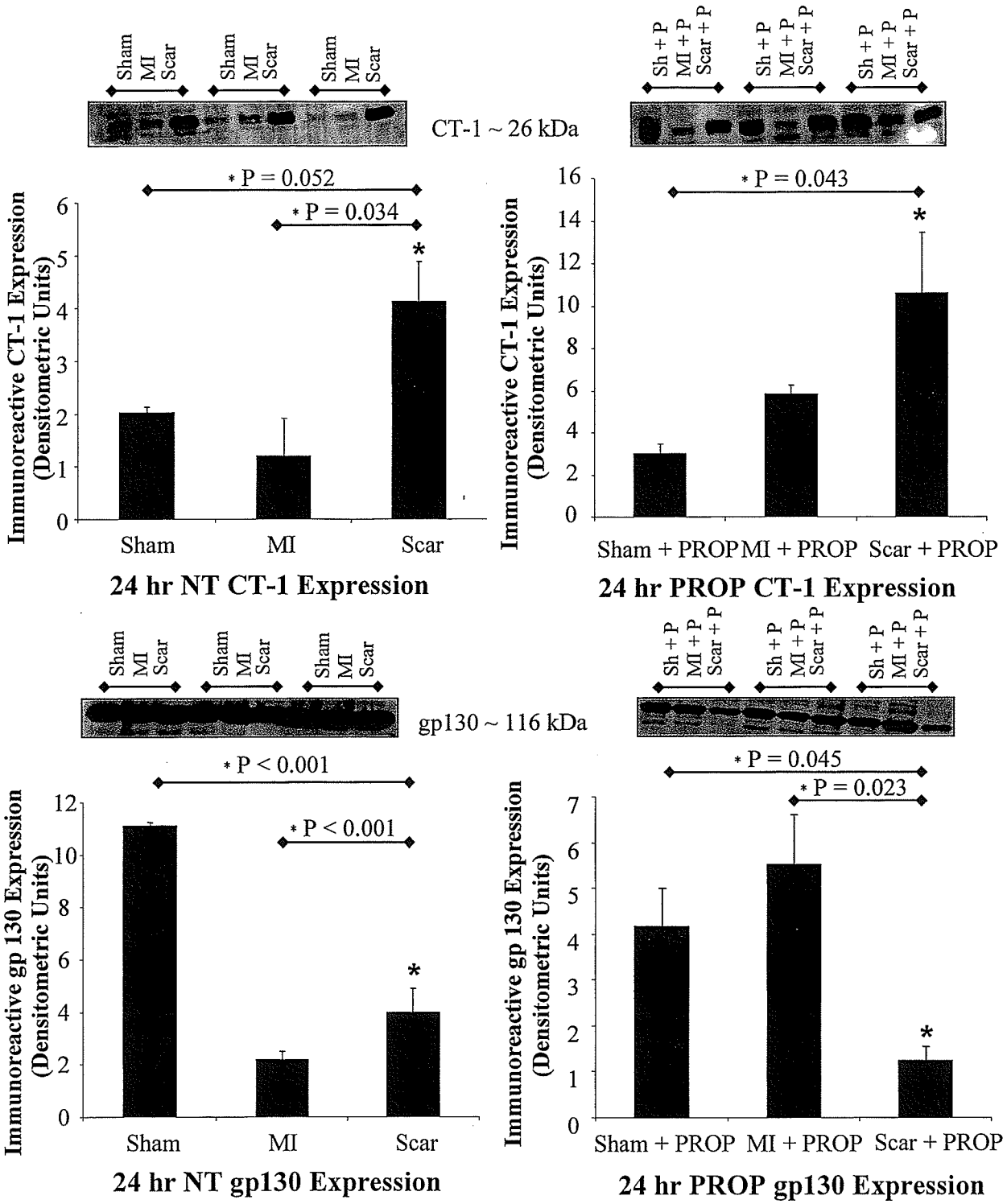


Figure 4. CT-1 and gp130 protein expression in control and propranolol treated 24 hr tissues after MI. Representative Western blots were used to determine protein expression in 10 μg cardiac tissue lysate with mouse monoclonal CT-1 and rabbit polyclonal gp130 antibody. Actin and Coomassie Blue were used as controls for equal protein loading. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM). Sh, Sham; P, Propranolol.

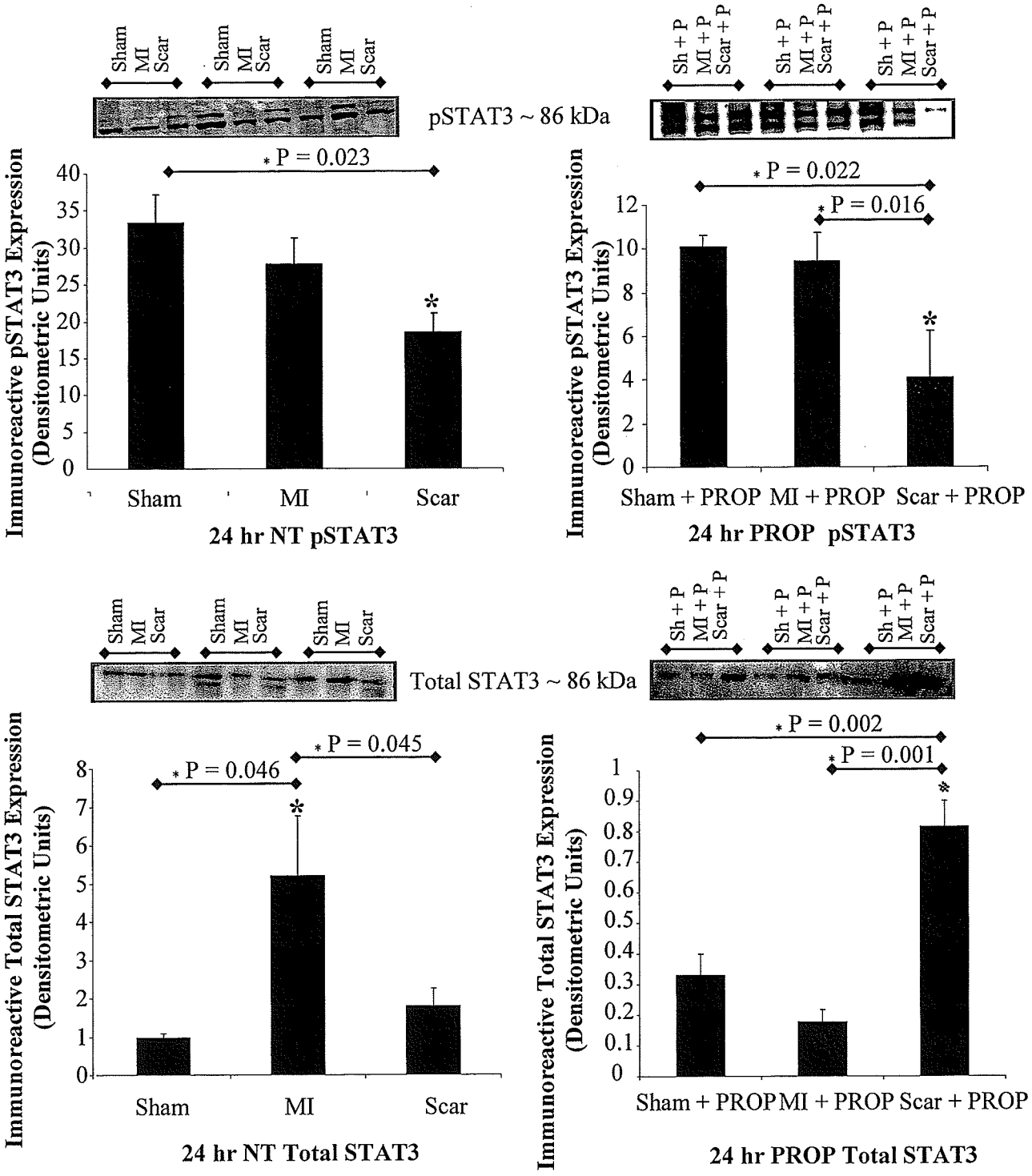


Figure 5. pSTAT3 and total STAT3 protein expression in control and propranolol treated 48 hr tissues after MI. Representative Western blots were used to determine protein expression in 10 µg cardiac tissue lysate with rabbit polyclonal pSTAT3 and mouse monoclonal total STAT3 antibody. Actin and Coomassie Blue were used controls for equal protein loading. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM). Sh, Sham; P, Propranolol.

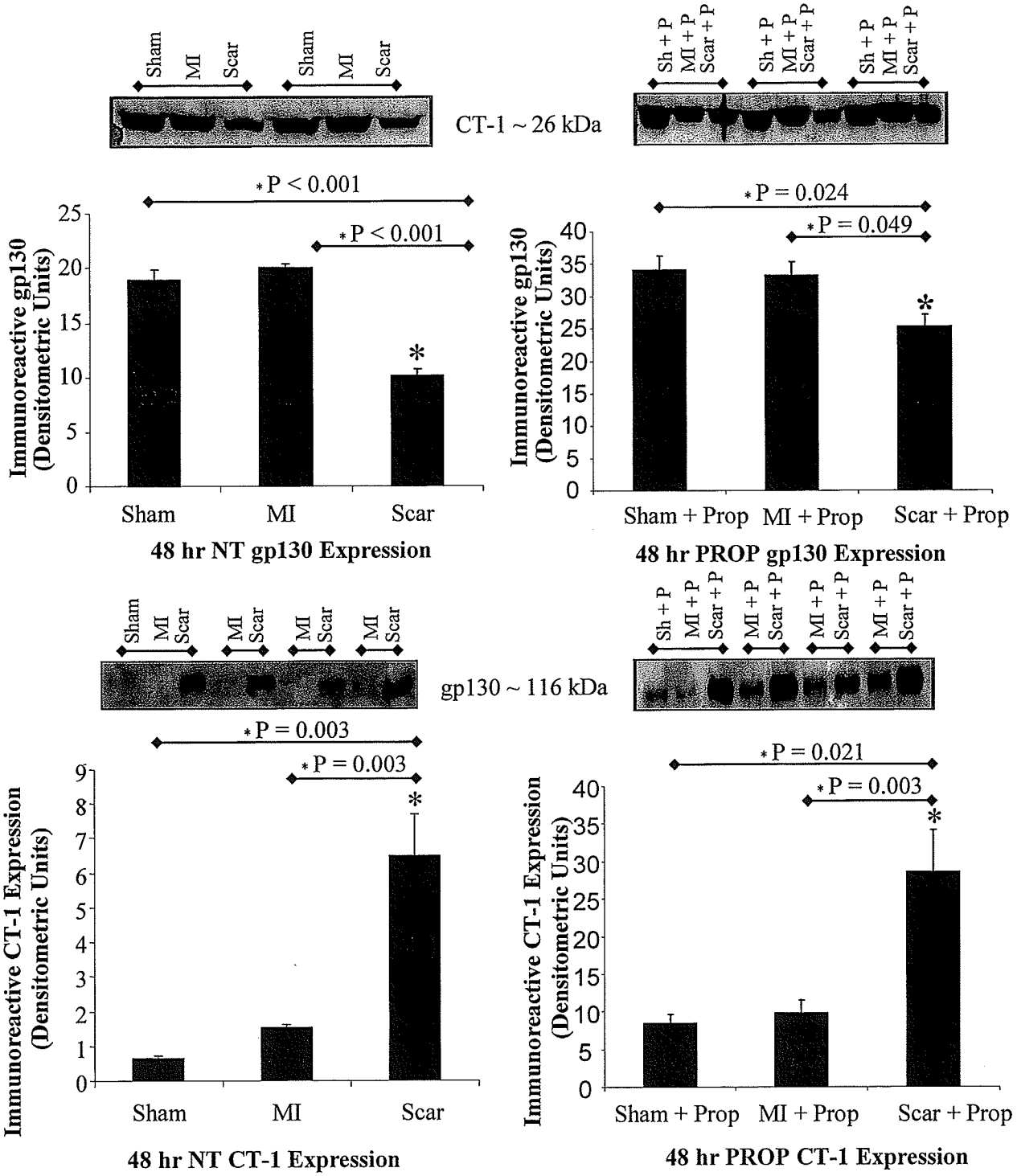


Figure 6. CT-1 and gp130 protein expression in control and propranolol treated 48 hr tissues after MI. Representative Western blots were used to determine protein expression in 10 μg cardiac tissue lysate with mouse monoclonal CT-1 and rabbit polyclonal gp130 antibody. Actin was used as a control for equal protein loading. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM). Sh, Sham; P, Propranolol.

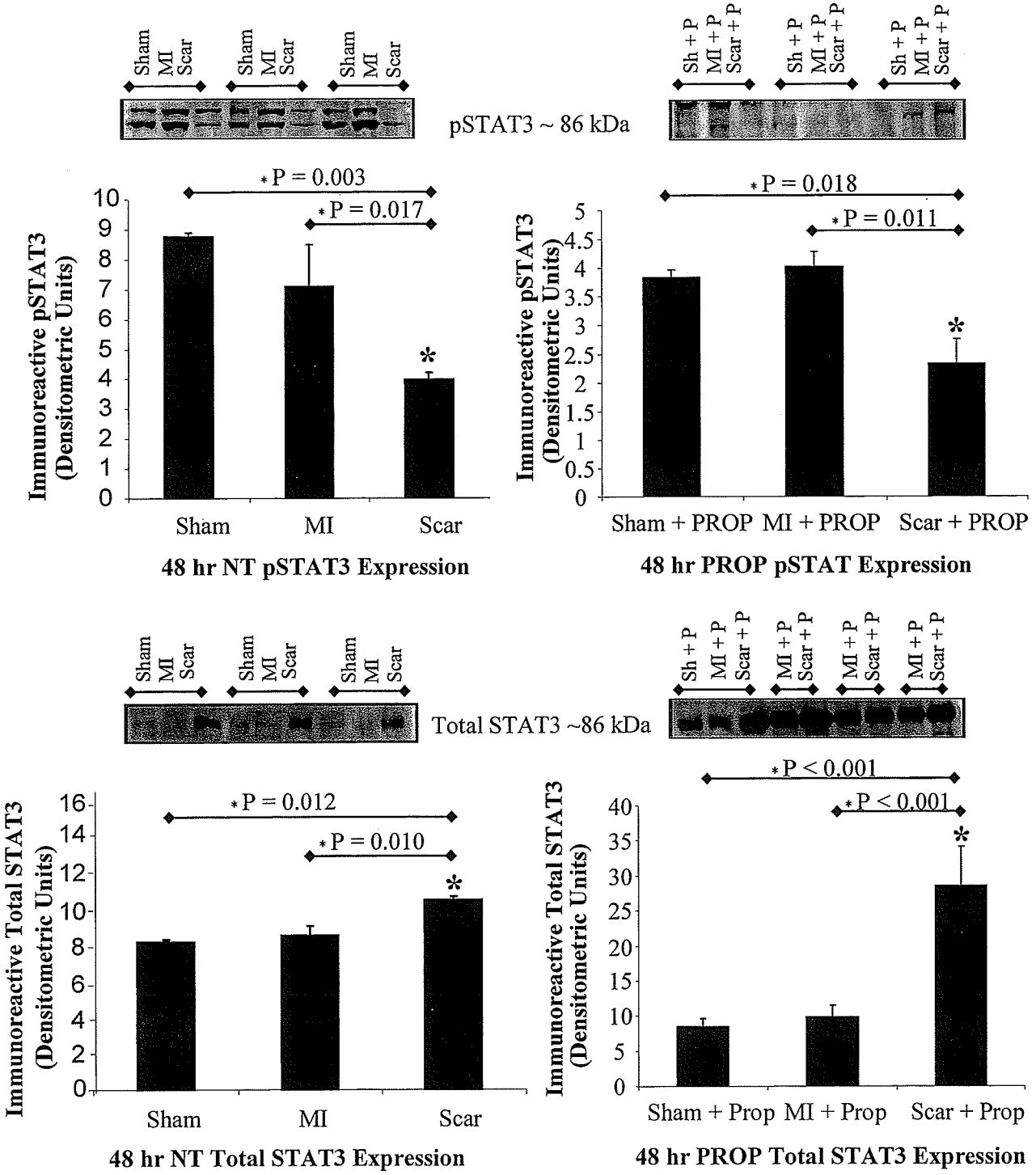


Figure 7. pSTAT3 and total STAT3 protein expression in 48 hr control and propranolol treated tissues following MI. Protein expression was determined in 10 μg cardiac tissue lysate by Western blot analysis with polyclonal rabbit pSTAT3 and monoclonal mouse total STAT3 antibody. Pictured are representative Western blots of protein expression, actin was used as a control for equal protein loading. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM). Sh, Sham; P, Propranolol.

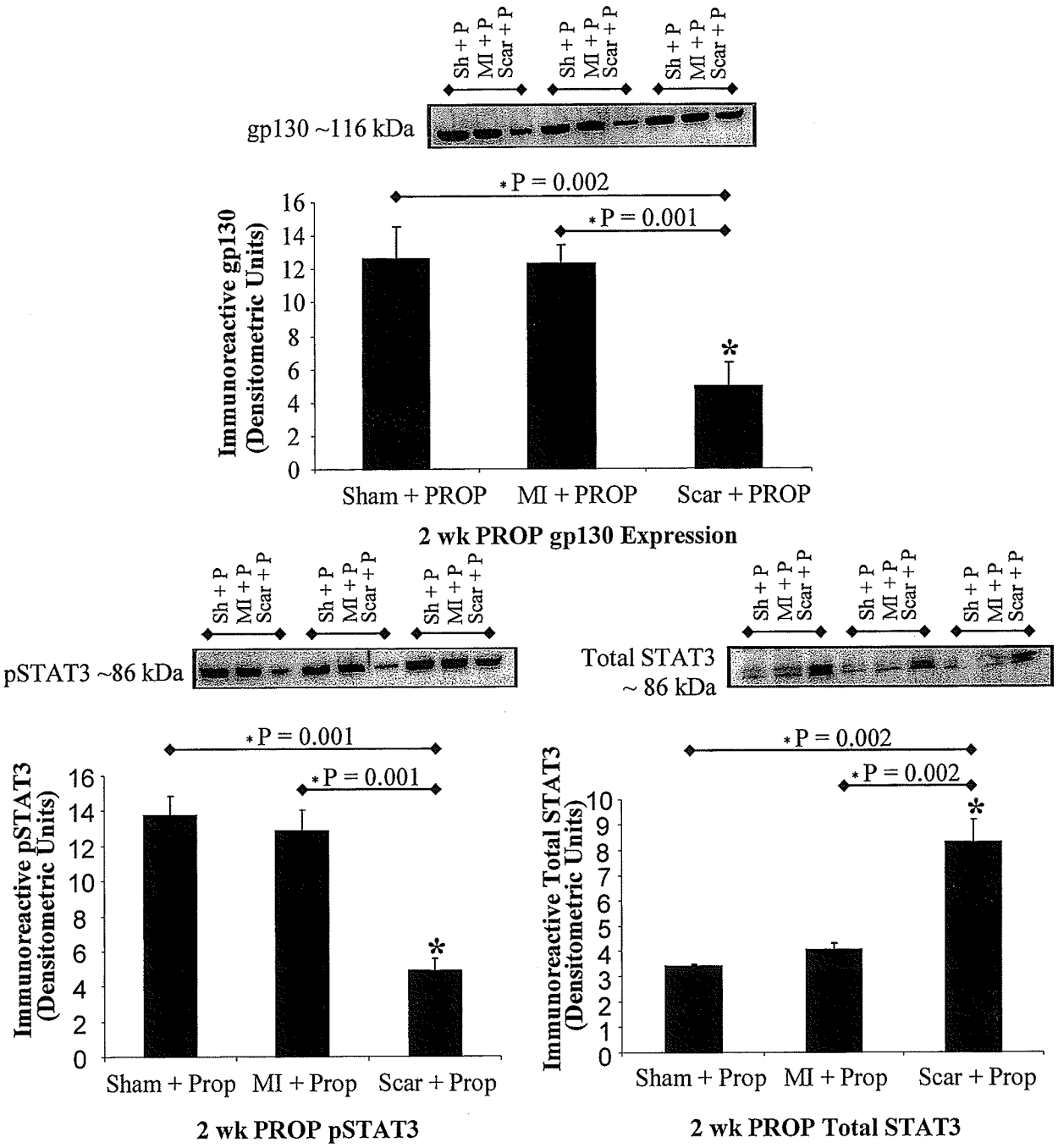


Figure 8: gp130, pSTAT3 and Total STAT3 protein expression in propranolol treated tissue samples 2 weeks after MI. Protein expression was determined in 10 µg cardiac tissue lysate by Western blot analysis with gp130, pSTAT3 or Total STAT3 antibody. Actin was used as a control for equal protein loading. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM). Sh, Sham; P, Propranolol.

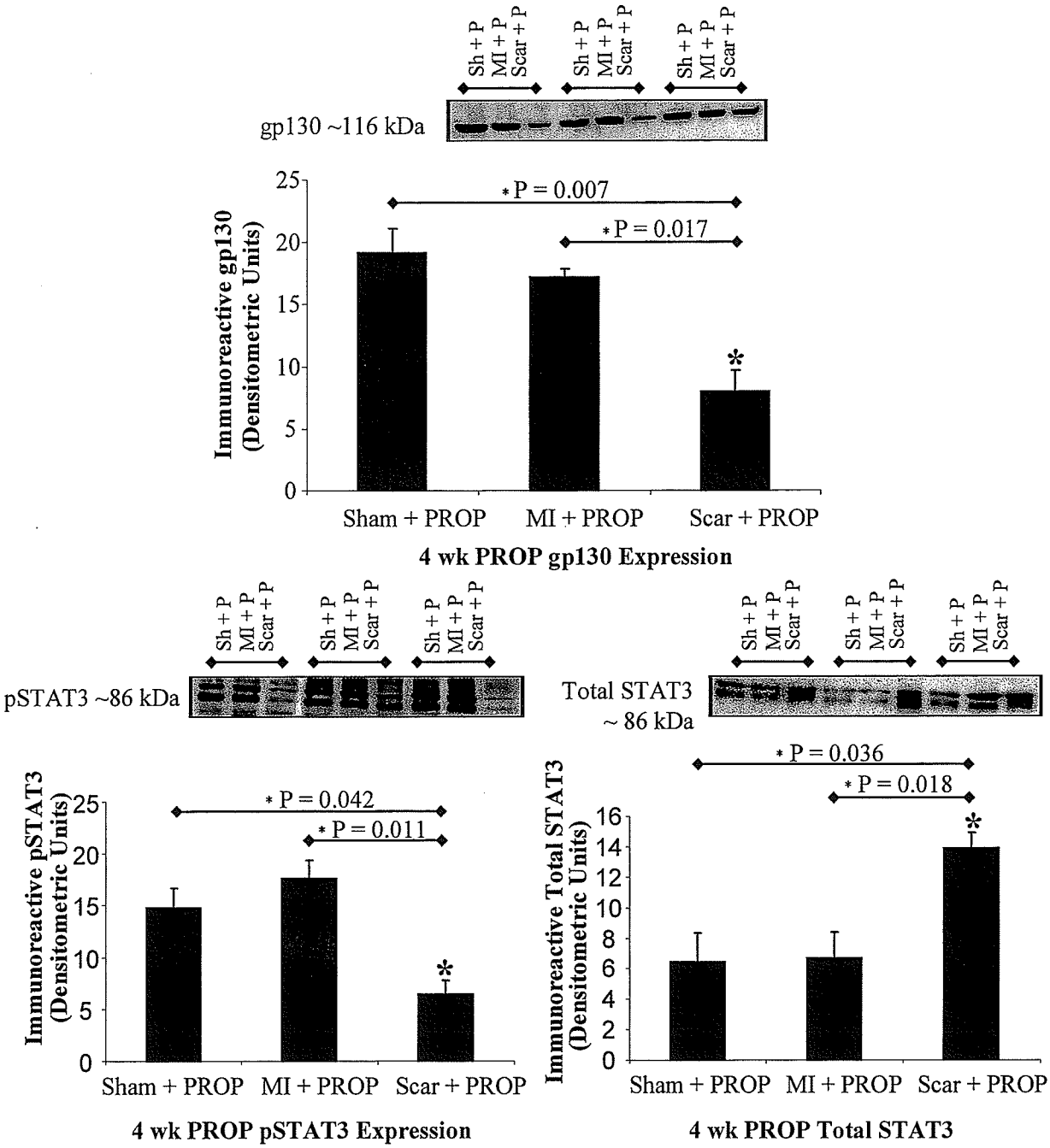


Figure 9. gp130, pSTAT3 and Total STAT3 protein expression in propranolol treated tissue samples 4 weeks after MI. Protein expression was determined in 10 μg cardiac tissue lysate by Western blot analysis with gp130, pSTAT3 or Total STAT3 antibody. Actin was used as a control for equal protein loading. The relative intensity of protein to control is expressed in arbitary densitometric units (mean ± SEM). Sh, Sham; P, Propranolol.

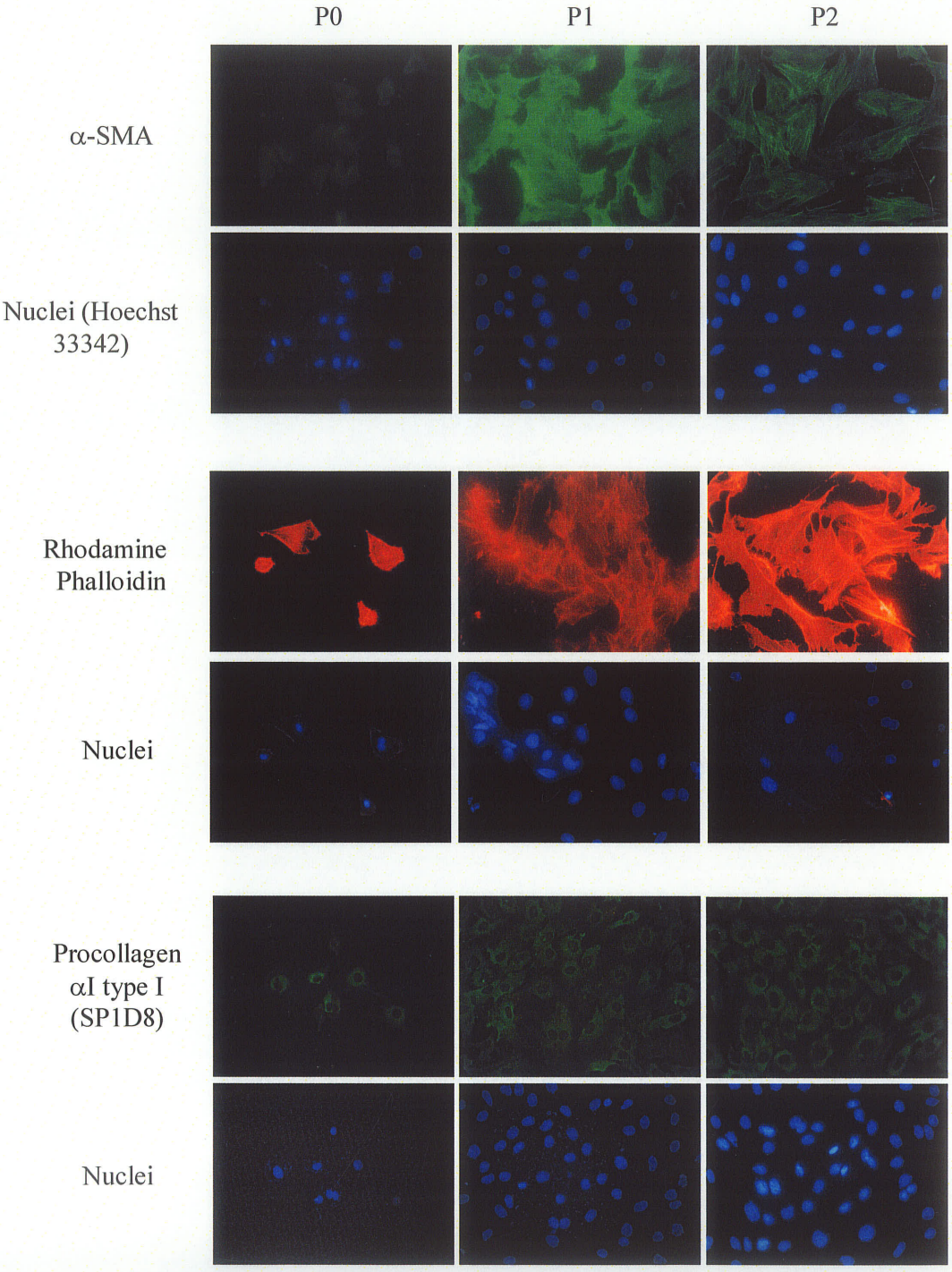
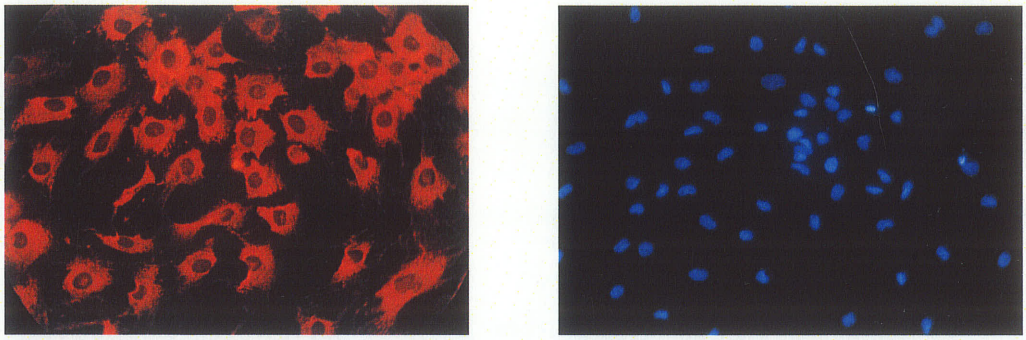
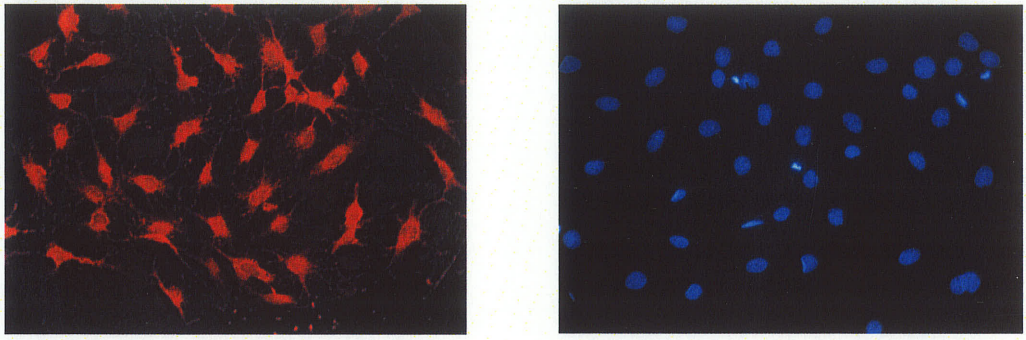


Figure 10. Phenotypic comparison of protein expression in adult rat cardiac fibroblasts at various stages of passage. Primary P0 cells were initally plated at low density and cultured for 3 days following isolation from the rat heart. Cells were passaged to P1 and allowed to grow for 5 days, then passaged again to P2. Cells were stained at each passage for immunoreactive expression of α–smooth muscle actin (α -SMA), rhodamine phalloidin (F-actin stain) and procollagen (SP1.D8). Cells were stained for nuclei using Hoescht 33342. Images are seen at 400 x magnification.

A. Control



B. 10 μM ISO



C. 20 μM ISO

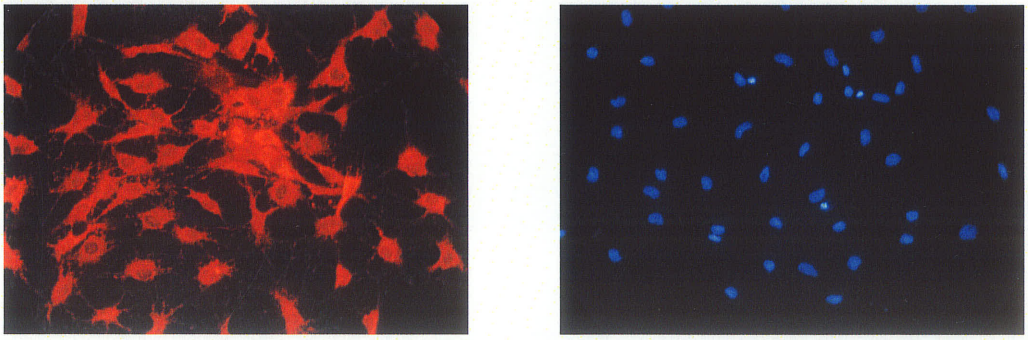
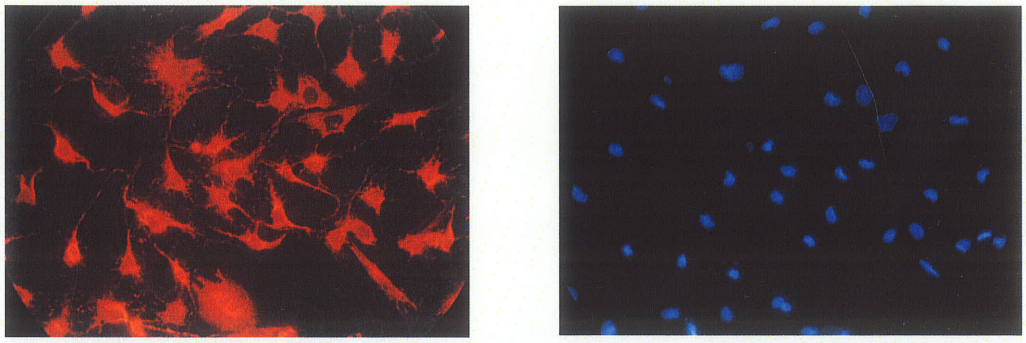
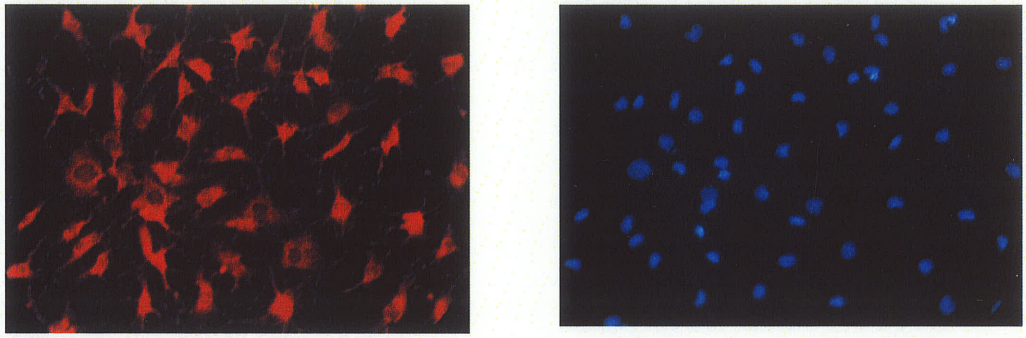


Figure 11. α-SMA expression in control, 10 and 20 μM isoproterenol treated P1 adult rat cardiac myofibroblasts. Expression of α–smooth muscle actin in isolated control (A) and isoproterenol treated ((B) 10 μM and (C) 20 μM) first passage (P1) adult rat cardiac fibroblasts (400 x). Hoescht stained nuclei are represented in blue.

A. 50 μM ISO



B. 100 μM ISO



C. 150 μM ISO

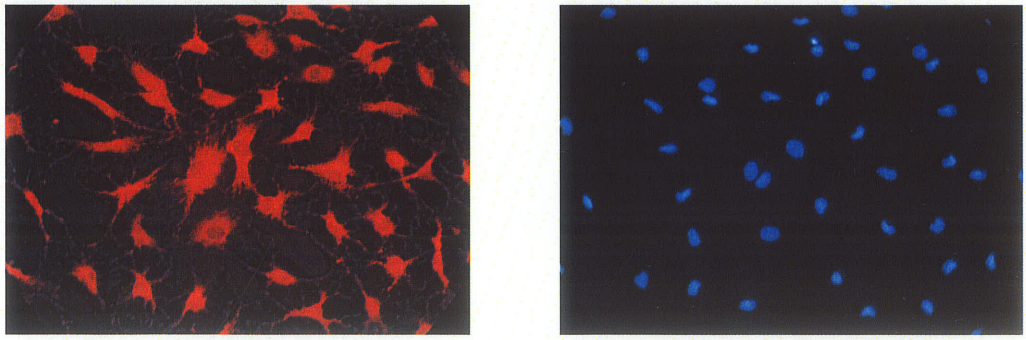


Figure 12. α-SMA expression in 50, 100 and 150 μM isoproterenol treated P1 adult rat cardiac myofibroblasts. Expression of α–smooth muscle actin in isoproterenol treated ((A) 50 μM, (B) 100 μM and (C) 150 μM) first passage (P1) adult rat cardiac fibroblasts (100 x). Hoescht stained nuclei are represented in blue.

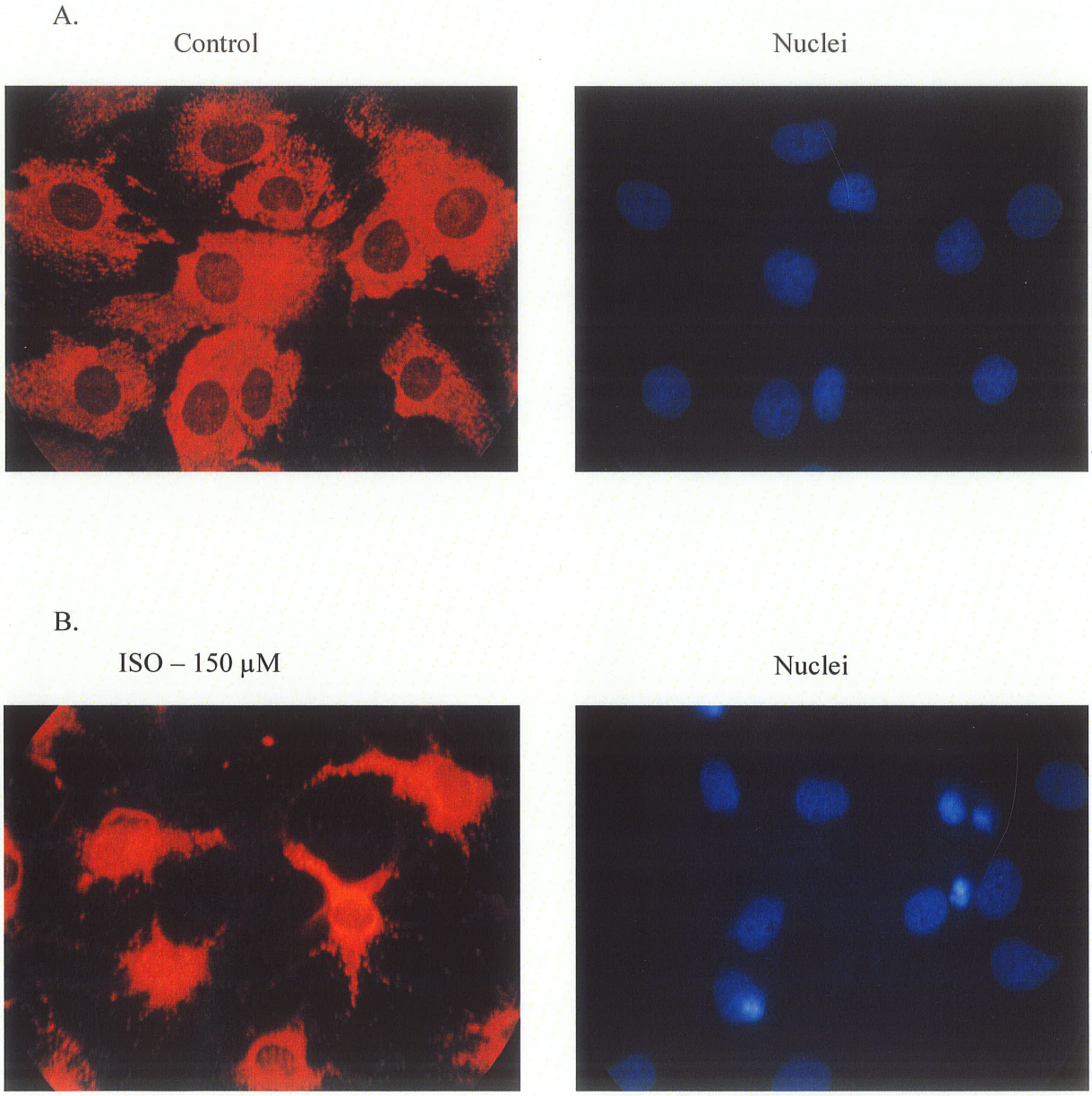


Figure 13. Magnified representation of α-SMA stained control and 150 µM isoproterenol treated P1 adult rat cardiac myofibroblasts. Expression of α–smooth muscle actin in isolated control (A) and isoproterenol treated (150 µM) first passage (P1) adult rat cardiac fibroblasts (400 x). Hoescht stained nuclei are represented in blue.

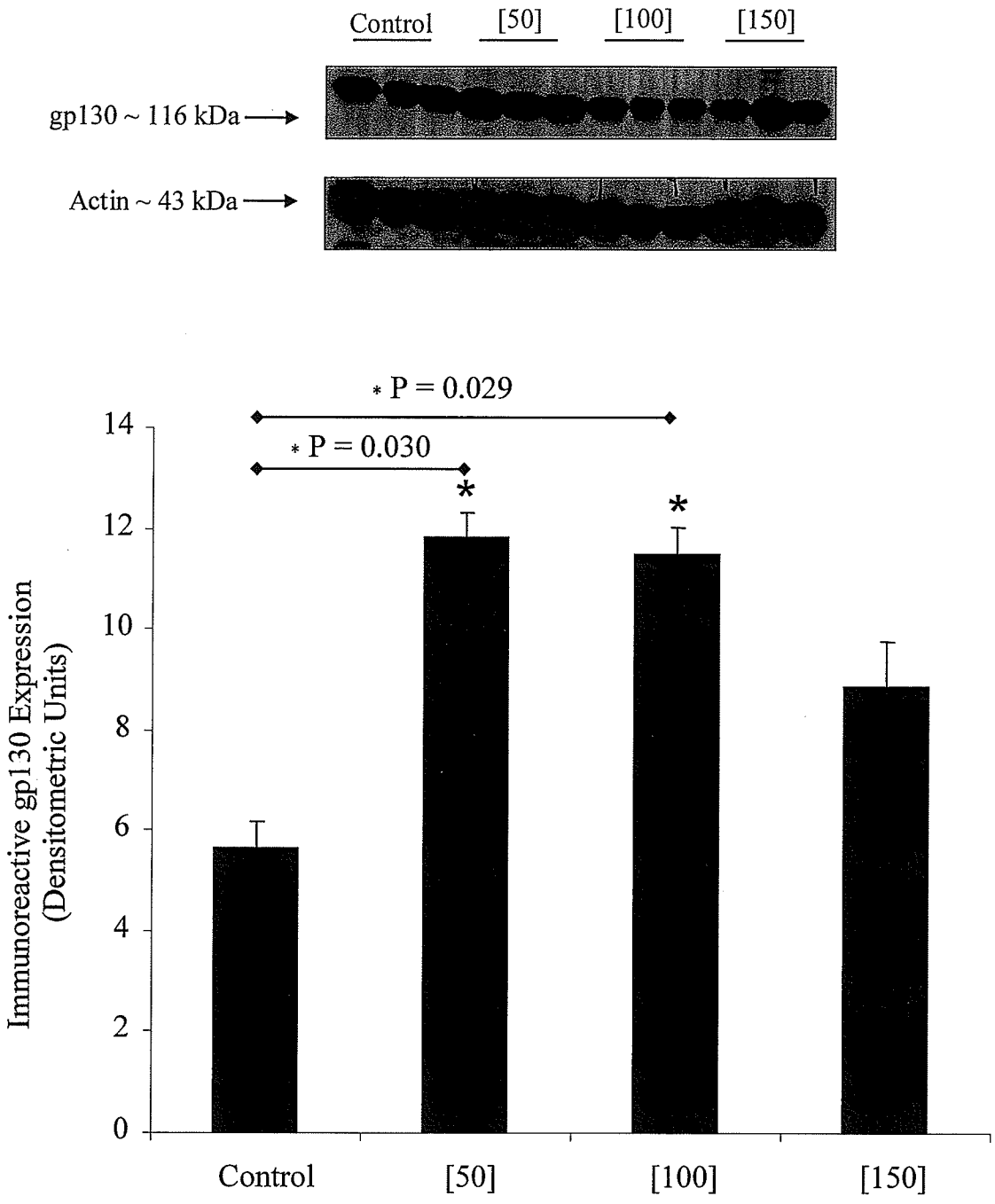


Figure 14: Expression of gp130 protein in control, 50, 100 and 150 μM isoproterenol treated P1 adult rat cardiac myofibroblasts. Western blot analysis of gp130 expression in isolated cell lysates of control and isoproterenol treated (50-150 μM) first passage (P1) adult rat cardiac myofibroblasts.

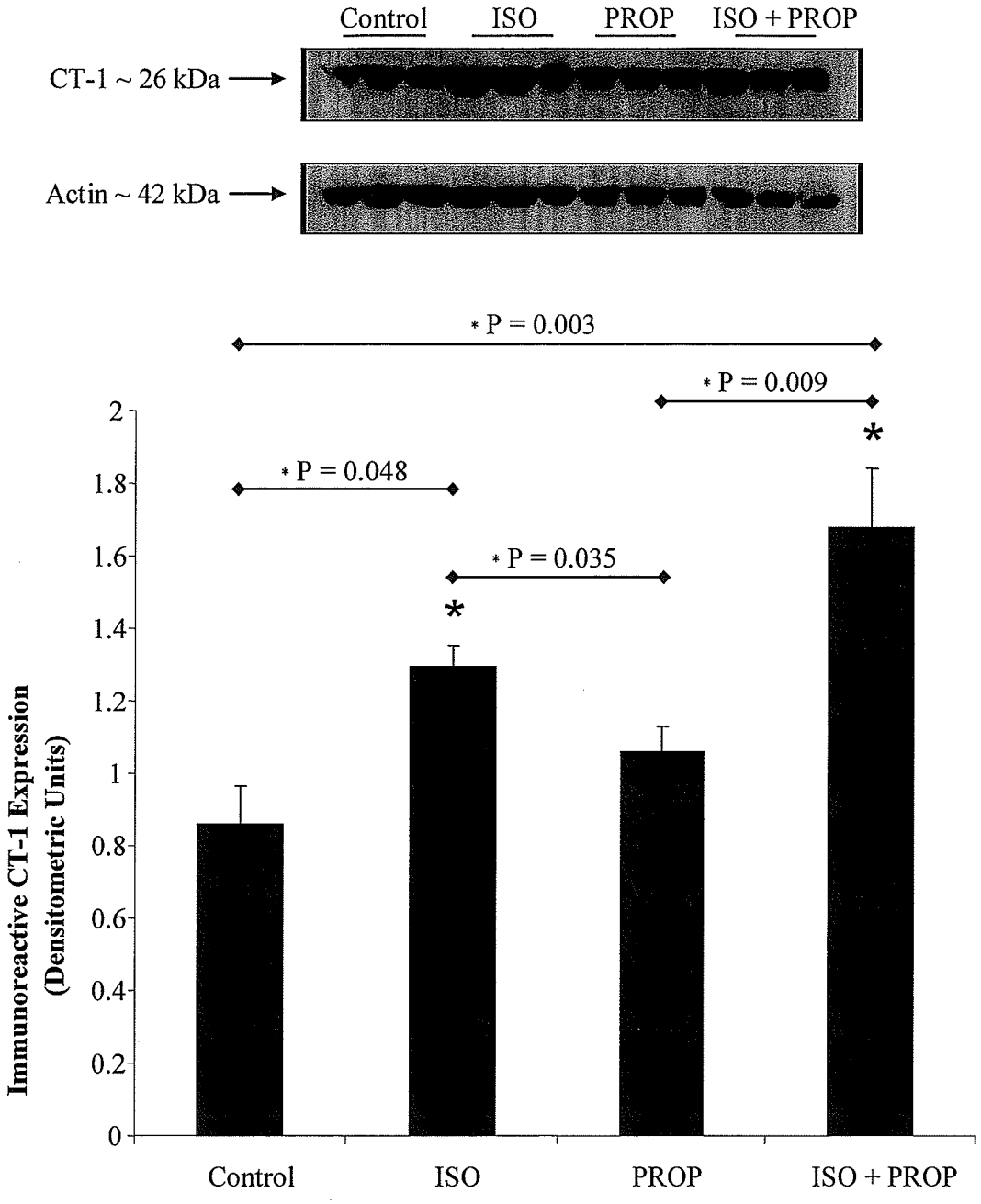


Figure 15. Protein expression of CT-1 in control, isoproterenol, propranolol, and isoproterenol and propranolol treated P1 adult rat cardiac myofibroblasts. Western blot analysis of cardiotrophin-1 expression in isolated cell lysates of control, isoproterenol (100 μM), propranolol (100 μM) and isoproterenol plus propranolol (100 and 100 μM) treated first passage (P1) adult rat cardiac myofibroblasts.

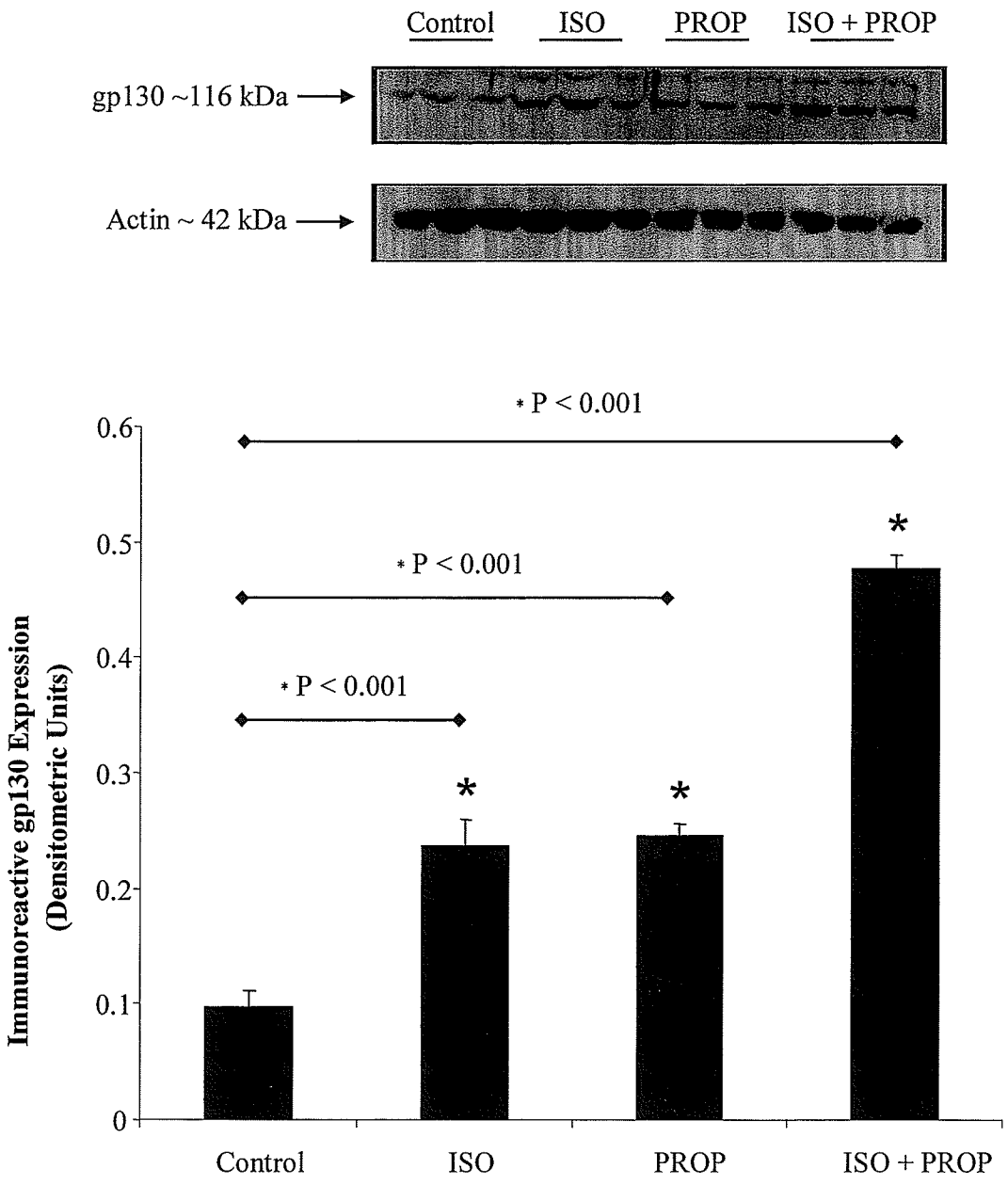


Figure 16. Protein expression of gp130 in control, isoproterenol, propranolol, and isoproterenol and propranolol treated P1 adult rat cardiac myofibroblasts. Western blot analysis of gp130 expression in isolated cell lysates of control, isoproterenol (100 μM), propranolol (100 μM) and isoproterenol plus propranolol (100 and 100 μM) treated first passage (P1) adult rat cardiac myofibroblasts.

IV) RESULTS

Cardiotrophin-1 is a novel chemokine expressed by cardiac myofibroblasts and shown to be both neuro and cardioprotective (48; 76; 101; 102; 145). Previous work from our lab has shown that CT-1 is upregulated in the post-MI adult rat heart (45). In Figure 1, CT-1 expression is significantly upregulated in the infarct scar over sham and remnant viable LV (labeled MI) in all of the 2 week control, losartan and propranolol treated samples. The level of CT-1 protein in the propranolol treated group is significantly upregulated compared to untreated control. There is no significant difference in CT-1 expression in 4 week control or losartan treated samples, however there is significant upregulation of CT-1 protein in the 4 week treated propranolol treated scar compared to both sham and the control treated tissue group (Figure 2). After 8 weeks, CT-1 is upregulated in the infarct scar compared to both sham and MI in the control and losartan treated tissue samples, but there is no significant difference between these two sample groups (Figure 3). Data from an 8 week propranolol treated group was not available in the study period.

Cardiotrophin-1 signals through the gp130 receptor complex. Downstream signaling of G-protein coupled receptors is modulated by the activation of STAT3 to its phosphorylated state (pSTAT3). The amount of CT-1, gp130, phosphorylated and total STAT3 was determined in 24 and 48 hr, 2 and 4 week propranolol treated tissue samples. At 24 hrs after MI, CT-1 protein is upregulated in the infarct scar in both control and propranolol treated tissues, however, expression is significantly upregulated in the propranolol treated groups compared to untreated controls (Figure 4). gp130 expression at 24 hrs post-MI is slightly but not significantly downregulated in the infarct scar

compared to both sham and MI (Figure 4). The protein expression of pSTAT3 and Total STAT3 is reduced in the untreated group, and the pSTAT3 is reduced and Total STAT3 upregulated in the propranolol treated 24 hr sample groups post-MI (Figure 5).

In 48 hr samples, the expression of CT-1 was significantly upregulated in the infarct scar of both control and propranolol treated samples, with CT-1 expression significantly upregulated in the propranolol group over untreated control (Figure 6). gp130 protein is significantly decreased in the infarct scar of both untreated and propranolol treated tissues (Figure 6). The expression of the phosphorylated active form STAT3 (pSTAT3) was significantly reduced in the infarct scar of both untreated and treated 48 hr tissue samples compared to sham and MI. Overall the expression of pSTAT3 in propranolol treated samples was significantly less than the expression in untreated tissues (Figure 7). In contrast, total STAT3 expression was upregulated in the infarct scar of both untreated and propranolol treated samples compared to sham and MI, but protein expression in propranolol treated tissues was significantly higher than that of untreated samples (Figure 7).

The expression of gp130, pSTAT3 and Total STAT3 was examined in 2 and 4 week treated tissues. β-blockade downregulates the expression of gp130 and pSTAT3, and upregulates the expression of Total STAT3 in the infarct scar compared to sham and MI (Figure 8 and 9).

Following myocardial infarction, fibroblasts transform into hypersecretory, contractile myofibroblasts capable of synthesizing matrix and helping maintain the integrity of the infarct scar (45; 126). The mechanisms behind the conversion of fibroblasts to myofibroblasts have been linked to mechanical tension as well as via the

production of local factors (29). We characterized the expression of various cell markers in P0, P1, P2 and P3 adult rat cardiac cells. Figure 10 shows a phenotypic comparison of the qualitative difference in cell expression of α-SMA, F-actin and procollagen (SP1.D8) using immunohistochemistry.

Isoproterenol stimulates β-adrenergic receptor activation and results in cellular toxicity. To approximate an acceptable dose of isoproterenol *in vitro* on P1 myofibroblasts, cells were treated with various concentrations (0-150 μM) and probed for α-SMA expression (Figures 11 - 13). Cell shape varied significantly with isoproterenol treatment, most notably upwards of 100 μM. To determine the presence of CT-1 receptors in P1 adult rat cardiac myofibroblasts following isoproterenol treatment of 50, 100 and 150 μM, cells were probed for gp130 protein expression (Figure 14). The effect of β–adrenergic stimulation on CT-1 and gp130 expression *in vitro* was assessed in P1 cardiac myofibroblasts treatment in response to administration of isoproterenol (100 μM), propranolol (100 μM) or isoproterenol (100 μM) in combination with propranolol (100 μM) (Figures 15 and 16). Isoproterenol induced activation of components of the β-adrenergic pathway resulted in a significant increase in cardiotrophin-1 expression. Administration of a combination of isoproterenol and propranolol resulted in an even greater increase in the expression of CT-1 protein *in vitro* (Figure 15). β-adrenergic stimulation results in increased expression of gp130 protein both isoproterenol and propranolol treatment *in vitro*, and is 4-fold upregulated following the administration of a combination of isoproterenol and propranolol.

V) DISCUSSION

Our ongoing work is based on the general hypothesis that CT-1 participates in repopulation of the infarct scar by myofibroblasts and fibroblasts, in the early phase of post-MI wound healing. As part of that work and to further explore the putative relationship between CT-1 expression in the post-MI heart (45), we carried out the current study to determine whether chronic angiotensin type I receptor blockade or β-adrenergic blockade results in the increased expression of CT-1 *in vivo*. At 2, 4 and 8 weeks after MI, we found no significant increase in CT-1 protein expression in losartan-treated samples vs. untreated controls. This finding disproves our original hypothesis that angiotensin modulates CT-1 expression. Alternatively, we hypothesized that CT-1 protein expression may be under the regulation of β-adrenergic signaling largely based on the previous evidence of cAMP responsive elements in the 5' region of the CT-1 gene (47). In propranolol treated tissue samples, CT-1 protein expression was significantly upregulated in the infarct scar vs. control in 24 and 48 hr as well as 2 and 4 week experimental groups (post-MI heart) showing a similar trend to previously obtained data in the absence of treatment. However, CT-1 baseline expression was elevated in the 48 hr, 2 and 4 week propranolol treated samples vs. non-treated controls across all groups. This provides some evidence for the alternative hypothesis that β-adrenergic function may influence CT-1 expression. The *in vivo* studies indicate that chronic β-blockade may sensitize the β-adrenergic signal and lead to reciprocal activation by a complex and undefined mechanism (60).

The sympathetic nervous system and the renin-angiotensin system are two

neurohormonal compensatory mechanisms stimulated in heart failure. The initial beneficial hemodynamic effects of these systems are associated with progressively adverse effects on cardiac remodeling and in particular on cardiac fibrosis resulting from an imbalance in the degradation of type I and III collagens (8). We observed improved cardiac function in post-MI rats with angiotensin (data not shown). Long-term AT_1 blockade is associated with improved LV function and decreased cardiac fibrosis in the post-MI heart (54; 55; 62; 97; 98). CT-1 affects many of the processes important in mature scar formation: migration, proliferation and collagen synthesis (45). The relative novelty of cardiotrophin-1 means its specific role in post-MI wound healing is unclear. As a potent inducer of hypertrophy in neonatal cardiac myocytes (94), CT-1 is also a known cardioprotective agent (7; 77; 114). While it is present in low concentrations in plasma samples of patients with both ischemic and valvular heart disease, CT-1 expression has been correlated with the degree of LV systolic dysfunction (131; 132).

Previous observations by Zolk *et al.* showed that elevated expression of CT-1 mRNA and protein levels in the hearts of patients with end stage cardiomyopathy who underwent heart transplantation (153). In the human heart, the increased expression of CT-1 protein was accompanied by decreased expression of gp130 protein (153). Similarly, in the present study, gp130 protein expression is downregulated in untreated and propranolol treated scar vs. sham and viable LV (MI). This suggests the possibility that gp130 receptor downregulation plays a role in balancing enhanced CT-1 signaling within the heart.

Signaling by all members of the IL-6 family of cytokines activates intracellular tyrosine kinases of the Janus family which then phosphorylate transcription factors of the

STAT (signal transducers and activators of transcription) family (70). To date, there are relatively few publications discussing the roles of STAT3 in the post-MI heart. The activation of STAT3, in response to various pathophysiologic stimuli, has been shown to promote cardiomyocyte survival and hypertrophy as well as cardiac angiogensis suggesting that STAT3 is beneficial for the heart (57). Activation of the Jak/STAT pathway is a key step in the regulation of the downstream effects of CT-1 (56). In the post-MI heart, the level of pSTAT3 expression was downregulated while total STAT3 was upregulated at 48 hrs, 2 and 4 weeks vs. controls. This finding is of considerable interest as it is supported by previous work from Podewski *et al.*, who found that the phosphorylation levels of STAT3 were severely reduced in failing hearts (99), supporting the possibility that decreased activation of STAT3 may contribute to the development of heart failure. The slightly increased production of total STAT3 in our post-MI hearts may be the result of compensation for the decreased activity of STAT3.

First passage (P1) myofibroblasts were stimulated with isoproterenol to determine whether increased cAMP (as a result of β-adrenergic receptor activation) causes increased CT-1 and gp130 expression. Isoproterenol stimulation of murine heart myocytes has been previously shown to be associated with increased CT-1 mRNA (150) however the effect of isoproterenol stimulation on target proteins in cardiac myofibroblasts had yet to be examined. We found that isoproterenol stimulation resulted in rapidly elevated CT-1 protein expression (within 24 hrs) in adult rat cardiac myofibroblasts, and further, that gp130 protein expression is also increased when compared to controls. While β-adrenergic receptor activation results in elevated levels of protein, β-blockade alone had no effect on CT-1 expression within the infarct scar.

Therefore, CT-1 downregulation within the infarct scar is most likely under the control of an as yet unidentified alternative pathway that may be under the secondary influence of β-adrenergic activation. β-agonist and β-antagonist co-stimulation resulted in synergistic activation and increased protein expression in culture. This likely resulted because of the intrinsic sympathomimetic activity of some β-antagonist agents (34) whereby at the molecular level, these drugs can both activate and antagonize the β-adrenergic receptor.

The results from the cell culture and animal model studies differ significantly and there are several possibilities for this apparent discrepancy. In a myofibroblast culture under the current conditions, there are virtually no other cell types present, as determined by i) the isolation procedure itself and ii) culture conditions and passage procedures. For example, the specific media used promotes fibroblast survival when compared to cardiac myocytes (DMEM/F12). As with all serum-starved cultures, there are no other cytokines or factors present other than those administered or those that are generally secreted by the myofibroblasts themselves (regulated by control plate for baseline expression). This is very different from the complex interactions of multiple cytokines that may be released *in vivo* between multiple cell types. Further, *in vitro* experiments may be regulated in terms of the application and duration of cytokine administration. Cells are serum-starved in preparation for application, rendering them quiescent prior to the application of external cytokines. In addition, *in vitro* cell stimulation is short in duration, occurring over a period of 24 hrs, as opposed to long term *in vivo* studies with administration ranging from 24 hrs up to 4 weeks following infarction. The difference in duration of drug application may also play a role in the regulation and stabilization of cytokine production in the *in vivo* vs. *in vitro* model.

LIMITATIONS WITHIN OUR STUDIES

Normally in patients, drug treatment with β-blockade following myocardial infarction is administered in incremental doses. In this study, 30 mg/kg/day propranolol was administered through the drinking water, on the basis of previous evaluations of daily rat water consumption. On the basis of previous evaluations of daily water consumption, and via observations of a consistent and similar pattern of consumption during the treatment, we assume a consistent dose of propranolol was delivered to the post-MI propranolol treated rats. In future studies it may prove useful to practice the method of oral gavage to assure a more precise administration and dosage in the rat model.

The ability to formulate definite conclusions from results derived from the *in vivo* model is complicated by several factors. *In vivo* experiments are beneficial when attempting to determine the effects of a given treatment in a whole animal study. Unfortunately, the complexity of the interactions within this model can not be limited to the specific therapy alone. Within the 3-dimensional composition of the myocardial matrix, cells interact with one another as well as with other cell types, and are subject to the influence of external effects that do not exist in isolated 2-dimensional *in vitro* experimentation (24). It is, therefore, an easier and more scientifically sound practice to deduce direct cause and effect relationships from cell culture experiments.

FUTURE DIRECTIONS

In the current study, the expression of only one component of the heterdimeric receptor complex of gp130/LIF, through which CT-1 signals, has been examined. Previous work from Knight *et al.* determined the effect of inflammatory stimuli on the expression of LIF in lung fibroblasts. *In vitro* activation using the proinflammatory cytokine IL-1 β resulted in increased mRNA and the greatest level of increase in protein expression of LIF in fibroblasts compared to other lung cell types (71). The pattern of release of LIF suggested *de novo* synthesis in response to inflammatory stimuli activation (71). As it is currently unreported in the literature, it would prove beneficial to analyze the protein expression of LIF and its receptor *in vitro* in the cardiac myofibroblast as well as *in vivo* in the post-MI treated and untreated heart.

Specific blockade of the β_1-AR alone may allow investigation into the effects of the β_2-AR in the post MI rat heart. Recent evidence indicates that though β_1-ARs are predominately expressed in the heart (14; 75; 143), the β_2-receptor may play a larger role in the post-MI heart than was previously believed. The use of the drug carvedilol which has been shown to inactivate more β_2 than β_1 receptors (86) and has been associated with particular advantages in the treatment of comorbid conditions such as CAD, stroke, hypertension, renal failure, diabetes and atrial fibrillation, that can independently contribute to the progression of heart failure (125). Alternatively, examination of the possible role of the α-adrenergic receptor system on CT-1 regulation and expression in the post-MI heart could also help elucidate the pathways controlling this specific cytokine. Further isolation and analysis of specific components, beneficially or deleteriously involved in cardiac repair, is of tremendous benefit because it allows for a

greater understanding of the complex systems involved in the regulation of and ultimately providing novel pharmacologic treatments for post-MI wound healing.

Finally, it is important to note the key differences in results derived from *in vitro* and *in vivo* experimentation. In terms of scientific methodology, *in vitro* experimentation clearly allows for simplification of the complexities of multiple pathways activated in post-MI wound healing. However, in combination with *in vivo* work, it is also apparent that direct inference from cell culture to the whole animal model is not always possible or accurate. For this reason, in future experiments if at all possible, it may be best to continue first with *in vitro* experimentation to single out components of these complex pathways involved in cardiotrophin-1 expression and regulation, followed by later *in vivo* experimentation once specific receptor interactions have been discerned.

Reference List

1. Anversa P, Olivetti G and Capasso JM. Cellular basis of ventricular remodeling after myocardial infarction. Am J Cardiol 68: 7D-16D, 1991.

2. Aronow WS. Epidemiology, pathophysiology, prognosis, and treatment of systolic and diastolic heart failure. Cardiol Rev 14: 108-124, 2006.

3. Aumailley M and Gayraud B. Structure and biological activity of the extracellular matrix. J Mol Med 76: 253-265, 1998.

4. Blankesteijn WM, Creemers E, Lutgens E, Cleutjens JP, Daemen MJ and Smits JF. Dynamics of cardiac wound healing following myocardial infarction: observations in genetically altered mice. Acta Physiol Scand 173: 75-82, 2001.

5. Boersma E, Mercado N, Poldermans D, Gardien M, Vos J and Simoons ML. Acute myocardial infarction. Lancet 361: 847-858, 2003.

6. Bolli R, Dawn B and Xuan YT. Emerging role of the JAK-STAT pathway as a mechanism of protection against ischemia/reperfusion injury. J Mol Cell Cardiol 33: 1893-1896, 2001.

7. Bordet T, Lesbordes JC, Rouhani S, Castelnau-Ptakhine L, Schmalbruch H, Haase G and Kahn A. Protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic ALS mice. Hum Mol Genet 10: 1925-1933, 2001.

8. Bos R, Mougenot N, Findji L, Mediani O, Vanhoutte PM and Lechat P. Inhibition of catecholamine-induced cardiac fibrosis by an aldosterone antagonist. J Cardiovasc Pharmacol 45: 8-13, 2005.

9. Bouzegrhane F and Thibault G. Is angiotensin II a proliferative factor of cardiac fibroblasts? Cardiovasc Res 53: 304-312, 2002.

10. Brar BK, Stephanou A, Pennica D and Latchman DS. CT-1 mediated cardioprotection against ischaemic re-oxygenation injury is mediated by PI3 kinase, Akt and MEK1/2 pathways. Cytokine 16: 93-6, 2001.

11. Braunwald E. Heart Disease: A Textbook of Cardiovascular Medicine. 1969.

12. Braunwald E. Expanding indications for beta-blockers in heart failure. N Engl J Med 344: 1711-1712, 2001.

13. Braunwald E and Pfeffer MA. Ventricular enlargement and remodeling following acute myocardial infarction: mechanisms and management. Am J Cardiol 68: 1D-6D, 1991.

14. Bristow MR. beta-adrenergic receptor blockade in chronic heart failure. Circulation 101: 558-569, 2000.

15. Bristow MR, Feldman AM, Adams KF, Jr. and Goldstein S. Selective versus nonselective beta-blockade for heart failure therapy: are there lessons to be learned from the COMET trial? J Card Fail 9: 444-453, 2003.

16. Bristow MR and Long CS. Cardiotrophin-1 in heart failure. Circulation 106: 1430-1432, 2002.

17. Bristow MR, Minobe W, Rasmussen R, Hershberger RE and Hoffman BB. Alpha-1 adrenergic receptors in the nonfailing and failing human heart. J Pharmacol Exp Ther 247: 1039-1045, 1988.

18. Bristow MR, Sandoval AB, Gilbert EM, Deisher T, Minobe W and Rasmussen R. Myocardial alpha- and beta-adrenergic receptors in heart failure: is cardiac-derived norepinephrine the regulatory signal? Eur Heart J 9 Suppl H: 35-40, 1988.

19. Bristow MR, Shakar SF, Linseman JV and Lowes BD. Inotropes and beta-blockers: is there a need for new guidelines? J Card Fail 7: 8-12, 2001.

20. Chidsey CA, Harrison DC and Braunwald E. Augmentation of the plasma nor-epinephrine response to exercise in patients with congestive heart failure. N Engl J Med 267: 650-654, 1962.

21. Clark AL and Cleland JG. The control of adrenergic function in heart failure: therapeutic intervention. Heart Fail Rev 5: 101-114, 2000.

22. Cleutjens JP, Blankesteijn WM, Daemen MJ and Smits JF. The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. Cardiovasc Res 44: 232-241, 1999.

23. Cleutjens JP, Verluyten MJ, Smits JF and Daemen MJ. Collagen remodeling after myocardial infarction in the rat heart. Am J Pathol 147: 325-338, 1995.

24. Cukierman E, Pankov R, Stevens DR and Yamada KM. Taking cell-matrix adhesions to the third dimension. Science 294: 1708-1712, 2001.

25. Dahlof B, Devereux RB, Kjeldsen SE, Julius S, Beevers G, de FU, Fyhrquist F, Ibsen H, Kristiansson K, Lederballe-Pedersen O, Lindholm LH, Nieminen MS, Omvik P, Oparil S and Wedel H. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 359: 995-1003, 2002.

26. de Lemos JA, McGuire DK and Drazner MH. B-type natriuretic peptide in cardiovascular disease. Lancet 362: 316-322, 2003.

27. de GM, Catt KJ, Inagami T, Wright JW and Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev 52: 415-472, 2000.

28. DeFelice A, Frering R and Horan P. Time course of hemodynamic changes in rats with healed severe myocardial infarction. Am J Physiol 257: H289-H296, 1989.

29. Desmouliere A, Chaponnier C and Gabbiani G. Tissue repair, contraction, and the myofibroblast. Wound Repair Regen 13: 7-12, 2005.

30. Dixon IM, Lee SL and Dhalla NS. Nitrendipine binding in congestive heart failure due to myocardial infarction. Circ Res 66: 782-788, 1990.

31. Dobaczewski M, Bujak M, Zymek P, Ren G, Entman ML and Frangogiannis NG. Extracellular matrix remodeling in canine and mouse myocardial infarcts. Cell Tissue Res 324: 475-488, 2006.

32. Domanski MJ, Krause-Steinrauf H, Massie BM, Deedwania P, Follmann D, Kovar D, Murray D, Oren R, Rosenberg Y, Young J, Zile M and Eichhorn E. A comparative analysis of the results from 4 trials of beta-blocker therapy for heart failure: BEST, CIBIS-II, MERIT-HF, and COPERNICUS. J Card Fail 9: 354-363, 2003.

33. El-Adawi H, Deng L, Tramontano A, Smith S, Mascareno E, Ganguly K, Castillo R and El-Sherif N. The functional role of the JAK-STAT pathway in post-infarction remodeling. Cardiovasc Res 57: 129-138, 2003.

34. Ellison KE and Gandhi G. Optimising the use of beta-adrenoceptor antagonists in coronary artery disease. Drugs 65: 787-797, 2005.

35. Ertl G and Frantz S. Wound model of myocardial infarction. Am J Physiol Heart Circ Physiol 288: H981-H983, 2005.

36. Esler M and Kaye D. Measurement of sympathetic nervous system activity in heart failure: the role of norepinephrine kinetics. Heart Fail Rev 5: 17-25, 2000.

37. Feldman DS, Carnes CA, Abraham WT and Bristow MR. Mechanisms of disease: beta-adrenergic receptors--alterations in signal transduction and pharmacogenomics in heart failure. Nat Clin Pract Cardiovasc Med 2: 475-483, 2005.

38. Fletcher PJ, Pfeffer JM, Pfeffer MA and Braunwald E. Left ventricular diastolic pressure-volume relations in rats with healed myocardial infarction. Circ Res 49: 618-626, 1981.

39. Fonarow GC. Practical considerations of beta-blockade in the management of the post-myocardial infarction patient. Am Heart J 149: 984-993, 2005.

40. Fowler MB, Laser JA, Hopkins GL, Minobe W and Bristow MR. Assessment of the beta-adrenergic receptor pathway in the intact failing human heart: progressive receptor down-regulation and subsensitivity to agonist response. Circulation 74: 1290-1302, 1986.

41. Frangogiannis NG, Michael LH and Entman ML. Myofibroblasts in reperfused myocardial infarcts express the embryonic form of smooth muscle myosin heavy chain (SMemb). Cardiovasc Res 48: 89-100, 2000.

42. Frangogiannis NG, Shimoni S, Chang SM, Ren G, Dewald O, Gersch C, Shan K, Aggeli C, Reardon M, Letsou GV, Espada R, Ramchandani M, Entman ML and Zoghbi WA. Active interstitial remodeling: an important process in the hibernating human myocardium. J Am Coll Cardiol 39: 1468-1474, 2002.

43. Frangogiannis NG, Smith CW and Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res 53: 31-47, 2002.

44. Freed DH, Borowiec AM, Angelovska T and Dixon IM. Induction of protein synthesis in cardiac fibroblasts by cardiotrophin-1: integration of multiple signaling pathways. Cardiovasc Res 60: 365-375, 2003.

45. Freed DH, Cunnington RH, Dangerfield AL, Sutton JS and Dixon IM. Emerging evidence for the role of cardiotrophin-1 in cardiac repair in the infarcted heart. Cardiovasc Res 65: 782-792, 2005.

46. Funamoto M, Fujio Y, Kunisada K, Negoro S, Tone E, Osugi T, Hirota H, Izumi M, Yoshizaki K, Walsh K, Kishimoto T and Yamauchi-Takihara K. Signal transducer and activator of transcription 3 is required for glycoprotein 130-mediated induction of vascular endothelial growth factor in cardiac myocytes. J Biol Chem 275: 10561-6, 2000.

47. Funamoto M, Hishinuma S, Fujio Y, Matsuda Y, Kunisada K, Oh H, Negoro S, Tone E, Kishimoto T and Yamauchi-Takihara K. Isolation and characterization of the murine cardiotrophin-1 gene: expression and norepinephrine-induced transcriptional activation. J Mol Cell Cardiol 32: 1275-1284, 2000.

48. Ghosh S, Ng LL, Talwar S, Squire IB and Galinanes M. Cardiotrophin-1 protects the human myocardium from ischemic injury. Comparison with the first and second window of protection by ischemic preconditioning. Cardiovasc Res 48: 440-447, 2000.

49. Giles J. Beta-blockers tackle memories of horror. Nature 436: 448-449, 2005.

50. Gohlke P, Pees C and Unger T. AT2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. Hypertension 31: 349-355, 1998.

51. Goldman S and Raya TE. Rat infarct model of myocardial infarction and heart failure. J Card Fail 1: 169-177, 1995.

52. Gwechenberger M, Pacher R, Berger R, Zorn G, Moser P, Stanek B and Huelsmann M. Comparison of soluble glycoprotein 130 and cardiac natriuretic peptides as long-term predictors of heart failure progression. J Heart Lung Transplant 24: 2190-2195, 2005.

53. Hamanaka I, Saito Y, Yasukawa H, Kishimoto I, Kuwahara K, Miyamoto Y, Harada M, Ogawa E, Kajiyama N, Takahashi N, Izumi T, Kawakami R, Masuda I, Yoshimura A and Nakao K. Induction of JAB/SOCS-1/SSI-1 and CIS3/SOCS-3/SSI-3 is involved in gp130 resistance in cardiovascular system in rat treated with cardiotrophin-1 in vivo. Circ Res 88: 727-732, 2001.

54. Hao J, Wang B, Jones SC, Jassal DS and Dixon IM. Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and in vitro. Am J Physiol Heart Circ Physiol 279: H3020-H3030, 2000.

55. Hedrich O, Patten RD and Denofrio D. Current Treatment Options for CHF Management: Focus on the Renin-Angiotensin-Aldosterone System. Curr Treat Options Cardiovasc Med 7: 3-13, 2005.

56. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F and Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 334: 297-314, 1998.

57. Hilfiker-Kleiner D, Hilfiker A and Drexler H. Many good reasons to have STAT3 in the heart. Pharmacol Ther 107: 131-137, 2005.

58. Hirota H, Chen J, Betz UA, Rajewsky K, Gu Y, Ross J, Jr., Muller W and Chien KR. Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. Cell 97: 189-198, 1999.

59. HSF. Heart and Stroke Foundation of Canada. 2006. Online Resource. Ref Type: Generic

60. Janssen PM, Schillinger W, Donahue JK, Zeitz O, Emami S, Lehnart SE, Weil J, Eschenhagen T, Hasenfuss G and Prestle J. Intracellular beta-blockade: overexpression of Galpha(i2) depresses the beta-adrenergic response in intact myocardium. Cardiovasc Res 55: 300-308, 2002.

61. Jin H, Yang R, Keller GA, Ryan A, Ko A, Finkle D, Swanson TA, Li W, Pennica D, Wood WI and Paoni NF. In vivo effects of cardiotrophin-1. Cytokine 8: 920-926, 1996.

62. Ju H, Zhao S, Davinder SJ and Dixon IM. Effect of AT1 receptor blockade on cardiac collagen remodeling after myocardial infarction. Cardiovasc Res 35: 223-232, 1997.

63. Jugdutt BI, Khan MI, Jugdutt SJ and Blinston GE. Effect of prolonged inotropic stimulation on ventricular remodeling during healing after myocardial infarction in the dog: mechanistic insights. J Am Coll Cardiol 27: 1787-1795, 1996.

64. Jugdutt BI, Schwarz-Michorowski BL and Khan MI. Effect of long-term captopril therapy on left ventricular remodeling and function during healing of canine myocardial infarction. J Am Coll Cardiol 19: 713-721, 1992.

65. Kanekar S, Borg TK, Terracio L and Carver W. Modulation of heart fibroblast migration and collagen gel contraction by IGF-I. Cell Adhes Commun 7: 513-523, 2000.

66. Kawano H, Do YS, Kawano Y, Starnes V, Barr M, Law RE and Hsueh WA. Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. Circulation 101: 1130-1137, 2000.

67. Kjeldsen SE, Dahlof B, Devereux RB, Julius S, Aurup P, Edelman J, Beevers G, de FU, Fyhrquist F, Ibsen H, Kristianson K, Lederballe-Pedersen O, Lindholm LH, Nieminen MS, Omvik P, Oparil S, Snapinn S and Wedel H. Effects of losartan on cardiovascular morbidity and mortality in patients with isolated systolic hypertension and left ventricular hypertrophy: a Losartan Intervention for Endpoint Reduction (LIFE) substudy. JAMA 288: 1491-1498, 2002.

68. Kjeldsen SE and Julius S. Hypertension mega-trials with cardiovascular end points: effect of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. Am Heart J 148: 747-754, 2004.

69. Kloner RA. Natural and unnatural triggers of myocardial infarction. Prog Cardiovasc Dis 48: 285-300, 2006.

70. Knight DA, D'Aprile AC, Spalding LJ, Goldie RG and Thompson PJ. Leukaemia inhibitory factor (LIF) upregulates excitatory non-adrenergic non-cholinergic and maintains cholinergic neural function in tracheal explants. Br J Pharmacol 130: 975-979, 2000.

71. Knight DA, Lydell CP, Zhou D, Weir TD, Robert SR and Bai TR. Leukemia inhibitory factor (LIF) and LIF receptor in human lung. Distribution and regulation of LIF release. Am J Respir Cell Mol Biol 20: 834-841, 1999.

72. Kunisada K, Negoro S, Tone E, Funamoto M, Osugi T, Yamada S, Okabe M, Kishimoto T and Yamauchi-Takihara K. Signal transducer and activator of transcription 3 in the heart transduces not only a hypertrophic signal but a protective signal against doxorubicin-induced cardiomyopathy. Proc Natl Acad Sci U S A 97: 315-319, 2000.

73. Kunisada K, Tone E, Fujio Y, Matsui H, Yamauchi-Takihara K and Kishimoto T. Activation of gp130 transduces hypertrophic signals via STAT3 in cardiac myocytes. Circulation 98: 346-352, 1998.

74. Kuwahara K, Saito Y, Harada M, Ishikawa M, Ogawa E, Miyamoto Y, Hamanaka I, Kamitani S, Kajiyama N, Takahashi N, Nakagawa O, Masuda I and Nakao K. Involvement of cardiotrophin-1 in cardiac myocyte-nonmyocyte interactions during hypertrophy of rat cardiac myocytes in vitro. Circulation 100: 1116-24, 1999.

75. Lamba S and Abraham WT. Alterations in adrenergic receptor signaling in heart failure. Heart Fail Rev 5: 7-16, 2000.

76. Latchman DS. Cardiotrophin-1 (CT-1): a novel hypertrophic and cardioprotective agent. Int J Exp Pathol 80: 189-196, 1999.

77. Liao Z, Brar BK, Cai Q, Stephanou A, O'Leary RM, Pennica D, Yellon DM and Latchman DS. Cardiotrophin-1 (CT-1) can protect the adult heart from injury when added both prior to ischaemia and at reperfusion. Cardiovasc Res 53: 902-910, 2002.

78. Lindsey ML, Mann DL, Entman ML and Spinale FG. Extracellular matrix remodeling following myocardial injury. Ann Med 35: 316-326, 2003.

79. Linsell CR, Lightman SL, Mullen PE, Brown MJ and Causon RC. Circadian rhythms of epinephrine and norepinephrine in man. J Clin Endocrinol Metab 60: 1210-1215, 1985.

80. Lopez B, Gonzalez A, Lasarte JJ, Sarobe P, Borras F, Diaz A, Barba J, Tomas L, Lozano E, Serrano M, Varo N, Beloqui O, Fortuno MA and Diez J. Is plasma cardiotrophin-1 a marker of hypertensive heart disease? J Hypertens 23: 625-632, 2005.

81. Lowes BD, Gilbert EM, Abraham WT, Minobe WA, Larrabee P, Ferguson D, Wolfel EE, Lindenfeld J, Tsvetkova T, Robertson AD, Quaife RA and Bristow MR. Myocardial gene expression in dilated cardiomyopathy treated with beta-blocking agents. N Engl J Med 346: 1357-1365, 2002.

82. Maisel AS, Krishnaswamy P, Nowak RM, McCord J, Hollander JE, Duc P, Omland T, Storrow AB, Abraham WT, Wu AH, Clopton P, Steg PG, Westheim A, Knudsen CW, Perez A, Kazanegra R, Herrmann HC and McCullough PA. Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. N Engl J Med 347: 161-167, 2002.

83. Mascareno E, El-Shafei M, Maulik N, Sato M, Guo Y, Das DK and Siddiqui MA. JAK/STAT signaling is associated with cardiac dysfunction during ischemia and reperfusion. Circulation 104: 325-329, 2001.

84. Masur SK, Dewal HS, Dinh TT, Erenburg I and Petridou S. Myofibroblasts differentiate from fibroblasts when plated at low density. Proc Natl Acad Sci U S A 93: 4219-4223, 1996.

85. Mayo Clinic. Myocardial Infarction. 2006. Online Resource.
Ref Type: Generic

86. Molenaar P, Christ T, Ravens U and Kaumann A. Carvedilol blocks beta2- more than beta1-adrenoceptors in human heart. Cardiovasc Res 69: 128-139, 2006.

87. Muller-Newen G. The cytokine receptor gp130: faithfully promiscuous. Sci STKE 2003: E40, 2003.

88. Negoro S, Kunisada K, Fujio Y, Funamoto M, Darville MI, Eizirik DL, Osugi T, Izumi M, Oshima Y, Nakaoka Y, Hirota H, Kishimoto T and Yamauchi-Takihara K. Activation of signal transducer and activator of transcription 3 protects cardiomyocytes from hypoxia/reoxygenation-induced oxidative stress through the

upregulation of manganese superoxide dismutase. Circulation 104: 979-981, 2001.

89. Ng DC, Court NW, dos Remedios CG and Bogoyevitch MA. Activation of signal transducer and activator of transcription (STAT) pathways in failing human hearts. Cardiovasc Res 57: 333-346, 2003.

90. Omland T, Persson A, Ng L, O'Brien R, Karlsson T, Herlitz J, Hartford M and Caidahl K. N-terminal pro-B-type natriuretic peptide and long-term mortality in acute coronary syndromes. Circulation 106: 2913-2918, 2002.

91. Omura T, Yoshiyama M, Takeuchi K, Kim S, Iwao H, Yamagishi H, Toda I, Teragaki M, Akioka K and Yoshikawa J. Angiotensin blockade inhibits SIF DNA binding activities via STAT3 after myocardial infarction. J Mol Cell Cardiol 32: 23-33, 2000.

92. Opie L. Beta Blocking Agents. In: Drugs for the Heart, edited by Gersh BJ. Philadelphia: W.B. Saunders Company, 2001, p. 1-32.

93. Pelouch V, Dixon IM, Golfman L, Beamish RE and Dhalla NS. Role of extracellular matrix proteins in heart function. Mol Cell Biochem 129: 101-120, 1993.

94. Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh SM, Darbonne WC, Knutzon DS, Yen R, Chien KR and . Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. Proc Natl Acad Sci U S A 92: 1142-1146, 1995.

95. Pennica D, Shaw KJ, Swanson TA, Moore MW, Shelton DL, Zioncheck KA, Rosenthal A, Taga T, Paoni NF and Wood WI. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. J Biol Chem 270: 10915-22, 1995.

96. Pfeffer MA and Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation 81: 1161-1172, 1990.

97. Pfeffer MA, Lamas GA, Vaughan DE, Parisi AF and Braunwald E. Effect of captopril on progressive ventricular dilatation after anterior myocardial infarction. N Engl J Med 319: 80-86, 1988.

98. Pfeffer MA, Pfeffer JM, Steinberg C and Finn P. Survival after an experimental myocardial infarction: beneficial effects of long-term therapy with captopril. Circulation 72: 406-412, 1985.

99. Podewski EK, Hilfiker-Kleiner D, Hilfiker A, Morawietz H, Lichtenberg A, Wollert KC and Drexler H. Alterations in Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling in patients with end-stage dilated cardiomyopathy. Circulation 107: 798-802, 2003.

100. Port JD and Bristow MR. Altered beta-adrenergic receptor gene regulation and signaling in chronic heart failure. J Mol Cell Cardiol 33: 887-905, 2001.

101. Railson J, Lawrence K, Stephanou A, Brar B, Pennica D and Latchman D. Cardiotrophin-1 reduces stress-induced heat shock protein production in cardiac myocytes. Cytokine 12: 1741-1744, 2000.

102. Railson JE, Liao Z, Brar BK, Buddle JC, Pennica D, Stephanou A and Latchman DS. Cardiotrophin-1 and urocortin cause protection by the same pathway and hypertrophy via distinct pathways in cardiac myocytes. Cytokine 17: 243-253, 2002.

103. Rauch U, Osende JI, Fuster V, Badimon JJ, Fayad Z and Chesebro JH. Thrombus formation on atherosclerotic plaques: pathogenesis and clinical consequences. Ann Intern Med 134: 224-238, 2001.

104. Robinson TF, Factor SM and Sonnenblick EH. The heart as a suction pump. Sci Am 254: 84-91, 1986.

105. Robledo O, Fourcin M, Chevalier S, Guillet C, Auguste P, Pouplard-Barthelaix A, Pennica D and Gascan H. Signaling of the cardiotrophin-1 receptor. Evidence for a third receptor component. J Biol Chem 272: 4855-63, 1997.

106. Ruilope LM, Rosei EA, Bakris GL, Mancia G, Poulter NR, Taddei S, Unger T, Volpe M, Waeber B and Zannad F. Angiotensin receptor blockers: therapeutic targets and cardiovascular protection. Blood Press 14: 196-209, 2005.

107. Sadoshima J. Novel AT(1) receptor-independent functions of losartan. Circ Res 90: 754-756, 2002.

108. Sano M, Fukuda K, Kodama H, Pan J, Saito M, Matsuzaki J, Takahashi T, Makino S, Kato T and Ogawa S. Interleukin-6 family of cytokines mediate angiotensin II-induced cardiac hypertrophy in rodent cardiomyocytes. J Biol Chem 275: 29717-29723, 2000.

109. Schieffer B, Wirger A, Meybrunn M, Seitz S, Holtz J, Riede UN and Drexler H. Comparative effects of chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on cardiac remodeling after myocardial infarction in the rat. Circulation 89: 2273-2282, 1994.

110. Schmidt B, Drexler H and Schieffer B. Therapeutic effects of angiotensin (AT1) receptor antagonists: potential contribution of mechanisms other than AT1 receptor blockade. Am J Cardiovasc Drugs 4: 361-368, 2004.

111. Schmidt B and Schieffer B. Angiotensin II AT1 receptor antagonists. Clinical implications of active metabolites. J Med Chem 46: 2261-2270, 2003.

112. Schroers A, Hecht O, Kallen KJ, Pachta M, Rose-John S and Grotzinger J. Dynamics of the gp130 cytokine complex: a model for assembly on the cellular membrane. Protein Sci 14: 783-790, 2005.

113. SELYE H, BAJUSZ E, GRASSO S and MENDELL P. Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 11: 398-407, 1960.

114. Sheng Z, Pennica D, Wood WI and Chien KR. Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. Development 122: 419-28, 1996.

115. Shiojima I, Aikawa M, Suzuki J, Yazaki Y and Nagai R. Embryonic smooth muscle myosin heavy chain SMemb is expressed in pressure-overloaded cardiac fibroblasts. Jpn Heart J 40: 803-818, 1999.

116. Sica DA. Alpha1-adrenergic blockers: current usage considerations. J Clin Hypertens (Greenwich) 7: 757-762, 2005.

117. Sica DA. Pharmacotherapy review: Angiotensin receptor antagonists. J Clin Hypertens (Greenwich) 7: 681-684, 2005.

118. Skeberdis VA. Structure and function of beta3-adrenergic receptors. Medicina (Kaunas) 40: 407-413, 2004.

119. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76-85, 1985.

120. Smits JF, van Krimpen C, Schoemaker RG, Cleutjens JP and Daemen MJ. Angiotensin II receptor blockade after myocardial infarction in rats: effects on hemodynamics, myocardial DNA synthesis, and interstitial collagen content. J Cardiovasc Pharmacol 20: 772-8, 1992.

121. Soonpaa MH and Field LJ. Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ Res 83: 15-26, 1998.

122. Soriano SF, Serrano A, Hernanz-Falcon P, Martin de AA, Monterrubio M, Martinez C, Rodriguez-Frade JM and Mellado M. Chemokines integrate

JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. Eur J Immunol 33: 1328-1333, 2003.

123. Squires CE, Escobar GP, Payne JF, Leonardi RA, Goshorn DK, Sheats NJ, Mains IM, Mingoia JT, Flack EC and Lindsey ML. Altered fibroblast function following myocardial infarction. J Mol Cell Cardiol 39: 699-707, 2005.

124. St John SM, Lee D, Rouleau JL, Goldman S, Plappert T, Braunwald E and Pfeffer MA. Left ventricular remodeling and ventricular arrhythmias after myocardial infarction. Circulation 107: 2577-2582, 2003.

125. Stroe AF and Gheorghiade M. Carvedilol: beta-blockade and beyond. Rev Cardiovasc Med 5 Suppl 1: S18-S27, 2004.

126. Sun Y, Kiani MF, Postlethwaite AE and Weber KT. Infarct scar as living tissue. Basic Res Cardiol 97: 343-347, 2002.

127. Sun Y and Weber KT. Cardiac remodelling by fibrous tissue: role of local factors and circulating hormones. Ann Med 30 Suppl 1:3-8: 3-8, 1998.

128. Sun Y and Weber KT. Infarct scar: a dynamic tissue. Cardiovasc Res 46: 250-6, 2000.

129. Suzuki Y, Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V and Egido J. Inflammation and angiotensin II. Int J Biochem Cell Biol 35: 881-900, 2003.

130. Talwar S, Squire IB, Downie PF, Davies JE and Ng LL. Plasma N terminal pro-brain natriuretic peptide and cardiotrophin 1 are raised in unstable angina. Heart 84: 421-4, 2000.

131. Talwar S, Squire IB, Downie PF, O'Brien R J, Davies JE and Ng LL. Elevated circulating cardiotrophin-1 in heart failure: relationship with parameters of left ventricular systolic dysfunction. Clin Sci (Colch) 99: 83-88, 2000.

132. Talwar S, Squire IB, O'brien RJ, Downie PF, Davies JE and Ng LL. Plasma cardiotrophin-1 following acute myocardial infarction: relationship with left ventricular systolic dysfunction. Clin Sci (Lond) 102: 9-14, 2002.

133. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C and Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 3: 349-363, 2002.

134. Touyz RM and Berry C. Recent advances in angiotensin II signaling. Braz J Med Biol Res 35: 1001-1015, 2002.

135. Tsuruda T, Jougasaki M, Boerrigter G, Huntley BK, Chen HH, D'Assoro AB, Lee SC, Larsen AM, Cataliotti A and Burnett JC, Jr. Cardiotrophin-1 stimulation of cardiac fibroblast growth: roles for glycoprotein 130/leukemia inhibitory factor receptor and the endothelin type A receptor. Circ Res 90: 128-134, 2002.

136. Unger T. Pharmacological properties of angiotensin II antagonists: examining all the therapeutic implications. J Renin Angiotensin Aldosterone Syst 2 Suppl 2: S4-S7, 2001.

137. Vracko R and Thorning D. Contractile cells in rat myocardial scar tissue. Lab Invest 65: 214-227, 1991.

138. Wang B, Ansari R, Sun Y, Postlethwaite AE, Weber KT and Kiani MF. The scar neovasculature after myocardial infarction in rats. Am J Physiol Heart Circ Physiol 289: H108-H113, 2005.

139. Weber KT. Cardiac interstitium in health and disease: the fibrillar collagen network. J Am Coll Cardiol 13: 1637-1652, 1989.

140. Weber KT. Extracellular matrix remodeling in heart failure: a role for de novo angiotensin II generation. Circulation 96: 4065-4082, 1997.

141. Weber KT, Jalil JE, Janicki JS and Pick R. Myocardial collagen remodeling in pressure overload hypertrophy. A case for interstitial heart disease. Am J Hypertens 2: 931-940, 1989.

142. Weber M and Hamm C. Role of B-type natriuretic peptide (BNP) and NT-proBNP in clinical routine. Heart 92: 843-849, 2006.

143. Weber MA. The role of the new beta-blockers in treating cardiovascular disease. Am J Hypertens 18: 169S-176S, 2005.

144. Weisman HF, Bush DE, Mannisi JA, Weisfeldt ML and Healy B. Cellular mechanisms of myocardial infarct expansion. Circulation 78: 186-201, 1988.

145. Wen TC, Rogido MR, Moore JE, Genetta T, Peng H and Sola A. Cardiotrophin-1 protects cortical neuronal cells against free radical-induced injuries in vitro. Neurosci Lett 387: 38-42, 2005.

146. Willems IE, Havenith MG, De Mey JG and Daemen MJ. The alpha-smooth muscle actin-positive cells in healing human myocardial scars. Am J Pathol 145: 868-75, 1994.

147. Wollert KC and Chien KR. Cardiotrophin-1 and the role of gp130-dependent signaling pathways in cardiac growth and development. J Mol Med 75: 492-501, 1997.

148. Wollert KC, Taga T, Saito M, Narazaki M, Kishimoto T, Glembotski CC, Vernallis AB, Heath JK, Pennica D, Wood WI and Chien KR. Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy. Assembly of sarcomeric units in series VIA gp130/leukemia inhibitory factor receptor-dependent pathways. J Biol Chem 19;271: 9535-9545, 1996.

149. Xuan YT, Guo Y, Han H, Zhu Y and Bolli R. An essential role of the JAK-STAT pathway in ischemic preconditioning. Proc Natl Acad Sci U S A 98: 9050-9055, 2001.

150. Yin F, Li P, Zheng M, Chen L, Xu Q, Chen K, Wang YY, Zhang YY and Han C. IL-6 family of cytokines mediates isoproterenol-induced delayed STAT3 activation in mouse heart. J Biol Chem 2003.

151. Yoshida K, Taga T, Saito M, Suematsu S, Kumanogoh A, Tanaka T, Fujiwara H, Hirata M, Yamagami T, Nakahata T, Hirabayashi T, Yoneda Y, Tanaka K, Wang WZ, Mori C, Shiota K, Yoshida N and Kishimoto T. Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. Proc Natl Acad Sci U S A 93: 407-411, 1996.

152. Zimmer HG, Gerdes AM, Lortet S and Mall G. Changes in heart function and cardiac cell size in rats with chronic myocardial infarction. J Mol Cell Cardiol 22: 1231-1243, 1990.

153. Zolk O, Ng LL, O'brien RJ, Weyand M and Eschenhagen T. Augmented expression of cardiotrophin-1 in failing human hearts is accompanied by diminished glycoprotein 130 receptor protein abundance. Circulation 106: 1442-1446, 2002.