NOTE TO USERS

Differences in Atrial vs. Ventricular Remodeling in Dogs with Ventricular Tachypaced-induced Congestive Heart Failure

By

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ABSTRACT

Background: Congestive Heart Failure (CHF) causes arrhythmogenic remodeling in both atria and ventricles, but potential differences between atrial and ventricular remodeling in CHF have not been studied.

Methods and Results: We examined atrial and ventricular tissues from dogs with CHF induced by ventricular tachypacing (VTP, 240/min) for 0 (control) or 24 hrs, 1, 2, or 5 wks. Tissue angiotensin-II concentration (ELISA) increased to steady state at 24 hrs, and was significantly higher in LA than LV. VTP caused tissue apoptosis, inflammatory-cell infiltration and cell-death, with maximum changes in LA being transient and larger than in LV. MAP kinase activation (Western blot) was rapid (within 24 hrs) in LA, but smaller and slower (p38, JNK) or non-significant (ERK) in LV. The 25-kDa activated form of TGFβ1, a particularly important profibrotic mediator in atria, increased significantly (Western blot) in LA at 24 hrs and 1 wk, but was not changed in LV. Substantial fibrosis developed in LA, but was much less important in LV.

Conclusions: There are qualitative and quantitative differences in LA and LV remodeling in experimental CHF, with important potential consequences for underlying mechanisms and therapeutic approaches.

Key words: Congestive Heart failure, atrial remodeling, and ventricular remodeling.

RESUMÉ

Introduction: L'insuffisance cardiaque congestive (ICC) cause du remodelage arrythmogène dans les oreillettes et les ventricules, mais les différences potentielles entre le remodelage auriculaire et le remodelage ventriculaire ne sont pas étudiées.

Méthodes et Résultats: Nous avons examiné les tissus auriculaire et ventriculaire des chiens avec ICC induite par tachycardie ventriculaire (TV, 240/min) pour 0 (control) ou 24 hrs, 1, 2, ou 5 semaines. La concentration d'angiotensine-II tissulaire (ELISA) avait cru jusqu'à un état stable après 24 hrs, et était significativement plus élevée dans l'OG que dans le VG. La TV a causé apoptose, infiltration des cellules inflammatoire, et mort cellulaire avec des changements maximums dans l'OG. Ces changements étaient transitoires et plus grands dans l'OG que dans le VG. L'activation des MAP kinases (Western blot) était rapide (pendant 24 hrs) dans l'OG, mais petite et lente (p38, JNK) ou non significative (ERK) dans le VG. La forme activée 25-kDa du TGFβ1; un médiator profibrotique particulièrement important dans les oreillettes, avait cru significativement (Western blot) dans l'OG après 24 hrs et 1 semaine, mais n'avait pas changé dans le VG. Une fibrose substantielle s'était développée dans l'OG, mais était moins importante dans le VG.

Conclusions: Il y a des différences qualitatives et quantitatives entre le remodelage auriculaire et ventriculaire dans ICC expérimentale, avec des conséquences importantes et potentielles pour les mécanismes impliqués et les approches thérapeutiques.

Mots Clés: L'insuffisance cardiaque congestive, remodelage auriculaire, et remodelage ventriculaire.

CONTRIBUTIONS OF AUTHORS

The candidate, Nessrine Hanna, is the main author of the article enclosed, as she made the majority of experimental work and analysis. Sophie Cardin's role was training and technically assisting in the research. Dr.Leung, the third author, performed the autopsy procedure and took the pathological parameters at the time of autopsy. Dr.Nattel is the main director of the research; he was supervising, guiding and directing the research.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AF	Atrial fibrillation
ANF	Atrial natriuretic factor
ANP	Atrial natriuretic peptide
AT1	Type I angiotensin receptor
AT2	Type II angiotensin receptor
AT3	Type III angiotensin receptor
AT4	Type IV angiotensin receptor
ATP	Adenosine triphosphate
CAMS	Cell adhesion molecules
CHF	Congestive heart failure
CHF-STAT	Congestive heart failure of survival trial of antiarrhythmic therapy
cNOS	Constitutive nitric oxide synthase
CSAID	Cytokine-suppressive anti-inflammatory drug
CT-1	Cardiotrophin-1
DIAMOND	Danish investigation of arrhythmia and mortality on Dofetilide
ECG	Electrocardiogram
ELISA	Enzymatic Immunoassay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular-signal regulated protein kinase
ET-1	Endothelin-1

GDP	Guanine diphosphate
GPCR	G protein coupled receptor
Grb2	Growth receptor binding protein
GTP	Guanine triphosphate
HF	Heart failure
HPS	Hematoxylin-phloxin-safran
ICC	Insuffisance cardiaque congestive
IGF-1	Insulin-like growth factor-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun NH2-terminal protein kinase
LA	Left atria
LAP	Latency associated peptide
LTBP	Latent TGF binding protein
LV	Left ventricle
MAPK	Mitogen activated protein kinase
MAPKAP	Mitogen activated protein kinase activated protein
MEK	Mitogen activated protein kinase
MEKK	Mitogen activated protein kinase kinase
NO	Nitric oxide
OG	Oreillette gauche
PAK	P-21 activated kinase
PARP	Poly (ADP-ribose) polymerase

PE	Phenylephrine
ROS	Reactive oxygen species
SAPK	Stress activated protein kinase
Shc	Src-homology protein
SMADS/MAD	Mothers against dpp
Sos	Son-of-sevenless
TGFβ	Transforming growth factor β
TNF-α	Tumor necrosis factor α
TUNEL	Terminal dUTP nicked-end labeling
TV	Tachycardie ventriculaire
VG	Ventricule gauche
VTP	Ventricular tachypacing
WBC	White blood cell
STAT	Signal transducer and activator of transcription
CHOP	C/EBP homologous protein
EGF	Epidermal growth factor
PDGF	Platelet-derived growth factor

1. Introduction

1.1 Heart Failure

1.1.1 Definition

There are few clinical syndromes that have undergone both the conceptual and epidemiologic transformation that has occurred with heart failure (HF). For many years the heart was defined as a pump that imparts the energy needed to "lift" fluid from a low-pressure system (the veins) to one at higher pressure (the arteries). Impairment of the cardiac pump, therefore, can have one or both of two consequences. On one hand, there can be inadequate forward flow of blood from the heart in to the aorta and pulmonary artery; on the other hand, there can be inadequate emptying of the venous reservoirs, which causes blood to back up behind the heart [1].

Although the concept of a heart being a pump persists in some circles and is generally correct, we now know the syndrome of heart failure is far more complex. Heart failure is not a disease but instead represents the final pathway by which a number of disorders damage the heart so as to cause disability and premature death. These disorders include coronary disease, hypertension, valvular disorders, and a diverse group of heart muscle diseases referred to as the cardiomyopathies [1-3]. Furthermore, because this syndrome establishes a number of vicious cycles, heart failure begets more heart failure. Regardless of etiology, heart failure is accompanied by abnormalities in both the contractile machinery and the membrane systems that regulate the cardiac cycle, so that this syndrome can also be defined in terms of the disordered biochemical and biophysical processes that impair myocardial contractility and relaxation. However, the molecular composition of the heart is also changed in most of heart failure patients, so that even these biochemical definitions are inadequate. Furthermore, molecular abnormalities differ in different forms of heart failure, and change with time as this condition progresses. The failing

heart is generally a dying heart because the growth response that leads to hypertrophy, which increases the mass of the failing heart, appears also to cause premature death of cardiac myocytes. In the adult heart, myocyte death is a calamity because the cells that are lost cannot be replaced. It is largely for this reason that heart failure is a progressive condition, with a prognosis that is worse than that of most common malignancies [1-3].

Katz [1] provided a definition of heart failure that recognizes not only the impaired organ physiology and altered cell biochemistry but also the progressive changes that cause rapid deterioration of the heart. He defines heart failure as a *clinical syndrome in which heart disease reduces cardiac output, increases venous pressures, and is accompanied by molecular abnormalities that cause progressive deterioration of the failing heart and premature myocardial cell death*[1].

1.1.2 Classification of heart failure

Several classifications of heart failure are commonly used in clinical practice. Here we mention only 3 classifications: Backward and Forward Failure, Systolic and Diastolic dysfunction, and Right and Left Failure.

1.1.2.1 Backward and Forward Failure. Like any pump, the heart has only two ways to fail: inadequate emptying of the venous reservoirs (often called backward failure) and reduced ejection of blood under pressure into the aorta and pulmonary artery (forward failure). The causes of backward failure of the left heart include mitral stenosis, in which narrowing of the mitral valve orifice impedes venous return into a normal left ventricle, and hypertrophic cardiomyopathy, in which left ventricular cavity obliteration caused by inappropriate concentric hypertrophy reduces diastolic filling. Forward failure of the left ventricle can occur when a

mechanical obstruction inhibits ejection, as in aortic stenosis; when myocardial damage or weakness reduces systolic shortening, as occurs with myocarditis; or when a ventricle becomes scarred after a large myocardial infarction. Similar mechanisms can impair right ventricular ejection, although left ventricle dysfunction is by far the most common cause of heart failure [1].

1.1.2.2 Systolic and Diastolic Dysfunction. These terms refer to the impaired ability of the heart to eject (systolic dysfunction) and to fill (diastolic dysfunction).

Chronic systolic dysfunction of the left ventricle, which from an architecture standpoint describes a dilated, thin-walled heart (eccentric hypertrophy), is usually caused by diseases that damage or weaken the myocardium. Chronic diastolic dysfunction, which describes a non-compliant, thick-walled ventricle with normal, or even reduced cavity size (concentric hypertrophy), is commonly seen in patients with left ventricular hypertrophy secondary to aortic stenosis or long-standing, inadequately treated systemic hypertension [1,2].

1.1.2.3 Right and Left Heart Failure. The distinction between the right and left heart failure is especially useful in patients with congenital and valvular heart disease, in whom a narrowed or leaky valve, or an intra-cardiac shunt, can affect predominantly the right or the left side of the heart. In developed countries, where the major etiologies of heart failure are coronary and hypertensive heart disease, left heart failure is especially common [1].

1.1.3 Clinical Manifestation

Heart failure patients usually report shortness of breath, fatigue, and intolerance to physical activity [1-4]. The shortness of breath (dyspnea) generally reported by patients with left heart failure is due in large part to the increased work required to ventilate the congested lungs

that become stiff and inelastic. Whereas the normal work of breathing is barely perceived, elevated pulmonary venous pressure, by filling the lungs with water, increases respiratory effort to such a point that it cannot be ignored. The resulting difficulty in breathing, called dyspnea is exacerbated by weakness of the respiratory muscles [5].

The fluid transudated through the pulmonary capillaries in left heart failure appears first in the interstitium, where it can be carried into the systemic veins via the lymphatics. This interstitial edema occurs mainly in the lower lobes of the lung because of gravity, which explains the common appearance of a crackling sound (rales) and thin horizontal lines seen in the lung in the chest radiograph (called Kerley B lines). Another radiologic feature of left heart failure is "cephalization" of the pulmonary venous shadows, which occurs when increased pulmonary venous pressure expands the veins supplying the upper lobes of the lungs; this reduces the normal difference in diameter between the vessels and the pulmonary veins supplying the lower lobes, which are normally engorged by the effects of gravity on the pressure distribution in these thin-walled vessels [1].

Dyspnea becomes more severe when the patient lies down (orthopnea). This occurs because elevation of legs increases venous return to the right heart, which by increasing blood flow into the lungs and lung stiffness exacerbates the symptom of dyspnea. As backward failure of the left heart becomes more severe, the amount of fluid transudated into the pulmonary interstitium begins to exceed the ability of the lymphatics to carry this out of the lungs. The result is fluid accumulation in the air spaces, which interferes with gas exchange. Because oxygen is much less water soluble than carbon dioxide, the major abnormality is arterial hypoxia. Before the availability of powerful diuretics, end-stage heart failure led eventually to pulmonary edema, in which the patient literally drown in fluid that fills the lungs [1].

Dropsy occurs when fluid is to transudated into the soft tissues and body cavities as in severe pulmonary hypertension in backward failure of the right heart. This condition is generally avoided by modern diuretics, which blunt the inappropriate fluid retention by the kidneys in these patients [1]. By replacing backward with forward failure, these drugs have changes the major clinical manifestations of this syndrome. As a result, fatigue, rather than fluid retention, is generally the primary cause of disability in these patients [6].

Fatigue was once attributed to reduced perfusion of the skeletal muscle, now it is apparent that fatigue is not a direct consequence of low cardiac output. Instead, the major cause of this symptom is a skeletal muscle myopathy that, while initiated by forward failure, is due to structural and molecular changes in these muscles. Skeletal muscle abnormalities include atrophy, rapid appearance of acidosis during exercises, accelerated phosphocreatine consumption, loss of mitochondria and oxidative enzymes, changes in fiber type and alteration in myosin isoforms [6,7].

1.1.4 Incidence, Prognosis, and Mortality

The incidence of heart failure has increased due, in part, to the aging population and improvements in the diagnosis and treatment of myocardial infarction, major cause of heart failure in the developed countries [8]. In Canada, heart failure affects 1-2% of the population [9]. New York heart association classified heart failure patients into 4 classes depending on the severity of the disease. First class includes HF patients with ability to do physical activity. Class II includes HF patients who feel fatigue, palpitations, dyspnea or angina during exercise but not at rest. Class III consists of HF patients who feel discomfort even during their ordinary physical activity but not at rest. Class IV includes HF patients who feel discomfort at rest and during a

minimal physical activity [3]. Survival after the onset of symptoms in most clinical trials of therapy for heart failure is short[1]. Longer periods of time, however, often elapse between the onset of cardiac overloading and the appearance of clinical syndrome that is recognized as heart failure. Patients with a modest hemodynamic overload can probably remain asymptomatic for many years, often more than a decade [1,2]. However, once symptoms develop, average survival is about 5 years [1]. The 1-year mortality rates in New York class III/IV heart failure patients range from 11 to 44% [9].

In developed countries, where left ventricular damage is caused by myocardial infarction, survival can be predicted from the severity of the left ventricular dysfunction, which in these patients is readily estimated by measuring the ejection fraction (systolic dysfunction). In the usual clinical trial that enrolls patients with low ejection fraction, who are generally men with an average age of 60 years, 50% survival rate is about 5 years. Diastolic dysfunction is common in elderly women, aged 70 years and older, whereas systolic dysfunction is more seen in men whose average age is about 60years. Survival rate is better in patients with diastolic dysfunction than in those with systolic dysfunction, and in women than in men [1].

1.2 Progression from compensation to failure

The development of heart failure starts by a compensatory phase in which cardiac remodeling occurs. Cardiac remodeling is defined as "genome expression, molecular, cellular, and interstitial changes that are manifested clinically as changes in size, shape, function of the heart after an injury" [10]. The process of cardiac remodeling is influenced by hemodynamic load, neurohormonal activation, hypertrophy of cardiac myocytes, presence of cytokines, cell necrosis and apoptosis, and fibrosis. Cardiac remodeling has been described as both an adaptive and

maladaptive process, with the adaptive component enabling the heart to maintain function in response to pressure or volume overloading in the acute phase of cardiac injury. Increments in load, such as those seen in mitral insufficiency, modulate remodeling of the ventricle to maintain forward flow often after cardiac injury such as myocardial infarction, continued remodeling may be viewed as an adverse phenomenon that leads to progressive decompensation. Progressive remodeling can always be considered deleterious and is associated with poor prognosis. There are no data to indicate the transition from possible adaptive to maladaptive remodeling occurs or how this might be identified in patients. The occurrence of such transition and its time course may be expected to vary greatly. However, once established beyond a certain phase, it is likely that remodeling actually contributes to the progression of heart failure [10]. Thus, the adaptive mechanisms that develop early in cardiac remodeling in response to an initial insult or injury will ultimately lead to an unnatural growth process of the heart, a hypertrophic reaction to altered loading conditions, and expression of genetic programs that are largely designed to maintain robust growth of both cardiac myocytes and the interstitial matrix. The accumulation of interstitial matrix leads to heightened chamber stiffness compromising the performance of the heart and contributing to arrhythmogenesis [3].

In the following sections, I will discuss the role of mitogen- activated protein kinases (MAP kinases), cytokines and neurohormonal factors in cardiac remodeling during development of heart failure.

1.2.1 Role of MAPK

MAPK or mitogen activated protein kinases, are serine threonine protein kinases that play pivotal roles in a variety of cell functions in many cell types. These enzymes are phosphorylated,

in response to growth-promoting stimuli or cellular stresses such as hyperosmotic shock, inflammation, ischemia, and presence of endotoxins or genotoxic stress [11,12]. Once phosphorylated, they cross through large pores in the nuclear membrane and phosphorylate, and therefore modulate the activity of nuclear transcription factors that modify gene expression. The vast majority of defined substrates for MAPK are transcription factors. However, MAPK have the ability to phosphorylate many other substrates including other protein kinases, phospholipases, and cytoskeleton-associated proteins. Among the large family of MAPKs, there are three important subfamilies consisting of ERK1/2, P38 and SAPK. ERK1/2 (extracellular signal regulated protein kinase) is also called p44/42, which corresponds to their molecular weights (44kDa and 42kDa) respectively. SAPK (stress-activated protein kinase) is also called p54/46, or JNK (c-Jun NH2-terminal protein kinase) after one of its substrates, c-Jun. P38 corresponds to its molecular weight, which is 38kDa [11, 12].

The MAPK signaling can be initiated in the heart by G protein coupled receptors (angiotensin II, endothelin-1, and adrenergic receptors), receptor tyrosine kinases (insulin-like growth receptor, PDGF receptor, EGF receptor, and fibroblast factor receptor), peptide growth factors, cell adhesion molecules, neurohormonal mediators, and inflammatory cytokines[11]. Common to MAP kinase pathways is activation of a serine/threonine kinase often referred to as *MAP kinase kinase kinase or MEKK or sometimes abbreviated as MKKK.* This name refers to the biological role of this protein kinase, which is to phosphorylate another kinase, called *MAP kinase kinase or MEK or MKK.* The latter then phosphorylates a third kinase, *MAP Kinase,* which crosses the nuclear membrane to regulate gene expression. Different *MAP kinase kinase kinases*, *MAP kinase kinases* are involved in activation of ERK1/2, P38 and SAPK MAP kinases (Figure 1).

ERK MAP kinase mediates signals that stimulate cell growth and proliferation, which are generally initiated by ligand binding to tyrosine kinase receptors. This causes the receptors to form an aggregate that, by activating their latent tyrosine kinase activity, autophosphorylates the receptor. This phosphorylation reaction begins a series of aggregations, in which signaling proteins bind to one another. Autophosphorylation allows the receptors first to bind adaptor proteins such as Shc and Grb2. The result is a multiprotein aggregate that is anchored along the inner surface of the plasma membrane. This receptor-adaptor protein aggregate then interacts with Sos, which activates Ras by exchanging its bound GDP for GTP. Many other signaling cascades can activate Ras, which is central to many systems, which control growth and proliferation [12].

The next reaction in the MAP kinase pathway occurs when the Ras-GTP complex activates a MAP kinase kinase kinase MEKK such as Raf-1. The activated Raf-1 then phosphorylates and activates a MAP kinase kinase called MEK-1and MEK-2 (MAP Kinase /ERK kinase). Activated MEK-1/2 phosphorylates ERK1/2 MAP kinase, which then translocates to the nucleus to phosphorylate literally dozens of different transcription factors, such as STAT (signal transducer and activator of transcription), Elk1, Ets1 and c-Myc, which regulate different gene expression [1,12]. Several cytoplasmic proteins have been shown to be substrates for ERK1/2 [13]. Proteins known to be phosphorylated by ERK1/2 include the S6 kinase p90, cytosolic phospholipase A_2, and the juxta membrane region of the EGF receptor. Several microtubule-associated proteins (MAP) are also substrates for ERK1/2, including MAP-1, MAP-2, MAP-4, and Tau [12,13].

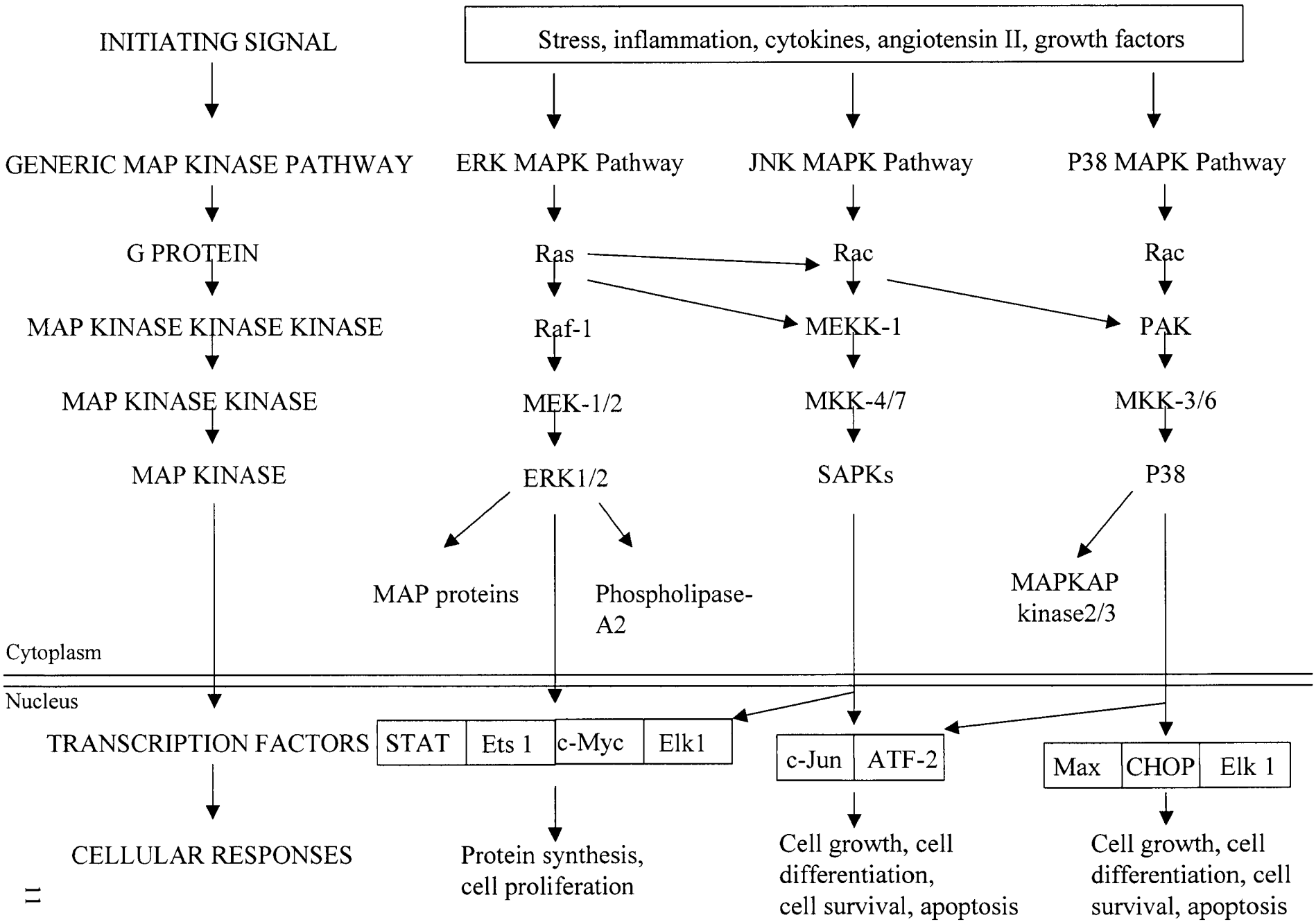
SAPK activation occurs in response to inflammatory cytokines, and to such stresses such as viral infection, radiation, and toxins. Rac, a low molecular weight GTP-binding protein of the Rho family that is related to Ras, activates SAPK. Rac activates MEKK-1 (MAP kinase kinase kinase); which is analogous to that of Raf-1. MEKK-1 then activates and phosphorylates SEK-1

or MKK-4 and MKK-7 (MAP kinase kinase), whose functions are analogous to that of MEK-1 and MEK-2. MKK-4 and MKK-7 then activate Jun kinase or JNK. JNK has many different substrates, which are exclusively transcription factors, such as ElK1, c-Jun, and ATF-2. Phosphorylation of c-Jun by JNK results in an increase in the formation of Jun/Jun homodimer and Jun/ATF2 heterodimers that transactivate at cAMP response element (CRE)-like consensus sequences within a variety of gene promoter regions (including that of c-Jun itself) [11, 12].

P38 has 2 abundant isoforms in the heart, p38α and p38β. Activation of p38, in general, occurs in response to cellular stresses including inflammatory cytokines, osmotic shock, and UV light. P38 is activated by Rac, which activates p-21 activated kinase (PAK), serine/threonine kinase. PAK activates and phosphorylates MKK-3, which activates p38α and MKK-6, which activates p38β. Activation of P38 by cellular stresses is associated with activation of the MAPK-activated protein (MAPKAP) kinase 2 and 3, which phosphorylates small heat shock proteins such as 25 and 27kDa heat shock proteins (Hsp25/27) [11]. MAPKAP kinase 2 may also directly regulate the activity of transcription factors (e.g. CRE binding protein) [12]. In the nucleus, P38 phosphorylates transcription factors such as ATF-2, Elk 1, CHOP (also known as GADD153), which is a member of c/EBP family of transcription factors, and Max, which forms heterodimers with c-Myc, a substrate for ERK1/2 MAP kinase [12].

1.2.1.1 MAPK and Cellular Stresses. JNK and P38 MAPK are sometimes referred to as "Stress-Responsive" MAPKs as they are activated by cellular stresses such as reactive oxygen species (ROS), hypoxia/reoxygenation, hyperosmotic shock, arsenite, and proinflammatory cytokines [11, 13].

Figure 1. Mitogen Activated Protein Kinases (MAPKs) Pathway



Probably the most relevant forms of cardiac stress in vivo are ischemia and ischemia/reperfusion, and it is clear that these stresses powerfully activate JNK and P38 MAP kinases in the intact isolated rat heart [14]. P38 MAPK and MAPKAPK2 are activated during ischemia, and their activation is sustained or increased during reperfusion. In contrast, JNK is not activated during global ischemia but is strongly activated during reperfusion phase [11].

Increased oxidative stress and production of reactive oxygen species (ROS), changes in homeostasis and metabolism and osmotic disturbances can be mediators of ischemia and/or ischemia/reperfusion. Studies have shown that oxidative stress, as exemplified by low concentrations of H_2O_2, activates JNK and P38 in perfused hearts and that DMSO (an OH^- scavenger) diminishes that activation [14].

It was shown that proinflammatory cytokines could induce activation of JNK and P38 and that inhibition of P38 by cytokine-suppressive anti-inflammatory drugs (CSAIDs) blocked the translation of the mRNA responsible for the expression of different cytokines including tumor necrosis factor α (TNF-α) [15].

1.2.1.2 MAPKs and Cardiac Hypertrophy. The hypertrophic response of the myocardium is an important pathophysiological adaptation that is associated with alterations in gene expression and cell morphology (increased sarcomeric assembly and myocyte profile).

G protein coupled receptor (GPCR) agonists such as angiotensin II, endothelin-1 (ET-1) and phenylephrine (PE), an α-adrenergic agonist, are powerful hypertrophic agonists in cultured neonatal cardiac myocytes and can activate JNK in cardiac myocytes [12] and in perfused rat heart [17]. It was shown that in response to these agonists, p38 and ERK1/2 become activated, as well, in cultured neonatal cardiac myocytes [11, 15] and perfused rat heart [16].

The role of ERK signaling in inducing cardiomyocyte hypertrophy is controversial. Several studies have reported that ERK signaling is necessary for effective endothelin-1 and phenylephrine induced cardiomyocyte hypertrophy [16]. Other studies with MEK1 inhibitor, PD908059, have suggested that a minimal or no requirement of ERKs in cardiac hypertrophy. One study even suggested that ERK activation in response to atrial natriuretic factor (ANF), a marker protein for cardiac hypertrophy, was associated with prevention of cardiomyocyte hypertrophy [15].

Several studies have shown that transfection of neonatal cardiac myocytes with constructs for constitutively active MEKK1 (a MEKK that preferentially activates the JNK pathway) in the presence or absence of MKK4 stimulates expression of ANF, β-myosin heavy chain, and skeletal muscle α-actin, which are associated with hypertrophy in the rat. Another study has shown that a dominant-negative JNK construct inhibits PE-induced ANF expression, further implicating the JNK cascade in the hypertrophic response [11]. A study by Izumi et al suggested that angiotensin II type 1 receptor is involved in the enhanced cardiac JNK activity in both the onset and the development of cardiac hypertrophy of hypertensive rats [17].

P38 MAPK has been proposed to affect cardiac hypertrophy. Transfection of neonatal cardiac myocytes with constitutively activated MKK3 or MKK6, both of which preferentially activate p38 MAPK, stimulates expression of ANF and skeletal muscle α-actin and increases myofibrillar assembly. Other studies have reported that the hypertrophic responses induced by PE, MKK3, or MKK6 are reduced by P38MAPK inhibitors [11].

1.2.1.3 MAPKs and Apoptosis. Apoptosis is one form of cell death. Cell death is an important determinant of cardiac remodeling because it causes a loss of contractile tissue,

compensatory hypertrophy of myocardial cells, and reparative fibrosis. Apoptosis in heart failure may be an important regulatory mechanism involved in the adaptive response to pressure overload in which initial apoptosis is linked to cardiac hypertrophy. As a result of altered loading conditions, the heart mass increases as the surviving myocytes become elongated or hypertrophied as part of an initial compensatory process to maintain stroke volume after the loss of contractile tissue. A reduction in the contractile machinery is a prominent feature in human cardiac remodeling whatever the origin and is accompanied by compensatory hypertrophy of myocytes and interstitial fibrosis [18].

Current classification of cell death is categorized in two separate entities, namely necrosis and apoptosis. In contrast to apoptosis, necrosis is characterized by the loss of energy–rich phosphates (creatine phosphate and ATP) and dysregulation of the transmembrane transport of solutes with secondary cell and organelle swelling. The rupture of intracellular and extracellular membranes finally leads to the demise of the myocyte and the release of reactive cellular contents, such as mitochondrial proteins, which evoke intense inflammatory and fibrotic reactions. As a result, cell death by necrosis evokes an inflammatory response, which is a promoter of fibrosis that occurs at end-stage heart failure and can lead to arrhythmias [19].

Defining the criteria for cell apoptosis include both morphological and biochemical markers that reflect a distinct pathophysiological mechanism of cell death. Morphologically, cells appear to be shrunken with intact membranes. At an advanced stage, several membrane blebs become visible that finally detaches to form individual apoptotic bodies. The nucleus diminishes in size and the chromatin condenses to high-density clumps with primarily marginal localization as evidenced by electron microscopy. At the biochemical level, the central feature of apoptosis is the activation of caspases, a distinct class of asparate-specific cysteine proteases, and the appearance of fragments of cellular target proteins for caspases (e.g. PARP, lamin,

fodrin). Secondary consequences of caspase activation are the exposure of phosphatidylserine on the outer leaflet of the plasma membrane and the internucleosomal cleavage of the genomic DNA. The latter feature is the basis for the two most widespread tests, TUNEL staining and the demonstration of DNA laddering on agarose gels, are used to provide evidence for apoptotic loss of cardiomyocytes in several myocardial diseases.

Apoptosis is associated with the expression of a number of regulatory genes commonly used as markers of apoptosis, including Bcl-2, an antiapoptotic gene, Fas, a proapoptotic gene, as well as others. In contrast to necrosis, apoptosis is commonly viewed by its requirement for de novo gene expression. Consequently, apoptosis is sensitive to mRNA or protein synthesis inhibitors. There is compelling evidence in support of reactive oxygen species (including superoxide and hydrogen peroxide)-induced apoptosis occurring not only during hypoxia or ischemia but also after overstretch and NO-induced apoptosis in the heart. Apoptosis plays an important role during development. During cardiac development, the rate of DNA fragmentation diminishes and is inversely related to bcl-2 levels [19,20].

Several studies have shown that ERK pathway may be protective or neutral against apoptosis. Insulin-like growth factor-1 (IGF-1), cardiotrophin-1 (CT-1), and catecholamines were each shown to exert their antiapoptotic effects, in part, by inducing ERK signaling [13]. In a model of ischemia/reperfusion in the intact heart, ERK 1/2 activation was shown to attenuate the amount of apoptosis subsequent to reperfusion injury [13]. Similarly, both baseline apoptosis and apoptosis induced by hydrogen peroxide were augmented by inhibition of MEK-1, the upstream regulator of ERK [21].

As for p38, overexpression of a constitutively active form of MKK3, the upstream activator of p38α, increased cell loss [22]. Furthermore, myocyte survival was improved by

overexpressing a dominant-negative mutant of the downstream kinase p38α, indicating a pro-apoptotic effect of MKK3/p38α pathway [22]. On the other hand, the signaling pathway involving MKK6 and p38β appears to protect against chemically induced apoptosis [23]. Interestingly, apoptosis and caspase activation were diminished by a non-specific p38 kinase inhibitor in an ischemia model in neonatal myocytes [24].

Similar to overexpression of MKK3, constitutively active SEK/MEKK1 (the upstream activator of JNK) induced apoptosis in isolated neonatal myocytes. Another study showed a correlation between JNK activation and apoptosis induced by ischemia/reperfusion in the rabbit heart [25]. While activation of ERK functions to protect cells from a variety of cellular stresses, activation of JNK and p38 MAPK induce apoptosis.

1.2.2 Role of Cytokines

Cytokines are a large family of peptides that includes inflammatory cytokines and peptide growth factors. Tumor necrosis factor α (TNF-α), interleukins, interferons, and lymphotoxins are examples of inflammatory cytokines [26]. Peptide growth factors include prolactin, erythropoietin, and transforming growth factor-β(TGFβ). In the following sections, we will discuss inflammatory cytokines in general, and TGFβ as an example of a peptide growth factor.

1.2.2.1 Inflammatory Cytokines

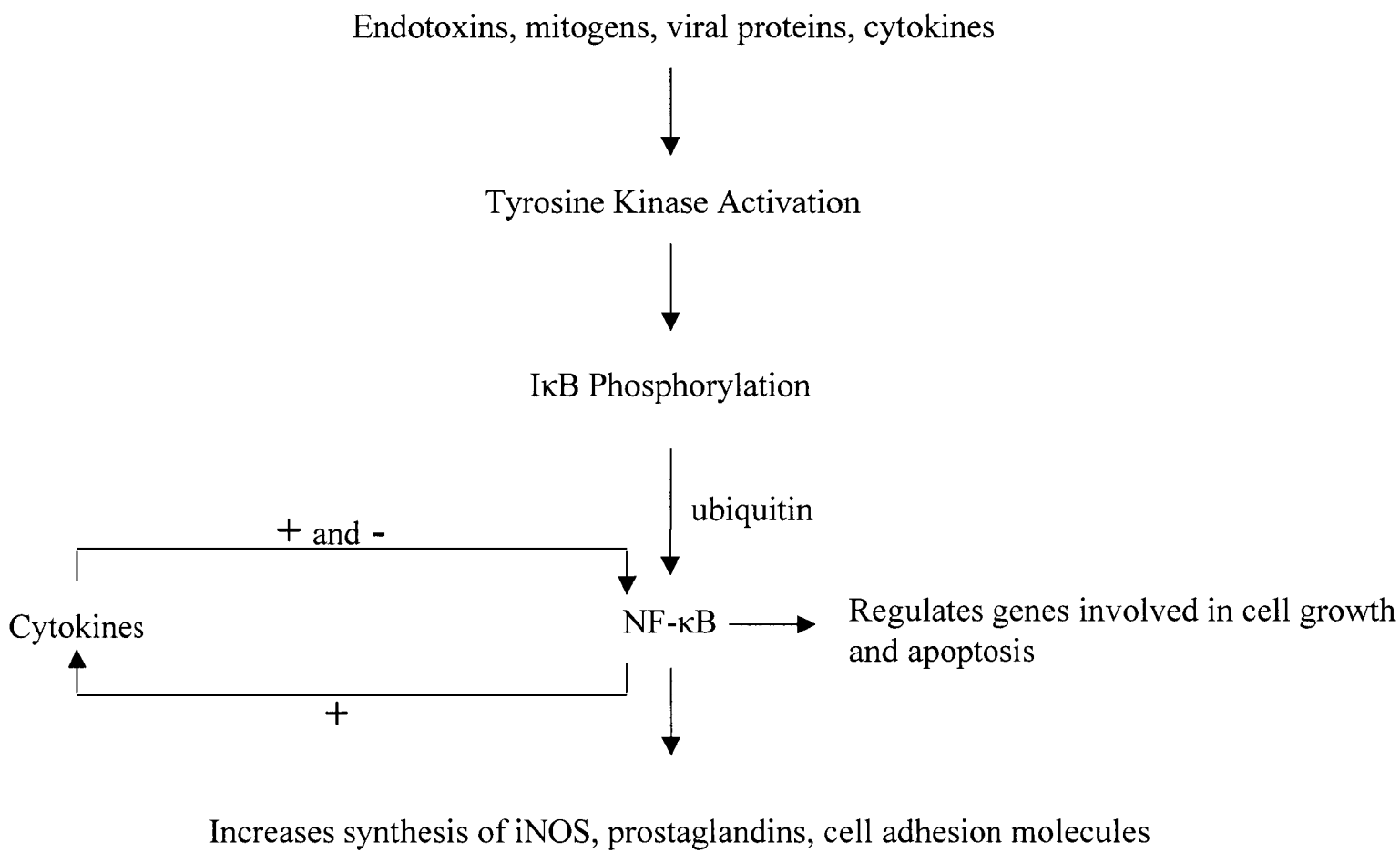
The biologic functions of cytokines extend far beyond their participation in inflammation. Functional diversity is even seen in a single cytokine; for example, TNF-α can activate members of a family of stress-activated protein kinases that have both growth promoting effects and the ability to initiate programmed cell death [27,28].

Control of cytokine synthesis: Inflammatory cytokines can be produced by both mononuclear cells that are attracted to sites of inflammation and by a variety of cell types, including cardiac myocytes. Cytokines are released by proteolytic cleavage of a protein precursor at the plasma membrane. Synthesis of these precursors is regulated by specific transcription factors that activate the genes that encode these peptides [26, 29].

Among the most important of the transcription factors that regulate cytokine production is NF-κB, which also stimulates the production of additional proteins that participate in immune and inflammatory responses. The latter include iNos, the inducible isoform of nitric oxide synthase, cyclooxygenase, which is important for prostaglandin synthesis, and cell adhesion molecules (CAMS) that attract leukocytes to areas of injury. In addition to its important role as an integrator of the inflammatory response, NF-κB regulates genes that are involved in cell growth and apoptosis [30,31].

The activity of NF-κB is stimulated by many factors, including endotoxins, mitogens, viral proteins, and the cytokines themselves. The latter activate NF-κB when they bind to their tyrosine kinase receptors. The stimulatory effect of cytokines is indirect, however, because NF-κB is not the substrate for these tyrosine kinases; instead, the substrate for phosphorylation is a protein called IκB. In its dephospho-form, IκB inhibits NF-κB. The ability of phosphorylation to reverse the inhibitory effects of IκB activates NF-κB. This occurs when phosphorylation allows IκB to bind ubiquitin in a reaction that leads to IκB degradation via proteolysis so as to reverse its inhibition of NF-κB activity. The liberated (activated) NF-κB then translocates to the nucleus, where it stimulates cytokine synthesis [29, 32] (Figure 2).

Figure 2. Activation of NF-κB and Cytokine Synthesis



The ability of cytokines to activate NF-κB and so to increase cytokine synthesis represents an example of a positive feedback in which a substance stimulates its own production. This amplification initiates a brief burst of cytokine activity. Additional negative feedback loops allow NF-κB to turn off cytokine production so as to prevent runaway signaling. Together, these tightly linked control mechanisms can shut down cytokine synthesis shortly after it is activated, even when the stimulating agents remain present [33].

Cytokine Receptors: Cytokine receptors are made up of an extracellular domain that contains the cytokine-binding site, and a membrane-spanning segment, and an intracellular (cytoplasmic) domain that transmits cytokine-induced signals to other proteins within the cytosol. The cytokine receptors, which lack intrinsic protein kinase activity, exert their regulatory efforts by activating tyrosine kinase reactions further downstream in their signaling cascades. The cytoplasmic domain of some cytokine receptors contains a 90 amino acid sequence that because it stimulates apoptosis, is sometimes refereed to as a "death domain" [26, 27].

The most important effect of the binding of a cytokine to its receptor is to cause the latter to aggregate, generally in trimers. In some cases, the active oligomers formed by the cytokine receptors recruit the coupling protein gp130 (also referred to as the β chain of the cytokine receptor), along with cytosolic tyrosine kinases. The aggregation of many cytokine receptors induces conformational changes in the cytoplasmic domain that cause the ligand-bound receptor to activate the intracellular tyrosine kinases, which mediate subsequent steps in their signaling cascades. Activated tyrosine kinases catalyze phosphorylation reactions that mediate down stream signaling; these cytokine-activated kinases also phosphorylate the cytokine receptors, gp130, and often the kinases themselves (autophosphorylation). These reactions generally amplify one another, and so provide additional examples of positive feedback loops. Termination

of these signals may occur when the phosphorylated proteins are dephosphorylated by special phosphatases, or by the actions of proteins that directly inhibit other steps in the cytokine-induced phosphorylation pathways [26, 27, 29, 32, 33].

Actions of Cytokines: Cytokines have the ability to protect the heart against ischemic injury, possibly by activating the synthesis of enzymes that destroy free oxygen radicals [34]. Cytokines expressed in ischemic and hypoxic myocardium also inhibit cell damage by increasing the expression of heat shock proteins in cardiac myocytes [35]. The ability of heat shock proteins to inhibit protein denaturation and stabilize cells in response to a variety of stresses, makes it likely that locally produced cytokines participate in adaptive, as well as maladaptive responses [35].

Another potentially beneficial consequence of cytokine production in the overloaded heart is the participation of these inflammatory mediators in signal transduction that stimulates cell growth [28]. Such a cytokine-mediated hypertrophic response would provide a beneficial short-term adaptation, although over the long-term, stimulation of mitogenic pathways eventually cause harm. Thus, although altered cytokine levels initially contribute to an adaptive growth response in the overloaded heart, with time, this response probably becomes maladaptive [36].

The cytotoxic effects of the cytokines are mediated in part by free radicals. These highly reactive chemical substances have been implicated as causes of ischemic and reperfusion injury, and have been suggested to contribute to negative inotropic effective (depressed myocardial contractility) as well as the progressive deterioration of the heart[31]. Because cells normally make small amounts of free radicals as byproducts of oxidative phosphorylation, the body has effective defenses that remove these toxic molecules. Another major source for these reactive compounds is nitric oxide (NO), a free radical gas that is formed by several different nitric oxide synthases. The low concentrations of NO produced by constitutive nitric oxide synthase (cNOS) mediate physiologic signaling and cause no damage. The much larger amounts of NO that are generated

by inducible nitric oxide synthase (iNOS) can result in toxic concentrations of reactive molecules such as nitrogen dioxide and peroxinitrites [37].

Inflammatory mediators increase the expression of iNOS. The large amounts of NO generated by iNOS contribute to the vasodilatation and depressed myocardial contractility seen in irreversible shock (delay in fluid replacement following hemorrhage leads to death even if the late transfusion restored the lost volume), activate NF-κB to increase cytokine production, and may contribute to a stimulus for cardiac myocyte hypertrophy [31, 37]. Although high concentrations of NO can be beneficial in attacking invading organisms, this inflammatory mediator also damages cells and can cause myocardial cell death [31, 32].

1.2.2.1.4 Cardiac Cachexia: A Systemic Inflammatory Response .The systemic inflammatory response, which attacks tissues through out the body, plays an important role in causing cardiac cachexia, a systemic wasting reaction that is due to mainly loss of adipose tissue that is especially severe in end-stage heart failure [1].

The modern era in understanding of cardiac cachexia began only a decade ago with the demonstration that circulating levels of TNF-α are elevated in the blood of patients with heart failure. This finding has been confirmed and extended by several reports that circulating levels of other cytokines are also elevated in heart failure, and that the extent of these elevations correlates with the severity of symptoms [30, 34, 36, 39].

1.2.2.1.5 Cardiac Cell Damage: A Local Inflammatory Response. A second feature of the inflammatory response in heart failure, in addition to the systemic response, occurs when cytokines are produced by the myocardium itself. In the beginning, this local response was found

to accompany inflammatory conditions such as viral infections of the heart and myocarditis. Later, elevated levels of cytokines in patients with dilated cardiomyopathy, whose clinical picture does not suggest viral or other infectious etiology, were detected indicating that these inflammatory mediators play a general role in heart failure [2,3]. Simple hemodynamic overloading can elevate levels of mRNA encoding TNF-α within 30 minutes after aortic banding in rat hearts, and within an hour, TNF-α protein levels increase 10-fold in both myocytes and nonmyocytes [38] indicating that this local inflammatory response is important in virtually all forms of heart failure.

Some cytokines such as interleukin-6 (IL-6) are synthesized by viable cardiac myocytes. Others such as IL-1β, which was found to be increased in chronic model of pressure overload, is produced mainly by macrophages suggesting that overload causes the myocardium to release chemotactic and other activating factors that recruit monocytes, which carry inflammatory mediators to the heart [39].

The likelihood that cytokines damage the failing heart is supported by the finding transgenic mice that overexpress TNF-α in their hearts exhibit a dilated cardiomyopathy that has many similarities to clinical heart failure [40] and that continuous infusion of TNF-α that achieve levels similar to those seen clinically can also cause a dilated cardiomyopathy [36] Now, it is acknowledged that heart failure is accompanied by a local inflammatory response in which cytokines are produced both by invading monocytes and locally by stressed cardiac myocytes [31, 36, 39].

1.2.2.2 TGFβ: A Peptide Growth Factor

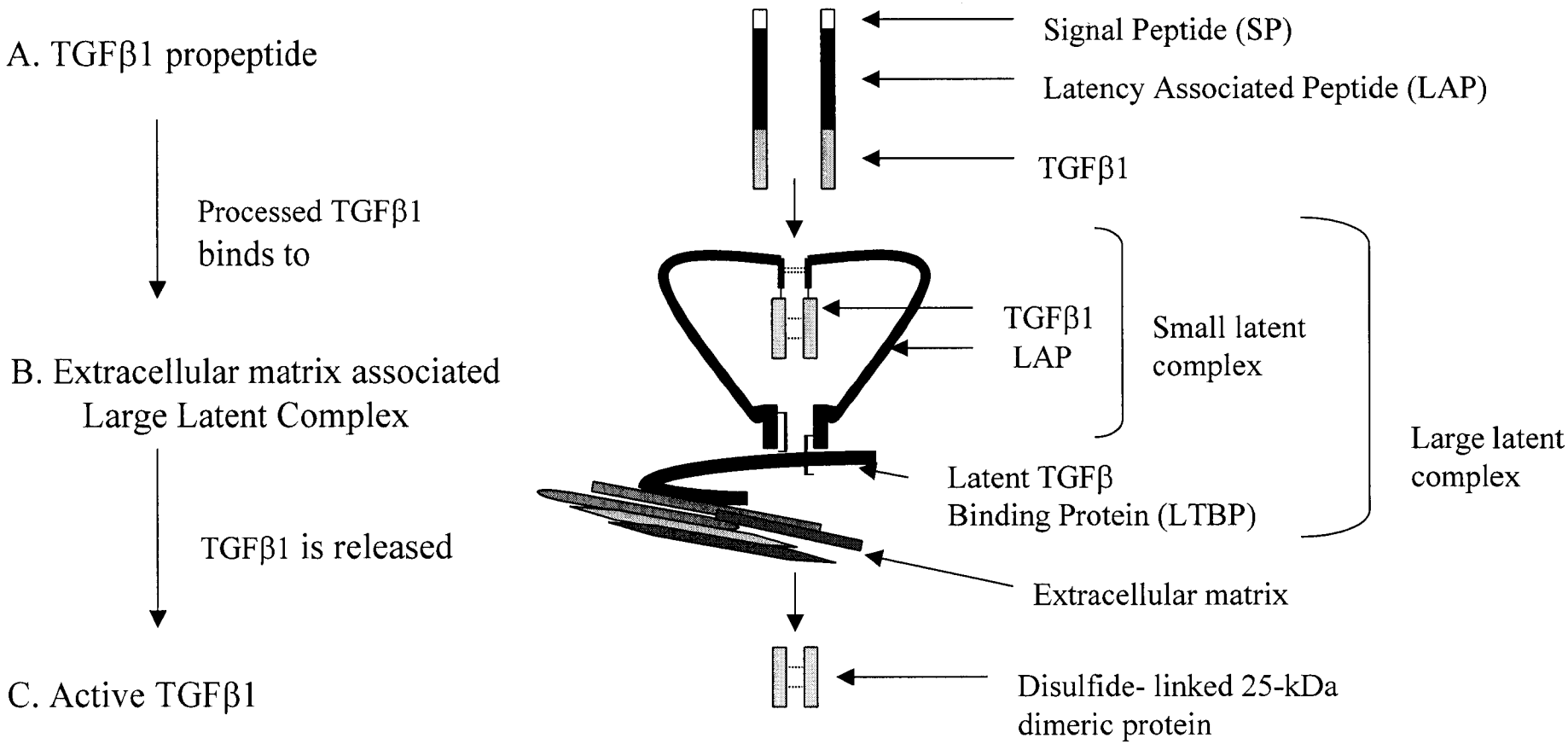
As mentioned before, the cytokine family is divided into inflammatory cytokines and peptide growth factors. Growth factors reach their receptors on the cell surface by way of the

extracellular fluid; their source can be distant cells (endocrine signaling), adjacent cells (paracrine signaling), or the same cells that produce the growth factor (autocrine signaling). The cellular effects of the peptide growth factors, like those of the inflammatory cytokines, begin when these peptides bind to membrane receptors. The ligand-bound receptors then form aggregates that activate either of two classes of protein kinase. In most cases, the substrate for these kinases is the amino acid tyrosine; a major exception is the TGFβ superfamily, which generally activates kinases that phosphorylate serine and threonine [1]. The following discussion highlights the actions of TGFβ and its effects on the heart.

TGFβ is a prototypical, multifunctional cytokine that was isolated from platelets and characterized 2 decades ago. The name is derived from the observation that TGFβ stimulates normal cells to grow in soft agar as though they had been virally transformed. In mammals the cytokine has 3 isoforms, TGFβ1, 2, and 3, whose biologic properties are nearly identical. The TGFβ1 gene is upregulated in response to tissue injury, and TGFβ1 is the isoform most implicated in fibrosis [41,42]. In this section the generic name TGFβ is used in discussing properties that are probably shared by all three isoforms; the specific isoform is mentioned, however, if it has been identified or used in a particular study.

TGFβ1 is disulfide- linked 25-kDa dimeric protein composed of 2 subunits linked by a disulfide bonds. It is synthesized as homodimeric propeptide, the precursor molecule, which is cleaved proteolytically to yield a dimeric peptide fragment called latency associated peptide (LAP) and the mature TGFβ1. This latency associated peptide remains noncovalenlty bound to the mature TGFβ1 rendering it latent and biologically inactive. This complex of LAP and mature TGFβ1 is called the small latent complex. The LAP binds to the latent TGF binding protein (LTBP), a member of the LTBP/fibrillin family, which binds to the extracellular matrix. Latent

Figure 3. Latent and Active TGFβ1



A. TGFβ1 is synthesized as homodimeric propeptide, which is cleaved proteolytically to yield a dimeric peptide fragment called latency associated peptide (LAP), the mature TGFβ1 and signaling peptide (SP). **B**. LAP remains noncovalenlty bound to the mature TGFβ1 forming small latent complex. The LAP binds to the latent TGF binding protein (LTBP),which binds to the extracellular matrix. TGFβ1 , LAP and LTBP form large latent complex. **C**.Active TGFβ1, a disulfide- linked 25-kDa dimeric protein

TGFβ1 is activated and all biological effects are exerted by active TGFβ1. Latent TGFβ1 is stored at cell surfaces and in the extracellular matrix and is converted to active TGFβ1 by transient acidification, cell-cell-interactions, and proteolytic processes [43, 44] (Figure 3).

TGFβ signaling depends on two classes of TGFβ receptors, called type I and type II, which operate together in forming aggregates that mediate signals initiated by the arrival TGFβ at the cell surface. There are type III receptors but they are membrane-anchored proteoglycans that have no signaling function but act to present TGFβ to other receptors. Type I and type II receptors include extracellular, membrane-spanning, and intracellular domains; both contain serine/threonine kinase catalytic activities, but only the type II receptors actually bind TGFβ [44, 45, 46].

TGFβ signaling begins when two of type II receptors bind to a homodimer formed by two TGFβ molecules. The tetrameric ligand-receptor complexes formed by the TGFβ dimer and the two type II receptors then recruit two type I receptors to form a heterohexamer that activates a latent serine/threonine kinase activity of the type II receptors. This stimulates type II receptors to phosphorylate the type I receptors, which activates the catalytic activity of type I receptors. This is a key to subsequent signaling by TGFβ because the activated type I receptors then catalyze a series of serine/threonine phosphorylations that propagate the TGFβ signal into the cell interior [44-46].

A major substrate for phosphorylation by the activated type I TGFβ receptors is a family of signaling transcription factors, called SMADS or MADS. Although each SMAD has a distinct function, all are composed of conserved amino- and carboxyl-terminal domains known as MH1 and MH2, respectively. Studies of the TGFβ signaling pathway have shown that upon activation of TGFβ1 type I receptor, SMAD2 and/or SMAD 3 transiently associate with the receptor and

are directly phosphorylated by the receptor kinase SMADs. The phosphorylated SMAD then forms a heteromeric complex with SMAD4, and this complex translocates from the cytoplasm to the nucleus, where they activate specific target genes through cooperative interactions with DNA and DNA-binding proteins [44-46], where they generally inhibit cell proliferation and induce fibrosis. By activating other substrates, TGFβ has the ability to inhibit cell proliferation and growth and to slow cell cycle [41, 42].

1.2.2.2.1 Role of TGFβ1 in wound healing and fibrosis. At the site of myocardial tissue injury, TGFβ1 is released from platelets, which contain high concentration of TGFβ1 and platelet –derived growth factor, and from inflammatory cells, mainly macrophages. Latent TGFβ1, bound locally to extracellular matrix, can also be activated after injury. In femtomolar concentrations, TGFβ1 is strongly chemotactic for neutrophils, T-cells, monocytes, and fibroblasts [47]. Moving to the site of injury, these cells become activated as they encounter higher (picomolar) concentration of TGFβ1. Monocytes begin secreting fibroblast growth factor, TNF-α, and IL-1, and fibroblasts increase their synthesis of extracellular matrix proteins such as collagens, fibronectin, and proteoglycans [39,41]. TGFβ1 inhibits the synthesis of the proteases for extracellular matrix proteins and stimulates the synthesis of protease inhibitors, so as to increase extracellular matrix deposition and prevents its degradation. TGFβ1 also increases the expression of cell-surface integrin receptors so that the cell-matrix interaction and matrix assembly are enhanced. The biologic effects of TGFβ1 are even amplified by autoinduction of its own production by these cells. TGFβ1 binds to proteoglycans in the matrix or near the cell surface and this binding may act to terminate the production of TGFβ1 after tissue repair is complete [41,42, 47, and 48].

During repeated injury, there is a continuous autoinduction of TGFβ1 leading to its overproduction and to a continuous production of extracellular matrix and subsequently, tissue fibrosis, which represents a pathologic extreme of the normal process of tissue repair. This overproduction of TGFβ1 overrides the normal termination signal and thereby perpetuates a vicious cycle of TGFβ1 overproduction leading to fibrosis [48].

Myocardial fibrosis is probably one of the major biological determinant of mortality in cardiac diseases including CHF, severe arrhythmias, and sudden death. In fibrosis, there is a greater collagen content, and a rigid type I collage replaces the more elastic type III collagen enhancing myocardial stiffness. Fibrosis can generate arrhythmias because it generates myocardial electrical heterogeneity, and hampers systolic ejection by rendering the myocardium heterogeneous [19,49,50].

It is common to identify two different types of fibrosis, namely, reparative and reactive fibrosis. Reparative fibrosis occurs as a reaction to a loss of myocardial material (due to cell death, after myocardial ischemia or senescence), and it is mainly interstitial. In contrast, reactive fibrosis is observed in the absence of cell loss as a reaction to inflammation and is primarily perivascular. Reactive fibrosis further extends into neighboring interstitial space. During cardiac remodeling, reactive and reparative fibrosis usually coexist [18].

Several stimuli have been suggested to increase the amount of fibrous tissue in the failing heart. One mechanism includes the action of many peptide growth factors that stimulate growth and proliferation, which can be secreted by myocytes and nonmyocytes. TGFβ1, a peptide growth factor as described earlier, is considered one of the major promoters of fibrosis. Angiotensin II provides another stimulus for fibrosis. This peptide acts directly to promote fibrosis [51], and indirectly to regulate the levels of additional signaling molecules such as TGFβ1

[52] that regulate tissue repair. Aldosterone, a steroid whose production is stimulated by the renin-angiotensin system, has been suggested to play a major role in stimulating fibrosis in the failing heart [55, 56]. Another mechanism that can lead to fibrosis in the failing heart is the inflammatory response. Locally produced cytokines can activate macrophages and monocytes, which then attract and activate fibroblasts. Perhaps the most important cause of this inflammatory response, and thus fibrosis of the failing heart, is necrosis, which evokes an inflammatory response that leads to a robust fibrotic reaction [47, 48].

1.2.3 Role of Neurohormonal Mediators

The neurohormonal mediators, a variety of chemical structures, are extracellular messengers that bind to specific receptors so as to modify intracellular signaling cascades not only in the heart but also in other organs and tissues, including the kidneys, blood vessels, and skeletal muscle. They are responsible for eliciting the hemodynamic defense reaction, whose main three components are salt and water retention, vasoconstriction, and cardiac stimulation [2, 3, 10]. Despite the persistence of injury or insult in the heart, the cardiac output and function heart can be maintained by compensatory mechanisms such as hypertrophy, neurohormonal factors, vasoconstriction, and fluid retention by the kidneys and heart dilatation. Although the ejection fraction continues to decline, the cardiac output and arterial pressure can be maintained by different mechanisms of adaptation. At the renal level, water and salt retention can help increase the cardiac output. The sympathetic system is activated by the baroreceptors and it stimulates, by the secretion of adrenaline and noradrenaline, an augmentation in the cardiac rhythm and contraction, and peripheral vasoconstriction [2, 3, 10]. The renin-angiotensin- aldosterone system is activated to induce both water retention and peripheral vasoconstriction [56]. Other hormones are

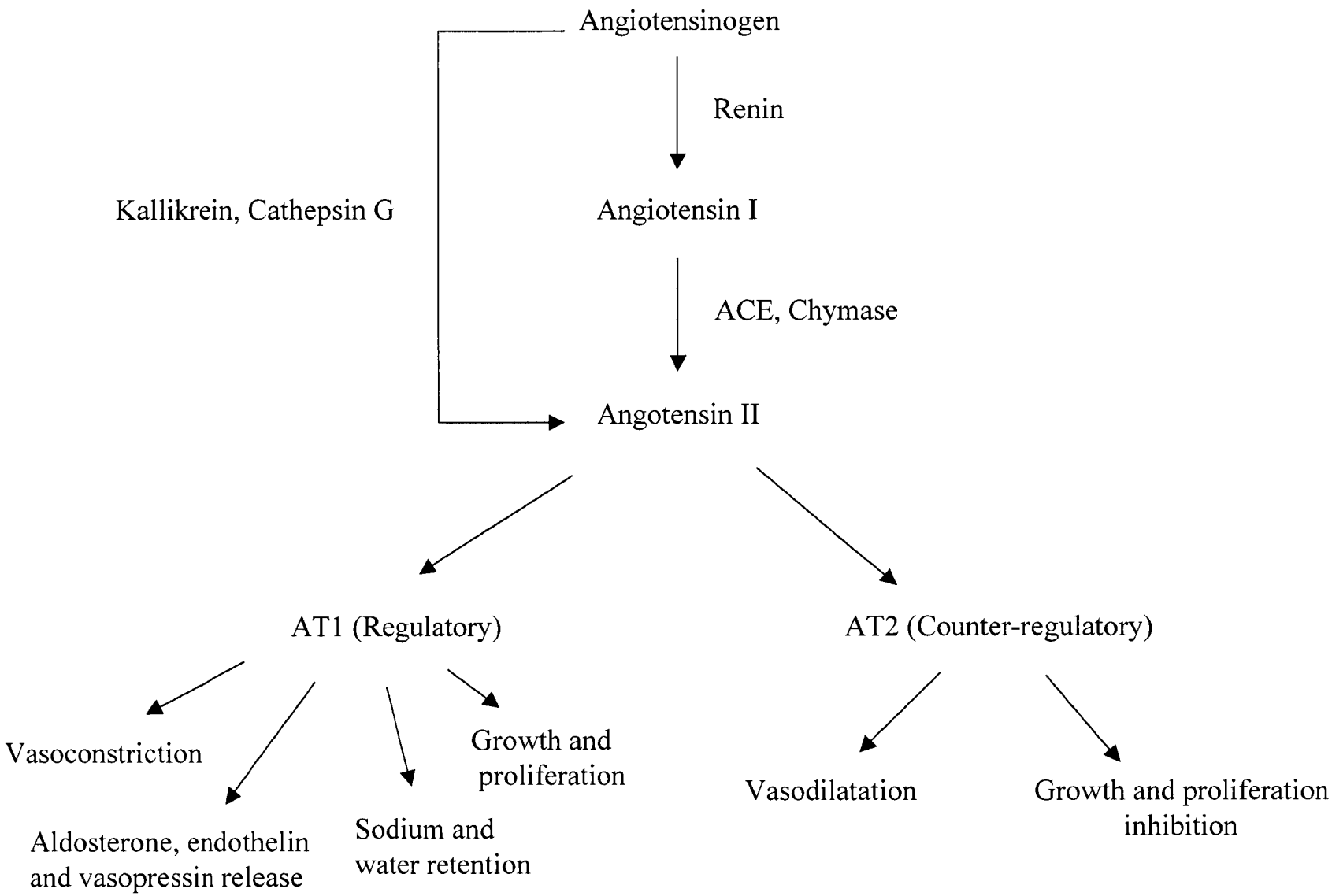
secreted in response to the hemodynamic imbalance such as vasopressin and endothelin. Vasopressin, an anti-diuretic hormone released by the hypothalamus in response in to the increase in the plasma osmolality, gives a feeling of thirst, induces vasoconstriction, and water retention by the kidneys. Endothelin expression is stimulated by the presence of other factors such as angiotensin II, catecholamines and vasopressin, which induce the release of its precursor, preproendothelin, which is cleaved into proendothelin and finally into endothelin. Endothelin induces peripheral vasoconstriction, increases myocardial contractility, stimulates aldosterone secretion, and inhibits sodium and water excretion by the kidneys [57].

In the long term, all these changes become highly maladaptive and responsible for progression of remodeling. In fact, augmentations in water and salt retention as well as peripheral vasoconstriction along with the elevated pressure increase the workload of the heart. In the long term, that will lead to the formation of edema and pulmonary congestion [2,3, 10].
In the following discussion, we will focus on angiotensin II and its effects in the failing heart.

1.2.3.1 Renin-Angiotensin System. Activation of the renin-angiotensin system plays a relatively minor role in the short-term response to exercise but it is of greater importance in restoring fluid volume after hemorrhage, which is an adaptive response. In heart failure, however, most of the long-term effects of angiotensin II are maladaptive [53, 54, and 56].

Two enzymatic cleavages convert the inactive precursor angiotensinogen into the active octapeptide angiotensin II. The first, proteolysis by renin, releases the decapeptide angiotensin I. Additional proteolytic reactions, notably that catalyzed by an enzyme generally referred to as angiotensin-converting enzyme (ACE), generates the octapeptide angiotenisn II. Angiotensin II can also be released from angiotensin I by chymase and synthesized directly from angiotensinogen by the proteolytic actions of kallikrein and cathepsin G. Furthermore,

Figure 4. Activation of Angiotensin II and its Receptor Subtypes

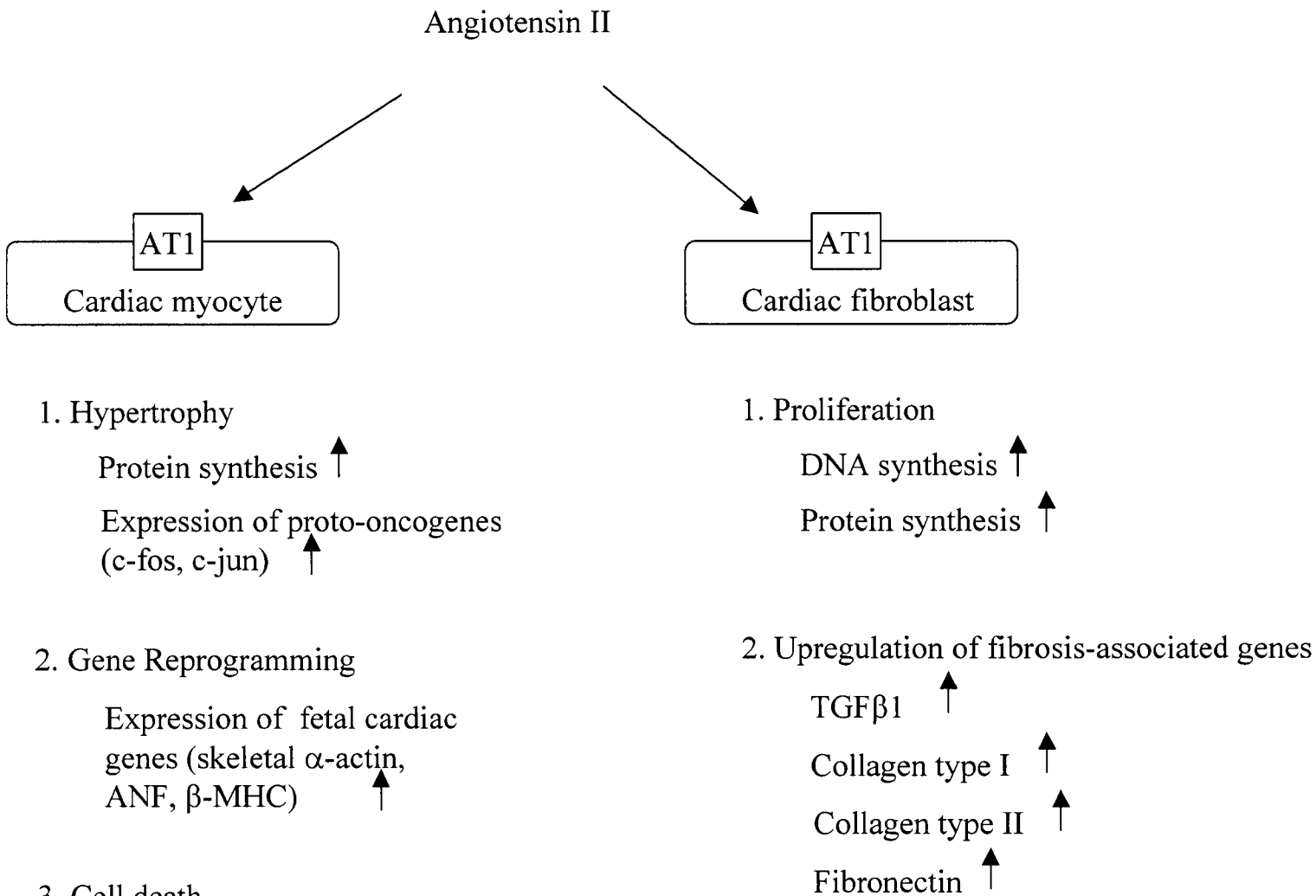


angiotensin II can undergo further proteolysis to generate additional biologically active peptides [56] (Figure 4).

Angiotensin II has 4 types of receptors: AT1, AT2, AT3, and AT4. The AT1 receptors, which predominate in the adult heart, are 7- transmembrane- spanning, G protein-coupled receptors that lack intrinsic tyrosine kinase activity. AT1 receptor is further subdivided into $AT1_A$ and $AT1_B$, which are similar with regard to amino acid sequence, pharmacological properties and tissue distribution patterns. AT1 receptor is ubiquitously and abundantly distributed in adult tissues, including blood vessel, heart, kidney, adrenal gland, liver, brain and lung. AT1 receptor mediates all the classic well-known effects of angiotensin II, such as elevation of blood pressure, vasoconstriction, aldosterone release from the adrenal gland, and renal sodium and water absorption (Figure 4). The AT1 receptors in the heart can be viewed as activator receptors; they increase in cardiac contractility, cause tissue fibrosis, stimulate cardiac myocyte hypertrophy and promote necrosis. The AT2 receptor is ubiquitously expressed in developing fetal tissues, suggesting a possible role in fetal development and organ morphogenesis. In contrast, AT2 receptor expression rapidly decreases after birth, and in the adult, expression of this receptor is limited mainly to the uterus, ovary, certain brain nuclei, heart and adrenal medulla. Angiotensin II activation of AT2 receptors generally has inhibitory effects that are opposite to those initiated by AT1 receptor activation; these include vasodilatation and growth inhibition (Figure 4). As for AT3 and AT4, their structures, their physiologic functions as well as their pharmacological properties remain poorly defined [56, 58].

Interestingly, Angiotensin II, via AT_1 receptor, activates a diversity of intracellular signaling cascades in neonatal rat cardiac myocytes, although the role of these signaling cascades in myocyte hypertrophy or gene reprogramming remains to be elucidated. Cardiac myocyte AT_1 receptor couples to a heterotrimeric G protein, G_q. As with other G protein-coupled receptors,

Figure 5. Actions of Angiotensin II in the Failing heart



Angiotensin II stimulates phosphatidylinositol-specific phospholipase C (PLC)-β isoform through G_q, and the activation of PLC causes increases in inositol triphosphate and diacylglycerol, which in turn lead to an increase in release of Ca^{2+} from intracellular stores and activation of protein kinase C (PKC), respectively [56]. Besides the above-mentioned basic cascades via G_q, it has been reported that Angiotensin II in neonatal rat cardiac myocytes activates tyrosine kinases (extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) [17, 59, 60] 70-kDa ribosomal S6 kinase (p70S6K), 90-kDa ribosomal S6 kinase (p90RSK) [61], p2lras [60], and phospholipases A_2 and D and increases phosphatidic acid and arachidonic acid [62].

Angiotensin II is a critical mediator of cellular changes associated with left ventricular hypertrophy, post myocardial infarction remodeling, and heart failure [54,56]. Angiotensin II can directly stimulate proliferation of cardiac fibroblasts and production of extracellular matrix proteins, such as collagen, which contributes to an increase in cardiac muscle stiffness and the development of diastolic dysfunction [19, 51, 59, and 63]. It increases the expression of mRNAs encoding for TGFβ1 [52] and there is cross talk between angiotensin II and SMAD proteins [64], which are phosphorylated by TGFβ1 and enter the nucleus where they generally inhibit cell proliferation and induce fibrosis [44-46] (Figure 5). In cardiac myocytes, angiotensin II induces expression of proto-oncogenes and fetal genes, and enhances protein synthesis leading to myocyte growth and hypertrophy [54, 63, 67]. It induces cell death [68] and increases the activity of MAPKs [17, 59, 60, 61], which play a role in cardiac hypertrophy [113, 17, 22] and apoptosis [21, 22, 25] (Figure 5).

Angiotensin II not only act on the target organs involved in the hemodynamic defense reaction but also regulates other mediators of the hemodynamic defense reaction. The latter interactions can also be of considerable clinical importance. For example, the ability of

angiotensin II to stimulate the secretion of aldosterone, vasopressin, catecholamines, and endothelin leads to regulatory amplifications that worsen many of the vicious cycles seen in heart failure patients [54, 56].

Chronic administration of ACE inhibitors, which prevents the conversion of angiotensin I to angiotensin II, or AT1 antagonists attenuates cardiac fibrosis, improves left ventricular function, prevents heart failure progression and improves survival in heart failure patients [19, 65, 66].

1.2.4 Experimental Models

Heart failure has been the topic of numerous studies, with different experimental models used. Heart failure can be induced subsequent to myocardial infarction. One approach used is ligature of the coronary artery, which generates ventricular ischemia and myocardial infarction, and after a longer period of time leads to development of heart failure [69]. In addition to ligature, other methods can be employed to induce myocardial infarction, such as coronary artery embolization with a catheter and microembolization by injection of microspheres. Heart failure can also be induced by pressure load, achieved by constriction of the pulmonary artery or the aorta, leading to stenosis in the pulmonary valve or the aortic valve, or by inducing hypertension by constricting the renal arteries. It is also possible to provoke volume loading, for example by creation of aorta-to-vena cava fistulae, aortic valve incompetence, and atrial septal defects [69]. Rapid ventricular pacing, achieved by implanting a pacemaker, is another approach for inducing heart failure. In the dog, for example, the tachycardia, induced by rapid pacing reaching 220 to 240 beats per minute for five weeks, leads to work overload and induces the development of progressive congestive heart failure [70]. Administration of drugs such as Adriamycin and streptozocin, induction of vitamin E deficiency or viral cardiomyopathy are all other approaches

used to develop a cardiomyopathy [69]. Finally, a large spectrum of transgenic mice, affecting a variety of important genes whether for the contractile system, neurohormones, or different molecules in the cellular signaling, were developed to identify the complicated pathological mechanisms implicated in the development of heart failure [71].

1.2.5 Atrial and Ventricular Remodeling in the Literature

There has been extensive investigation of the remodeling caused by CHF at the ventricular level. Much less information is available about the effects of CHF on atrial remodeling, despite the clinical importance of CHF as one of the most clinical causes of atrial fibrillation, most common sustained arrhythmia [72]. To date, Heinke et al [73] compared the number of apoptotic cells in the ventricular and atrial myocytes in ventricular tachypacing-induced heart failure in dogs. He found that the apoptotic ventricular myocytes in dogs subjected to 3-4 week of pacing is greater than apoptotic atrial myocytes. A study by Nakajima et al [74] has shown that overt fibrosis was observed only in the atria of TGFβ1-overexpressing mice, despite the presence of similar levels of active TGFβ1 in the transgenic atria and ventricles. Another study by Warnecke et al [75] has found that angiotensin type I receptor AT1 splice patterns differ distinctly between atria and ventricles and to a lesser degree between controls and failing hearts, suggesting differences in AT1 mRNA translation into protein in the various cardiac areas and under different pathophysiological conditions. Khadour et al [76] have reported that levels of endothelial NOS (eNOS) protein were enhanced in the left atria but not ventricles after 21 days of pacing, suggesting that NO production may be enhanced to counterbalance hypertrophy that develops during pacing-induced CHF and to participate in the hemodynamic defense reaction.

1.2.6 Arrhythmias in CHF

1.2.6.1Ventricular Arrhythmias. Many of the patients with CHF who die suddenly probably suffer a lethal ventricular arrhythmia. Ventricular arrhythmias are often divided into two classes: the bradyarrhythmias, in which the heart beats too slowly, and the tachyarrhythmias, in which ventricular rate is either too rapid to allow the heart to pump normally or else ventricular depolarization becomes so disorganized as to cause effective contractions to cease; the latter is ventricular fibrillation. Both of these arrhythmogenic mechanisms operate in the failing heart; both can cause death [77].

1.2.6.2 Atrial Arrhythmias. Congestive heart failure is one of the most common clinical causes of atrial fibrillation (AF), the most common sustained arrhythmia [72]. This arrhythmia is defined by a very rapid atrial rate (generally >400/min in humans) along with irregular atrial activation and a lack of a repetitive pattern of coordinated atrial activity on the electrocardiogram (ECG). AF is associated with a variety of complications, including thromboemboli resulting from coagulation in the relatively static atrial blood pool, a loss of the fine adjustment of ventricular rate to the body's precise metabolic needs, potential impairment of cardiac function (particularly if the ventricular response is rapid), and subjective symptoms like palpitations, dizziness, breathlessness, and chest pain [78, 79].

The prevalence of atrial fibrillation with a progressive degree of congestive heart failure is increasing, as judged by New York Heart Association functional class. Most evidence suggests that patients with heart failure and atrial fibrillation have a worse prognosis than patients with heart failure but no AF [78, 80]. Moreover, the presence of congestive heart failure has been identified as one of the most powerful independent predictors of atrial fibrillation, with a six-fold

increase in relative risk of its development. On the other hand, atrial fibrillation can cause or significantly aggravate symptoms of congestive heart failure in previously asymptomatic or well-compensated patients [72, 80]. In some patients, symptomatic dilated cardiomyopathy may develop over time entirely due to atrial fibrillation with rapid ventricular rates. Upon restoration of sinus rhythm, this type of "tachymyopathy" has been shown to be often reversible. Recent investigations of the physiologic and structural changes of the atrial myocardium ("electrical and structural remodeling") have shown that neurohumoral activation, fibrosis, and apoptosis are demonstrable with both diseases [19,50, 81]. On the other hand, experimental data suggest that experimental CHF strongly promotes the induction of sustained AF by causing interstitial fibrosis that interferes with local conduction [82, 83]. The substrates of AF in CHF are very different from those of atrial tachycardia–related AF, with important potential implications for understanding, treating, and preventing AF related to CHF [78, 80, 84].

Clinical studies show activation of the renin-angiotensin system and MAPKs, particularly ERK, in AF [85]. In canine CHF, atrial angiotensin concentrations are increased and MAPKs are activated [19, 50]. Enalapril attenuates these changes, reducing atrial fibrosis and AF promotion [19]. Transient activation of cell death pathways may also be important in the development of fibrosis [50].

1.2.6.2.1 Options for Therapy for patients with HF and AF. The continued cycle of HF predisposing to AF and AF exacerbating HF argues for interventions to break this cycle. Patients with severe heart failure and atrial fibrillation have reduced cardiac output and reduced peak exercise performance compared with patients with HF with sinus rhythm. This suggests that restoration or maintenance of sinus rhythm may contribute to improved survival. The data, however, to support interventions which maintain or restore sinus rhythm is lacking [78, 80].

Some randomized trials have demonstrated improved survival among patients with HF and AF who revert to sinus rhythm. For example, among patients enrolled in CHF-STAT, lower mortality was observed in patients with AF at baseline who converted to sinus rhythm on amiodarone compared with those who did not convert to sinus rhythm. Similarly, among the patients enrolled in the Danish Investigations of Arrhythmia and Mortality on Dofetilide (DIAMOND) congestive heart failure and myocardial infarction studies, restoration of sinus rhythm was associated with a significant reduction in mortality [78, 80].

Current therapeutic options for patients with HF with AF are varied. First-line options include rate control and anticoagulation or medical therapy to restore and maintain sinus rhythm. Quality of life improves with both rate control and rhythm control. Other therapies are available for patients who fail the initial management strategies. Atrioventricular node ablation and pacemaker implantation provide effective rate control and improve quality of life. Catheter ablation to restore and maintain sinus rhythm and implantable device-based therapies (pacing, pacemakers and implantable defibrillators with antitachycardia pacing and/or defibrillation therapies) also may be considered [80].

Our improved understanding of the pathophysiology of AF and HF and our increased understanding of their complex interactions may provide avenues for future interventions. For example, therapies that interfere with signal transduction or directly prevent ion channel alterations could prevent or reduce atrial remodeling [81]. The prevention of atrial remodeling may be useful to prevent development of AF substrate [50, 82, 84]. The effectiveness of angiotensin antagonism in reducing arrhythmogenic CHF-related atrial remodeling by reducing structural remodeling, namely interstitial fibrosis [82], may account for the efficacy of converting-enzyme inhibitors in preventing AF in myocardial infarction patients with left ventricular dysfunction [66].

Atrial histopathology in other AF-related clinical conditions is similar to that in experimental CHF, so angiotensin antagonism might have a broader-applicability in AF therapy [78]. The development of antiarrhythmia drugs specifically targeting the atrium may allow for antiarrhythmia therapy without the danger of ventricular proarrhythmia [80].

2. Questions raised and Hypothesis

Heart failure represents the final common pathway by which a number of disorders damage the heart so as to cause disability and premature death [1]. Heart failure, as well, begets atrial fibrillation, which is responsible for considerable morbidity and medical costs, and increased mortality in patients with congestive heart failure [8, 9]. It was previously shown that heart failure leads to structural remodeling in atria [82] and ventricles [10], therefore a pharmacological intervention that reduces this remodeling may prevent the progressive deterioration of the heart and the incidence of atrial fibrillation [50, 82]. Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical practice [81]. Ventricular tachypacing tachycardia (VTP)–induced Congestive heart failure (CHF) produces atrial interstitial fibrosis in dogs comparable to atrial pathology in clinical AF [82], and strongly promotes AF maintenance [19,50, 84]. The likelihood of AF increases with increasing extent of fibrosis, which follows a characteristic set of cellular responses [50]. Activation of renin-angiotensin system leads to cardiac fibrosis in hypertensive heart disease [17, 59], CHF, and myocardial infarction [19, 50, 63, 66, 85, 86]. Angiotensin II is an important mediator in CHF-induced structural remodeling [19, 50] and induces apoptosis in cardiomyocytes [68]. Apoptosis causes cell loss in CHF and may be a significant determinant of

prognosis. Preliminary observations from our lab have suggested that fibrosis may be greater in the atria than in the ventricles and that atrial apoptosis is transient and peaks at early stage of ventricular tachypacing in dogs (24hrs), however Cesselli et al [87] have shown that ventricular apoptosis is slow and progressive in the same model. These observations led us to propose the following questions: 1) is fibrosis different in atrial vs. ventricular remodeling in CHF? If so, 2) will cell death and apoptosis be different in atria vs. ventricles? 3) Is the inflammatory response different in atria vs. ventricles? 4) Are tissue angiotensin II and TGFβ1 implicated in atrial and ventricular remodeling in CHF? 5) Will studying the temporal evolution of CHF help us understand the underlying signaling mechanisms involved in atrial and ventricular remodeling?

Our working hypothesis was that there are differences in the mechanisms and consequences of atrial vs. ventricular remodeling in response to CHF, and this remodeling involves a series of events that implicates the renin-angiotensin system, cytokines, MAPKs, cell death and apoptosis, and finally development of fibrosis. To approach this hypothesis, we needed to determine the temporal evolution of molecular and histological changes in the atria and ventricles of dogs during ventricular tachypacing-induced congestive heart failure. Five groups of 6 dogs each were subjected to ventricular rapid pacing for 0, 24 hours, 1, 2, or 5 weeks. For each group, the atrial and ventricular tissues were examined separately. We determined the concentration of tissue angiotensin II by ELISA. The expression of the active form of TGFβ1 was measured by Western Blot. The expression of the phosphorylated (active) forms of P38, ERK1/2, and JNK MAPKs was evaluated by Western Blot. For detection of apoptosis we used TUNEL staining, a method of end labeling mediated by deoxynucleotidyl transferase enzyme. Finally, histological sections of the atria and ventricles were used to quantify fibrosis (Masson trichrome staining), the presence of inflammatory cells (HPS staining), and total cell death (HPS

staining) including both necrotic and apoptotic cardiac myocytes. The following article describes the results obtained and is submitted for revision to the journal Cardiovascular Research.

Differences in atrial versus ventricular remodeling in dogs with ventricular tachypaced-induced congestive heart failure

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Abstract

Nessrine Hanna, Sophie Cardin, Tack-Ki Leung, Stanley Nattel. Differences in atrial versus ventricular remodeling in dogs with ventricular tachypaced-induced congestive heart failure

Background: Congestive heart failure (CHF) causes arrhythmogenic remodeling in both atria and ventricles, but differences between atrial and ventricular remodeling in CHF have not been well characterized. **Methods and Results:** We examined atrial and ventricular tissues from dogs with CHF induced by ventricular tachypacing (220-240/min) for 0 (control) or 24 hours, or 1, 2, or 5 weeks. Histopathology was used to assess apoptosis, fibrosis, white blood cell infiltration and cell death, ELISA to measure angiotensin-II concentration and Western blot to evaluate protein expression. Ventricular tachypacing-induced CHF was associated with substantially more fibrosis in left atrium (maximum 10±1%, at 5 weeks) than in left ventricle (0.4±0.1% at 5 weeks, $P<0.01$ versus left atrium). Tissue angiotensin-II concentration increased to steady state in atrial tissue at 24 hours but increased more slowly in left ventricle, with a maximum that was significantly higher in atrium than ventricle. Ventricular tachypacing caused tissue apoptosis, inflammatory cell infiltration and cell death, with maximum changes in left atrium being faster, transient and larger than in left ventricle. Mitogen activated protein kinase activation (Western blot) was rapid (within 24 hours) in left atrium, but smaller and slower (p38, c-Jun N-terminal kinase) or non-significant (extracellular signal-related kinase) in left ventricle. The 25-kDa activated form of transforming growth factor β1, a particularly important profibrotic mediator in atrium, increased significantly (Western blot) in left atrium, from 2.6±0.6 (control) to 9.2±1.7 (24 hours) and 8.1±1.8 optical density units (1 week), but was not significantly changed in ventricle. **Conclusions:** There are qualitative and quantitative differences in atrial versus ventricular remodeling in experimental ventricular tachypacing-induced CHF, with potentially

important consequences for understanding underlying mechanisms and developing new therapeutic approaches.

Keywords: Arrhythmias; Heart failure; Remodeling; Angiotensin; Atrial fibrillation

1. Introduction

Myocardial remodeling is an important component of the congestive heart failure (CHF) syndrome [1]. Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical practice and CHF is one of the most important causes of AF [2]. Clinical AF is often associated with significant atrial interstitial fibrosis, and a similar pathological picture is produced, along with a susceptibility to the induction of long-lasting AF, by ventricular tachypacing-induced CHF in the dog [3]. Tachycardia-induced CHF is reversible upon cessation of tachypacing, and with reversal of CHF there is full recovery from CHF-associated atrial electrophysiological (4) and ionic (5) remodeling. However, atrial interstitial fibrosis persists (4, 5) and the ability to induce long-lasting AF remains, pointing to a central role for tissue fibrosis in the CHF-associated AF substrate. The likelihood of AF increases with increasing extent of fibrosis, which follows a characteristic set of cellular responses [6]. Ventricular tachypacing-induced CHF also induces ventricular remodeling [7], with some elements (e.g. development of apoptosis, chamber dilation, cell death) qualitatively similar to those observed with atrial remodeling, but also some apparent differences. We were unable to identify studies in the literature that compare the details of atrial versus ventricular remodeling in CHF. The present study was therefore designed to compare the time course and magnitude of changes in tissue angiotensin-II concentration, apoptosis, white-cell infiltration, cell death, fibrosis and activation of mitogen activated protein kinases and tumor-related growth-factor-β (TGFβ), with the use of matched left atrial and left ventricular samples from dogs with tachypacing-induced CHF. We have previously characterized a number of these

variables at the atrial level in the same model [6], but did not at the time plan to analyze ventricular changes and therefore had no stored ventricular tissues from the earlier study. We therefore performed all measurements at both the atrial and ventricular level for each dog in this new series of experimental animals, to eliminate potential contaminating effects of inter-animal, inter-investigator, seasonal and time-related differences on the results of the atrial versus ventricular remodeling comparison.

2. Materials and Methods

Five groups of mongrel dogs weighing 24-37 kg were subjected to ventricular tachypacing for: 0 (controls), 24 hours, 1, 2, and 5 weeks. Previously described techniques [3-6] were used to pace the right ventricle at 240 bpm for 3 weeks, with the rate then decreased to 220 bpm to limit mortality. On the study day, the pacemaker was deactivated. Dogs were then anaesthetized (morphine, 2 mg/kg sc; α-chloralose, 120 mg/kg i.v. loading dose, 29.25 mg/kg/h i.v. maintenance infusion) and ventilated. Body temperature was maintained at 37°C, and femoral arteries and veins were cannulated. A median sternotomy was performed and a fluid-filled catheter was used to obtain right atrial and left ventricular pressures. Dogs were sacrificed by α-chloralose overdose and hearts removed for subsequent analysis. For terminal-dUTP nick-end labeling (TUNEL) and histopathological studies, hearts were immersed into 10%-neutral buffered formalin and embedded in paraffin. For molecular biology analyses, hearts were snap-frozen in liquid nitrogen and kept at -80°C.

2.1. Histopathology

Lung wet weight to dry weight ratio was determined at autopsy as an indicator of total lung water. Left ventricular chamber diameter was also measured at autopsy. Samples were then taken from the left atrial posterior and inferior walls, and left ventricular anterior, lateral and posterior free walls, with 5-μm sections cut along longitudinal and transverse planes for each region. Since histopathological changes were found to be spatially consistent, results are presented as averages for all atrial and ventricular regions in each dog. Connective tissue content was quantified as previously described [3-6], with the use of SigmaScan (Jandel Scientific), as a percentage of surface area, excluding blood vessels, on Masson trichrome-stained sections. To analyze cell death, sections were stained with hematoxylin-phloxin-safran (HPS). Dead (acidophilic) and viable cells were counted in 15 transverse-section fields at 400 ×. HPS-stained longitudinal sections were used to quantify white-cell infiltration at high magnification (1000 ×) for 12 high-powered fields of each slide.

2.2. TUNEL Staining

Paraffin-embedded sections were deparaffinized, rehydrated, and saponified and marked with Biotin-dUTP via deoxynucleotidyl transferase (TdT, Boehringer-Mannheim). Slides were then incubated sequentially with 1:50 extravidin-fluoresceinisothiocyanate (FITC), 1:40 α-sarcomeric actin antibody, 1:100 tetramethylrhodamine (TRITC)-coupled anti-mouse IgG and propidium iodide to detect cardiomyocyte apoptosis.

2.3. Western blots

Protein extracts (200 μg) obtained as previously described [6, 8] were denatured and subjected to electrophoresis on 12 or 15% sodium dodecyl sulphate (SDS) polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (0.45 μm), blocked for 90 minutes with 5% nonfat dry milk in 0.1% Tween 80 / Tris-buffered saline and incubated overnight in primary antibody solutions. After 3 washes in 0.1% Tween 80 / Tris-buffered saline, membranes were incubated with horseradish peroxidase-conjugated primary antibodies in 5%- nonfat dry milk in Tween 80 / Tris-buffered saline for 90-120 minutes, followed by 4 washes in the same solution. Antibodies were detected with chemilumescence and quantified by laser densitometry. Each gel contained matched samples from control left atrium and ventricle, as well as from left atrium and ventricle from dogs with different durations of ventricular tachypacing, in order to ensure comparison between control and tachypaced dog tissues under identical handling and exposure conditions. Membranes were then stripped, re-blocked with 5%-nonfat dry milk for 2 hours and then re-probed with GAPDH as an internal standard. Values presented are ratios between target and GAPDH band density.

Primary antibodies were: rabbit anti-extracellular signal related kinase (ERK) polyclonal IgG, rabbit anti-p38 polyclonal IgG, rabbit anti-c-Jun N-terminal kinase (JNK) polyclonal IgG (Cell Signaling), rabbit anti-TGFß1 polyclonal IgG (Santa Cruz), and mouse anti-GAPDH (Research Diagnostics). Mitogen activated protein (MAP) kinases were probed with antibodies raised against phosphorylated forms, which detect only MAP kinases catalytically-activated by threonine- or tyrosine-phosphorylation, as well as with antibodies that detect total MAP kinase expression. No differences were seen in the response of 44 (ERK isoform 1) versus 42 (ERK isoform 2) kDa ERK and 54 versus 46 kDa JNK; therefore results are presented as average band densities of the 2 moieties.

2.4. Angiotensin-II

Tissue angiotensin-II concentration was measured by Enzyme-Linked ImmunoSorbent Assay (ELISA, Peninsula Laboratories) as previously described [8]. Absorbance at 450 nm was recorded, and concentration was calculated from a standard curve generated for each experiment.

2.5. Statistical analysis

All assays were based on separate parallel determinations for left ventricle versus left atrium in each heart, and no pooling of samples was used. Multiple group means were compared by analysis of variance. Dunnett's or Mann-Whitney U-tests were performed when the variance was homodecatic or heterodecatic respectively. Paired Student's *t*-test or Wilcoxon's signed-ranks test were used to compare mean left atrial with left ventricular values, based on data sets containing paired values obtained from matched tissue samples in each animal. Results are mean±S.E.M., and a 2-tailed $P<0.05$ was considered statistically significant.

3. Results

3.1. Time-dependent development of indices of CHF

Overall group characteristics and hemodynamic data are provided in Table 1. Indices reflecting left ventricular hypertrophy (increased heart weight/body weight ratio) and dilation (augmented left ventricular diameter) were evident by 1 week of ventricular tachypacing, whereas indices more reflective of decompensated CHF (elevated right atrial pressure, left ventricular end-diastolic pressure, increased lung wet weight/dry weight ratio) achieved statistically significant differences only at 5 weeks.

3.2. Tissue fibrosis

Under baseline conditions, very little fibrous tissue was apparent in either left atrial (Fig. 1A, left) or left ventricular (Fig. 1B, left) samples, whereas at 5 weeks there was clear tissue fibrosis (Fig. 1, right panels), which was particularly marked at the atrial level. Fibrous tissue content increased progressively during ventricular tachypacing in both atrium and ventricle (Fig. 1C), but the fibrous tissue content was substantially and significantly greater in left atrium than ventricle (e.g., at 5 weeks, 9.9±1.3% in left atrium versus 0.4±0.1% in left ventricle, $P<0.01$).

3.3. White cell infiltration

Figures 2A and 2B show HPS-stained left atrial and ventricular sections from a control (left) and 24-hour tachypaced (right) dog. After 24-hour ventricular tachypacing, the number of leukocytes in left atrial sections increased ~8-fold, accompanied by perceptible tissue edema and evident cell death. Changes in left ventricular tissue were subtler. The time course of leukocyte infiltration was different in atrial compared to ventricular tissue (Fig. 2C), with left atrium showing a rapid and transient ~10-fold rise to a peak value of 2.1±0.2 white blood cells/high-powered field at 24 hours, whereas in left ventricular tissue the leukocyte count showed smaller absolute changes (maximum 0.5±0.1 white blood cells/high-powered field at 1 week, $P<0.01$ versus maximum value in left atrium) but remained significantly increased over the entire 5-week ventricular tachypacing interval.

3.4. Cell death and apoptosis

Non-viable acidophilic cardiomyocytes were apparent as acidophilic, pink-staining cells in tissue sections from dogs exposed to ventricular tachypacing (Figs 2A, 2B, right panels). The

number of dead cells in left atrial sections peaked at 6.9±0.9/10^3 cardiomyocytes after 24-hour tachypacing (Fig. 3A), at which point atrial cell-death was much greater than ventricular, and decreased progressively thereafter. Cell-death count increases were slower but more sustained in left ventricular tissues, with a value (2.0±0.3/10^3 cardiomyocytes) at 5 weeks that was significantly greater than the atrial value at the same time point. The density of TUNEL-positive cells also increased transiently in left atrium, reaching a maximum of ~10-fold control at 24 hours, contrasting with a slower but more progressive increase in left ventricle (Fig. 3B).

3.5 Tissue angiotensin-II concentrations

Atrial angiotensin-II concentrations increased significantly after 24 hours of ventricular tachypacing, reaching ~4 times control values, and remained elevated thereafter (Fig. 4). Angiotensin-II concentrations increased to a lesser extent in left ventricle, with the increase achieving statistical significance only at 5 weeks, at ~2 times control values. Angiotensin-II concentrations were significantly greater in atrial than ventricular tissues at all tachypacing intervals studied.

3.6. MAP kinase expression

Figure 6 shows Western blots for phosphorylated and total MAPK expression in left atria and left ventricles in different pacing groups. Phosphorylated ERK expression in left atrium increased to a maximum after 24 hours and declined slowly thereafter (Fig. 6A). Ventricular tachypacing did not significantly affect phosphorylated ERK expression in left ventricular tissues. Phosphorylated JNK expression peaked at 24 hours in left atrium and then returned towards control values (Fig. 6B). A statistically significant increase in ventricular

phosphorylated JNK expression was only seen after 5 weeks of tachypacing. Unlike the transient changes in left atrial phosphorylated ERK and JNK expression, increases in atrial phosphorylated P38 expression were sustained over the full range of ventricular tachypacing intervals. There were no significant differences in left atrial versus left ventricular phosphorylated P38 expression over the observation period. Total ERK, p38, and JNK expression did not change significantly with ventricular tachypacing in either left atrium or ventricle (Figure 7).

3.7. TGFß1

Figure 8 shows Western blot data for TGFß1 expression. TGFβ exists in a membrane and matrix bound, inactive ~50-kDa form (in a complex including TGFβ1 and latency-associated peptide) and an activated ~25-kDa form [9]. Ventricular tachypacing increased the intensity of bands corresponding to the 25-kDa activated form in left atrial tissues (Fig. 6A), whereas changes in left ventricular bands were smaller. Left ventricular bands were generally less intense than left atrial following tachypacing (Fig. 6A). The time course of TGFβ1 changes varied somewhat among gels, with increases in atrial expression being clearest at 1 week in the example shown. Overall, mean 25-kDa TGFβ band densities increased ~3.5- and 3.1-fold at 24 hours and 1 week respectively in left atrial tissues (Fig. 6B), but changes in left ventricular samples were smaller and did not reach statistical significance. The density of the 50-kDa band was consistent over time, with no changes seen with tachypacing.

4.0. Discussion

In this study, we have compared directly atrial versus ventricular remodeling as a function of time in dogs with CHF induced by ventricular tachypacing. There were clear and consistent

differences between left atrial and left ventricular remodeling, with a variety of changes in left atrium (white cell infiltration, apoptosis, MAP kinase and TGFβ activation) being faster, quantitatively greater and more transient than in left ventricle, and with tissue fibrosis being much more intense in left atrial tissue.

4.1. Atrial versus ventricular remodeling in CHF

Many studies have considered ventricular remodeling in CHF (for an overview, see recent review by Gaballa and Goldman [10]. CHF-related atrial remodeling has great significance for the pathophysiology of AF and appears to be a useful paradigm for a broad range of clinical conditions in which AF is associated with structural remodeling [3]; however, there is relatively little information available about the development of atrial structural remodeling in experimental models. Patients with clinical AF and impaired left ventricular function have an increased rate of TUNEL-positivity indicating apoptosis, evidence of caspase-3 activation and of BCL-2 downregulation [11]. Atrial ERK activation [12] and changes in atrial angiotensin receptor expression suggestive of increased angiotensin-II stimulation (downregulation of angiotensin-1 and upregulation of angiotensin-2 receptors) [13] have been noted in AF patients. A variety of changes in atrial signaling systems have been noted in atrial samples from AF patients [14], but the extent to which the changes are due to AF per se, as opposed to being a consequence of underlying heart disease, is uncertain.

Atrial structural remodeling is prominent in ventricular tachypacing-induced experimental CHF, in which it appears to play a central role in the AF substrate and in associated atrial functional impairment [3-5, 15]. Both angiotensin-II dependent and independent pathways appear involved in the prominent atrial remodeling response [6], and there is evidence for a role

of matrix metalloproteinases [16,17]. Disintegrin metalloproteinases are also activated in CHF [18], and their expression is increased in atrial tissue samples from AF patients [19]. Activation of MAP kinase and apoptotic pathways are also demonstrable in animal models of CHF [6]. Inhibition of atrial apoptosis and ERK activation by converting enzyme inhibitors reduces, but does not eliminate, the development of atrial fibrosis, indicating the importance of additional pathways [6].

In the present study, we report for the first time detailed comparisons of atrial versus ventricular remodeling in experimental CHF induced by ventricular tachypacing, based on the analysis of measurements on matched atrial and ventricular tissue samples from each dog. Our results show important differences between atrial and ventricular tissue in the time course and magnitude of a variety of important remodeling-induced changes. A wide range of alterations, including leukocyte infiltration, cell death, apoptosis, and ERK and JNK activation, were larger in atrium, occurred earlier and showed a more transient profile compared to ventricle. These observations suggest a more acute and severe stress on the left atrium compared to the left ventricle. One possible explanation is that the thin-walled atrium is more severely affected than the ventricle by the acute hemodynamic stress imposed by severe ventricular tachyarrhythmia. In addition, tachypacing-induced CHF frequently causes at least moderate mitral regurgitation [3], which would cause an additional excess pressure load on the left atrium. The development of atrial fibrosis may help the atrium to adapt to these stresses by increasing atrial stiffness, possibly explaining the attenuation of changes in atrial cell death, leukocyte infiltration and apoptosis over time in parallel with the progressive development of atrial fibrosis. The relative lack of left ventricular fibrosis may be functionally important, because increased ventricular stiffness would be maladaptive in CHF. Although the maximum magnitude of a variety of responses were greater in the left atrium compared to the left ventricle, the transient nature of atrial changes

contrasted with more progressive and persistent alterations in the left ventricle, so that statistically significant increases in white cell infiltration, apoptosis, cell-death and phosphorylated JNK and p38 expression were noted in left ventricular but not atrial tissues after 5 weeks of tachypacing. Such progressive changes may contribute to continuing left ventricular deterioration in CHF.

MAP kinases play an important role in myocardial remodeling [20]. Recent work indicates that rabbits with 3-week tachypacing-induced CHF have increased ventricular expression of phosphorylated JNK and p38, but not of phosphorylated ERK [21]. We similarly failed to observe changes in ventricular ERK phosphorylation with ventricular tachypacing-induced CHF, but noted statistically significant increases in phosphorylated JNK and p38 expression at 5 weeks. In contrast, atrial phosphorylated ERK expression was significantly increased at 24 hours to 2 weeks after tachypacing onset, consistent with previous evidence for ERK activation at the atrial level in AF patients [12] and in previous studies of atrial tissues from dogs with CHF [6]. These results suggest that ERK activation may play a more important role in atrial than ventricular remodeling associated with CHF.

4.2. Potential significance of our findings

The prevention of atrial remodeling is becoming an interesting target for AF therapy, particularly given the serious potential side effects of atrial antiarrhythmic drugs [22]. Both angiotensin converting-enzyme inhibitors [6,8] and angiotensin receptor antagonists [23] reduce experimental CHF-related structural remodeling and AF promotion, and angiotensin converting enzyme inhibitors prevent the occurrence of AF in patients with left ventricular dysfunction [24,25]. Whereas a great deal of energy has been (appropriately) expended on defining the mechanisms of ventricular remodeling in CHF, much less attention has been paid to atrial

remodeling. A possible rationale for the lack of attention to atrial remodeling might be that it is unnecessary to specifically study atrial remodeling, if essentially the same mechanisms operate at the atrial and ventricular level. The present work demonstrates that this is not the case (i.e., that atrial and ventricular remodeling in CHF can differ both quantitatively and qualitatively), and that specific analyses of the mechanisms operative at the atrial level may be needed in order to understand atrial remodeling in detail and to identify potential novel therapeutic targets for the prevention of atrial structural remodeling and associated AF.

TGFβ is known to be an important profibrotic factor [26]. TGF-β1 mRNA concentrations are increased in human atrium by angiotensin-II [27]. Angiotensin receptor inhibition reduces TGFβ1 plasma concentrations and interstitial fibrosis in hypertensive kidney-transplant patients [28], and converting enzyme inhibition reduces TGFβ1 mRNA expression and ventricular fibrosis in a rat model of CHF [29]. Targeted cardiac TGFβ1 overexpression in mice produces prominent atrial fibrosis but no significant ventricular fibrosis, despite similar degrees of TGFβ1 overexpression in atria and ventricles [30]. Furthermore, TGFβ overexpressing mice show an enhanced susceptibility to AF [31]. Thus, TGF-β1 activation may be an important contributor to the development of AF-promoting atrial fibrosis in CHF and signaling events leading to increased TGFβ1 activity may be interesting targets for therapy to prevent arrhythmogenic atrial structural remodeling.

4.3. Potential limitations

The present study evaluated the time course of changes involved in atrial and ventricular remodeling in a well-defined experimental model of CHF, but extrapolation to clinical CHF should be cautious. The pathophysiology of CHF differs for different experimental and clinical

paradigms and, although sustained tachycardia can contribute to or cause clinical CHF [32], the pathophysiology of ventricular tachypacing-induced experimental CHF cannot be assumed to apply directly to clinical CHF. Studies comparing atrial and ventricular remodeling in other animal models of CHF would be of interest, as would analyses of the variables we studied in atrial and ventricular tissue samples from CHF patients. We have previously characterized the time course of atrial MAP kinase activation, leukocyte infiltration, apoptosis, and cell death caused by ventricular tachypacing-induced CHF in a different set of dogs [6]. All of these measurements were repeated in the animals used for the present study, however, because we felt that it was essential for an accurate atrial-ventricular comparison that all measurements be performed concurrently and in parallel with the use of left atrial and left ventricular tissues from the same animals. The changes observed in atrial tissue in the present study are in qualitative agreement with those noted in our earlier work. We also assessed additional indices in the present study that have not been previously evaluated in atrial remodeling, such as the expression of TGFβ1.

4.4. Conclusions

In this study, we compared atrial and ventricular remodeling in an experimental model of CHF, and found significant quantitative and qualitative differences. These differences may have important implications for understanding the mechanisms of cardiac remodeling in CHF and for the development of novel therapeutic approaches to the prevention of the development of the AF substrate.

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References

[1] Jessup M, Brozena S. Heart failure. N Engl J Med 2003;348:2007-2018.

[2] Ehrlich JR, Nattel S, Hohnloser SH. Atrial fibrillation and congestive heart failure: specific considerations at the intersection of two common and important cardiac disease sets. J Cardiovasc Electrophysiol 2002;13:399-405.

[3] Li D, Fareh S, Leung TK et al. Promotion of atrial fibrillation by heart failure in dogs: atrial remodeling of a different sort. Circulation 1999;100:87-95.

[4] Shinagawa K, Shi Y-F, Tardif JC, Leung T-K, **Nattel S**: Dynamic nature of the atrial fibrillation substrate during the development and reversal of heart failure in dogs. Circulation 105: 2672-2678, 2002.

[5] Cha TJ, Ehrlich JR, Zhang L, Shi YF, Tardif JC, Leung TK, **Nattel S**: Dissociation between ionic remodeling and ability to sustain atrial fibrillation during recovery from experimental congestive heart failure. Circulation (in press).

[6] Cardin S, Li D, Thorin-Trescases N, et al. Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and independent pathways. Cardiovasc Res 2003;60:315-325.

[7] Cesselli D, Jakonuik I, Barlucchi L et al. Oxidative stress-mediated cardiac cell death is a major determinant of ventricular dysfunction and failure in dog-dilated cardiomyopathy. Circ Res 2001;89:279-286.

[8] Li D, Shinagawa K, Pang L et al. Effects of angiotensin converting enzyme inhibition on the development of the atrial fibrillation substrate in dogs with ventricular tachypacing induced congestive heart failure. Circulation 2001;104:2608-2614.

[9] Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. J Cell Sci 2003;116:217-224.

[10] Gaballa MA, Goldman S. Ventricular remodeling in heart failure. J Card Fail. 2002;8(6 Suppl):S476-S485.

[11] Aime-Sempe C, Folliguet T, Rucker-Martin C et al. Myocardial cell death in fibrillating and dilated human right atria. J Am Coll Cardiol 1999;34:1577-1586.

[12] Goette A, Staack T, Rocken C et al. Increased expression of extracellular signal-regulated kinase and angiotensin-converting enzyme in human atria during atrial fibrillation. J Am Coll Cardiol 2000;35:1669-1677.

[13] Goette A, Arndt M, Rocken C et al. Regulation of angiotensin II receptor subtypes during atrial fibrillation in humans. Circulation 2000;101:2678-2681.

[14] Goette A, Lendeckel U, Klein HU. Calpains and cytokines in fibrillating human atria. Am J Physiol (Heart Circ Physiol) 2002;283:H264-H272.

[15] Shi Y, Ducharme A, Li D et al. Remodeling of atrial dimensions and emptying function in canine models of atrial fibrillation. Cardiovasc Res 2001;52:217-225.

[16] Boixel C, Fontaine V, Rucker-Martin C et al. Fibrosis of the left atria during progression of heart failure is associated with increased matrix metalloproteinases in the rat. J Am Coll Cardiol 2003;42:336-344.

[17] Hoit BD. Matrix metalloproteinases and atrial structural remodeling. J Am Coll Cardiol 2003;42:345-347.

[18] Hunt MJ, Aru GM, Hayden MR et al. Induction of oxidative stress and disintegrin metalloproteinase in human heart end-stage failure. Am J Physiol (Lung Cell Mol Physiol) 2002;283:L239-L245.

[19] Arndt M, Lendeckel U, Röcken C et al. Altered expression of ADAMs (A Disintegrin And Metalloproteinase) in fibrillating human atria. Circulation 2002;105:720-725.

[20] Ravingerova T, Barancik M, Strniskova M. Mitogen-activated protein kinases: a new therapeutic target in cardiac pathology. Mol Cell Biochem 2003;247:127-138.

[21] Schulz R, Aker S, Belosjorow S et al. Stress kinase phosphorylation is increased in pacing-induced heart failure in rabbits. Am J Physiol (Heart Circ Physiol) 2003;285:H2084-H2090.

[22] Nattel S. Therapeutic implications of atrial fibrillation mechanisms: can mechanistic insights be used to improve AF management? Cardiovasc Res 2002;54:347-360.

[23] Kumagai K, Nakashima H, Urata H et al. Effects of angiotensin II type 1 receptor antagonist on electrical and structural remodeling in atrial fibrillation. J Am Coll Cardiol 2003;41:2197-2204.

[24] Pedersen OD, Bagger H, Kober L et al. Trandolapril reduces the incidence of atrial fibrillation after acute myocardial infarction in patients with left ventricular dysfunction. Circulation 1999;100:376-380.

[25] Vermes E, Tardif JC, Bourassa MG et al. Enalapril decreases the incidence of atrial fibrillation in patients with left ventricular dysfunction: insight from the Studies Of Left Ventricular Dysfunction (SOLVD) trials. Circulation 2003;107:2926-2931.

[26] Roberts AB, Sporn MB, Assoian RK et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 1986;83:4167-4171.

[27] Kupfahl C, Pink D, Friedrich K et al. Angiotensin II directly increases transforming growth factor beta1 and osteopontin and indirectly affects collagen mRNA expression in the human heart. Cardiovasc Res 2000;46:463-475.

[28] el-Agroudy AE, Hassan NA, Foda MA et al. Effect of angiotensin II receptor blocker on plasma levels of TGF-beta 1 and interstitial fibrosis in hypertensive kidney transplant patients. Am J Nephrol 2003;23:300-306.

[29] Ma M, Watanabe K, Wahed MI et al. Inhibition of progression of heart failure and expression of TGF-beta 1 mRNA in rats with heart failure by the ACE inhibitor quinapril. J Cardiovasc Pharmacol 2001;38(Suppl 1):S51-S54.

[30] Nakajima H, Nakajima HO, Salcher O et al. Atrial but not ventricular fibrosis in mice expressing a mutant transforming growth factor-beta(1) transgene in the heart. Circ Res 2000;86:571-579.

[31] Olgin JE, Verheule S. Transgenic and knockout mouse models of atrial arrhythmias. Cardiovasc Res. 2002 May;54(2):280-6.

[32] Umana E, Solares CA, Alpert MA. Tachycardia-induced cardiomyopathy. Am J Med. 2003 Jan;114(1):51-5.

Figure Legends

Figure 1. A, Representative Masson trichrome stained sections of left atrial tissue from a control (CTL, left) and a 5-week ventricular tachypaced (5 W, right) dog. B, Representative Masson's trichrome stained sections of left ventricular tissue from the same control and 5-week tachypaced dogs as shown in A. C, Mean±S.E.M. connective tissue content (expressed as percentage of cross-sectional area) in left atrium and left ventricle in control, 24 hour (24 H), 1 (1 W), 2 (2 W), and 5 (5 W) week ventricular tachypaced dogs (n=6 dogs / ventricular tachypaced group, 5 for control). VTP=ventricular tachypacing.

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared to corresponding control group.

$^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ for left atrial versus left ventricular values at the corresponding time point.

Figure 2. A, HPS stained sections of representative left atrial sections from control (CTL) and 24-hour (24 H) ventricular tachypaced dogs. Arrows point to white blood cells (WBCs) and a dead cardiomyocyte. B, HPS stained sections of representative left ventricular sections from the same dogs as in A. C, Quantification of white blood cell infiltration based on mean number of white blood cells per high-powered field (HPF) in 12 sections each of left atrial and ventricular tissue per dog (mean±S.E.M., n=6 dogs / ventricular tachypaced group, 5 for control). VTP= ventricular tachypacing.

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared to corresponding control group.

$^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ for left atrial versus left ventricular values at the corresponding time point.

Figure 3. A, Cell death (quantified as the number of acidophilic cells / 1000 cardiomyocytes in 15 HPS stained sections at 400× magnification) in left atrium and left ventricle. B, Apoptosis in left atrium and left ventricle as quantified by the number of TUNEL-positive cells / 1000 cardiomyocytes. All data are mean±S.E.M., n=6 dogs / ventricular tachypaced group, 5 for control. VTP= ventricular tachypacing.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared to corresponding control group.
$^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ for left atrial versus left ventricular values at the corresponding time point.

Figure 4. Concentration of tissue angiotensin II in left atrium and left ventricle (pg of angiotensin-II per mg of cardiac tissue, by ELISA, mean±SEM, n=6 dogs / group). VTP= ventricular tachypacing.
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared to corresponding control group.
$^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ for left atrial versus left ventricular values at the corresponding time point.

Figure 5. Representative blots of phosphorylated and total MAPK. A, Phosphorylated ERK1/2 (average of 42 and 44 kDa band intensities) expression in left atrium and left ventricle. B, Total ERK1/2 in LA and LV. C, Phosphorylated JNK (based on average of 46 and 54 kDa band intensities) expression in LA and LV. D, Total JNK in LA and LV. E, Phosphorylated P38 expression in LA and LV. F. Total P38 in LA and LV.

Figure 6. Intensity of the phosphorylated MAPKs' bands. A, Phosphorylated ERK1/2 expression in LA and LV. B, Phosphorylated JNK expression in LA and LV.C, Phosphorylated P38 expression in LA and LV. All values are normalized to GAPDH

band intensity as measured on the same samples. All data are mean±S.E.M., n=6 dogs/ group. OD= optical density units.

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared to corresponding control group.

$^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ for left atrial versus left ventricular values at the corresponding time point.

Figure 7. Intensity of the total MAPKs' bands. A, Total ERK1/2 expression in LA and LV. B, Total JNK expression in LA and LV. C, Total P38 expression in LA and LV. No differences were seen in total MAP kinase expression. All values are normalized to GAPDH band intensity as measured on the same samples. All data are mean±S.E.M., n=6 dogs/ group. OD= optical density units.

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared to corresponding control group.

$^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ for left atrial versus left ventricular values at the corresponding time point.

Figure 8. A, Representative Western blots for TGF-ß1 showing the inactive, membrane-bound TGF-ß1 complex at ~50 kDa and active TGF-ß1 at 25 kDa. B, Mean±S.E.M. intensity of the 25-kDa bands (normalized to GAPDH) in left atrium and ventricle (n=6 dogs/group). OD= optical density units.

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ compared to corresponding control group.

$^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ for left atrial versus left ventricular values at the corresponding time point.

Table 1

Time-dependent development of indices of CHF

	CTL	24 hours	1 week	2 weeks	5 weeks
HR, bpm	87±17	89±4	90±4	84±6	107±14
HW/BW, %	0.64±0.02	0.72±0.06	0.89±0.01**	1.03±0.08**	1.00±0.03***
Lung ww/dw	3.6±0.3	4.0±0.2	5.4±1.1	5.6±0.4	6.9±0.9*
LVEDP, mmHg	5.9±0.6	5.9±0.3	6.1±0.6	7.0±0.8	18.6±2.8*
RAP, mmHg	3.0±0.4	3.9±0.5	4.3±0.6	3.6±0.3	5.9±0.9*
LV Diameter, cm	2.7±0.1	2.7±0.1	3.3±0.2*	3.4±0.1**	4.4±0.1***

HR, heart rate; HW/BW, heart weight as percentage of body weight; Lung ww/wt, lung wet weight o dry weight ratio; LVEDP, left ventricular end-diastolic pressure; RAP, right atrial pressure; LV, left ventricle.

*P<0.05, **P<0.01, ***P<0.001 versus CTL.

Figure 1

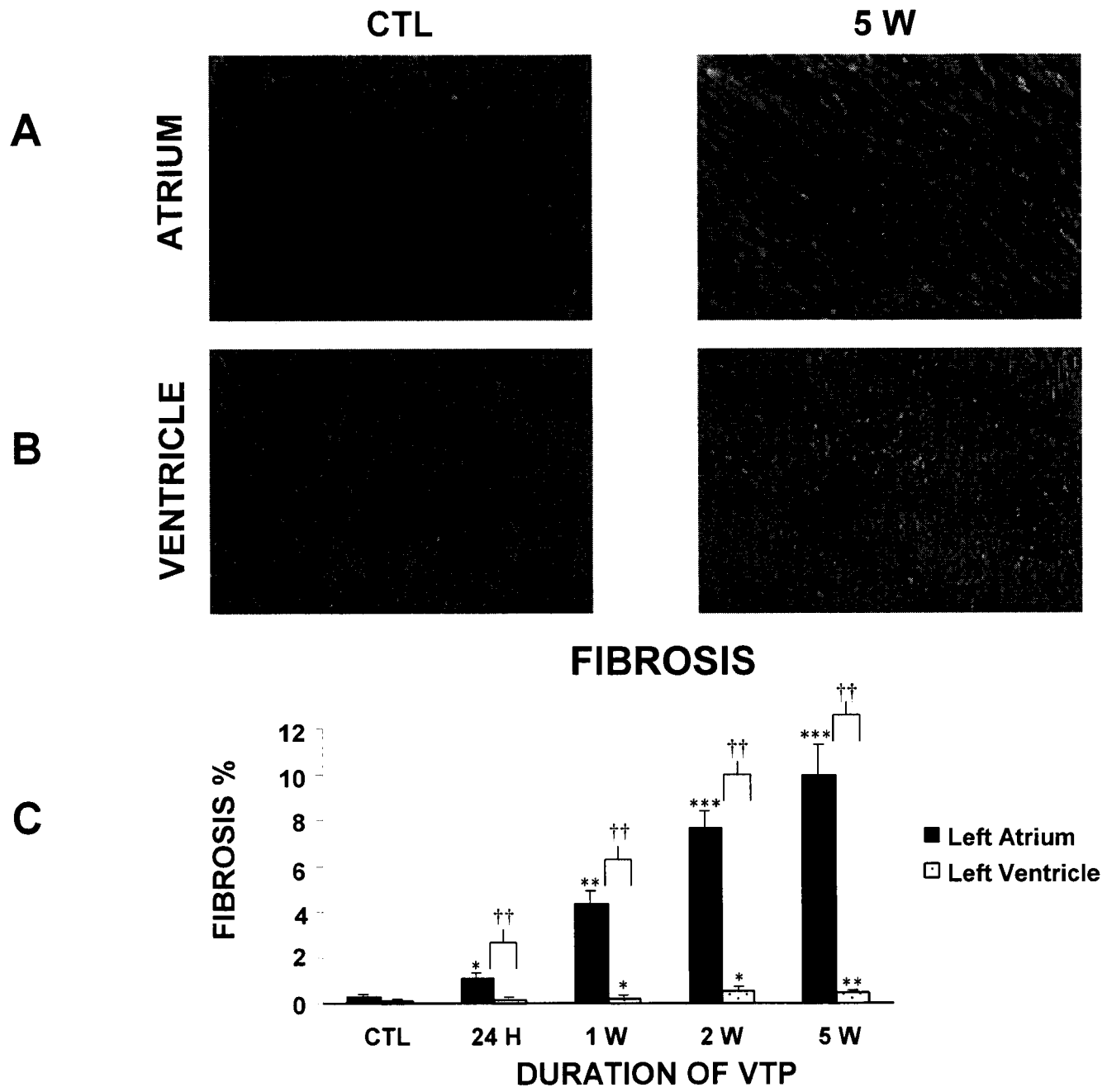

CTL
5 W
A
ATRIUM
B
VENTRICLE
C
FIBROSIS
FIBROSIS %
12
10
8
6
4
2
0
CTL
24 H
1 W
2 W
5 W
DURATION OF VTP
Left Atrium
Left Ventricle

Figure 2

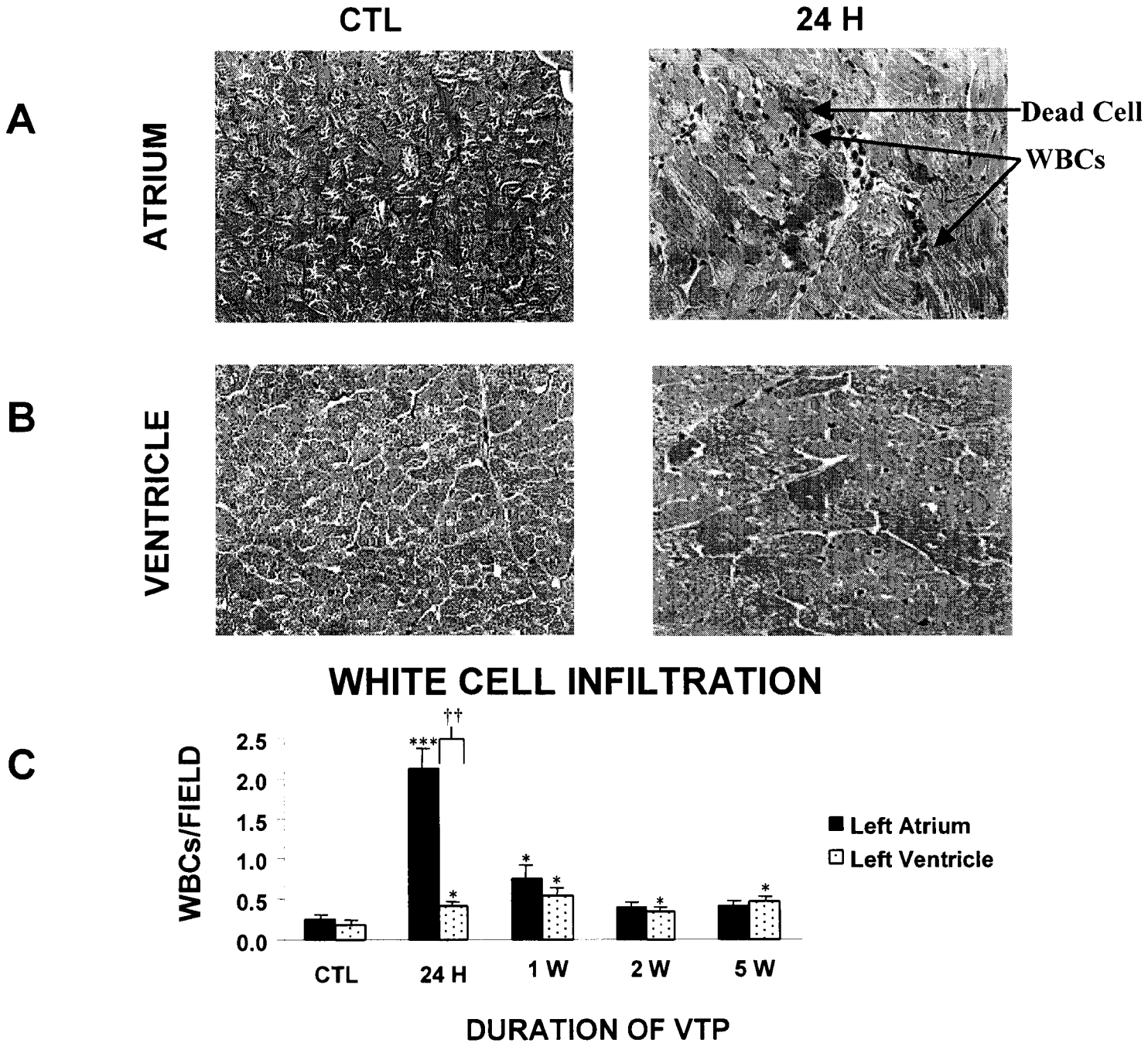

CTL
24 H
A
ATRIUM
Dead Cell
WBCs
B
VENTRICLE
WHITE CELL INFILTRATION
C
WBCs/FIELD
2.5
2.0
1.5
1.0
0.5
0.0
***
††
*
CTL
24 H
1 W
2 W
5 W
Left Atrium
Left Ventricle
DURATION OF VTP

Figure 3

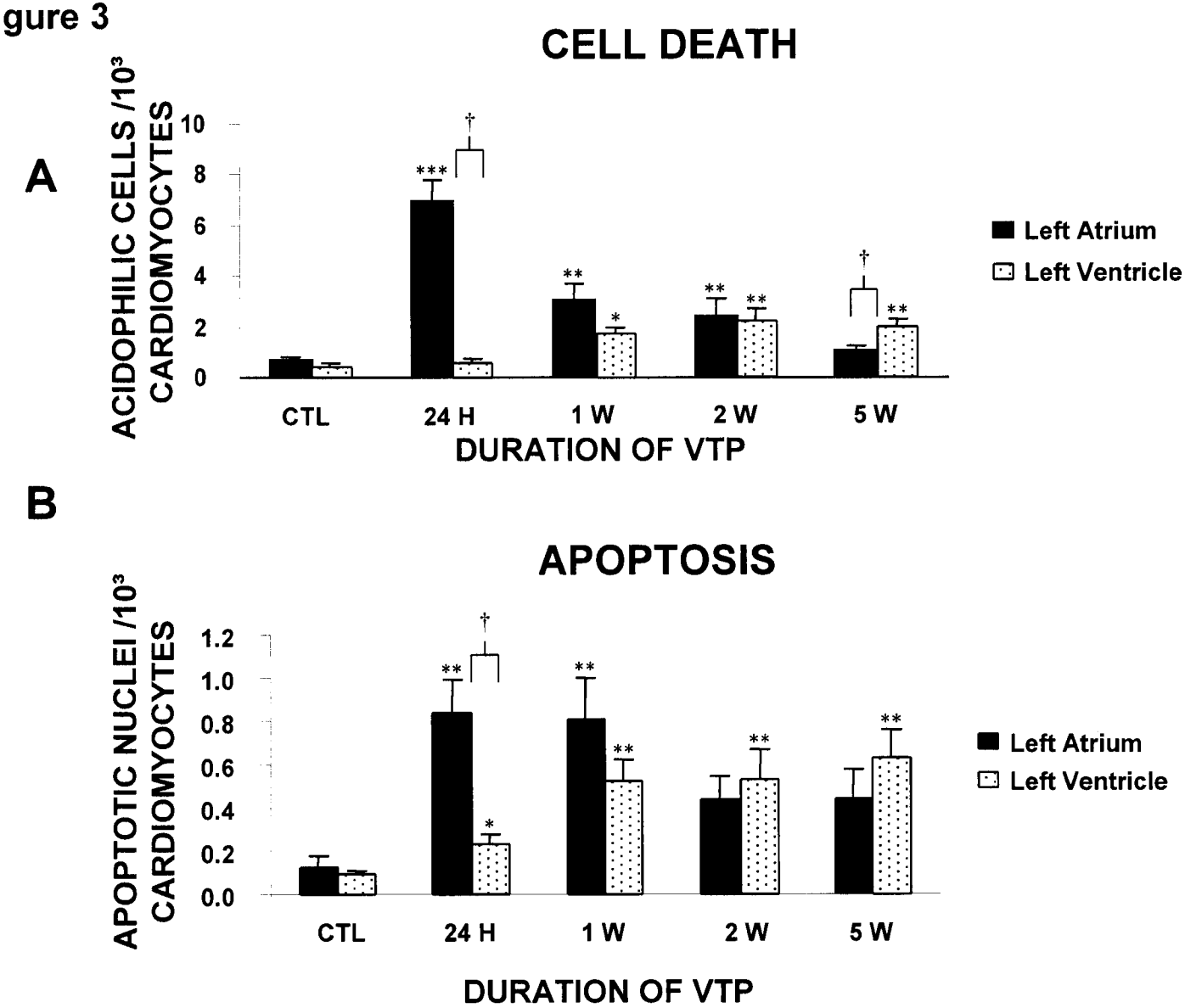

A
CELL DEATH
ACIDOPHILIC CELLS /10³ CARDIOMYOCYTES
10
8
6
4
2
0
***
†
**
*
**
**
†
**
CTL
24 H
1 W
2 W
5 W
DURATION OF VTP
Left Atrium
Left Ventricle
B
APOPTOSIS
APOPTOTIC NUCLEI /10³ CARDIOMYOCYTES
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
†
*
**
**
**
**
CTL
24 H
1 W
2 W
5 W
DURATION OF VTP
Left Atrium
Left Ventricle

Figure 4

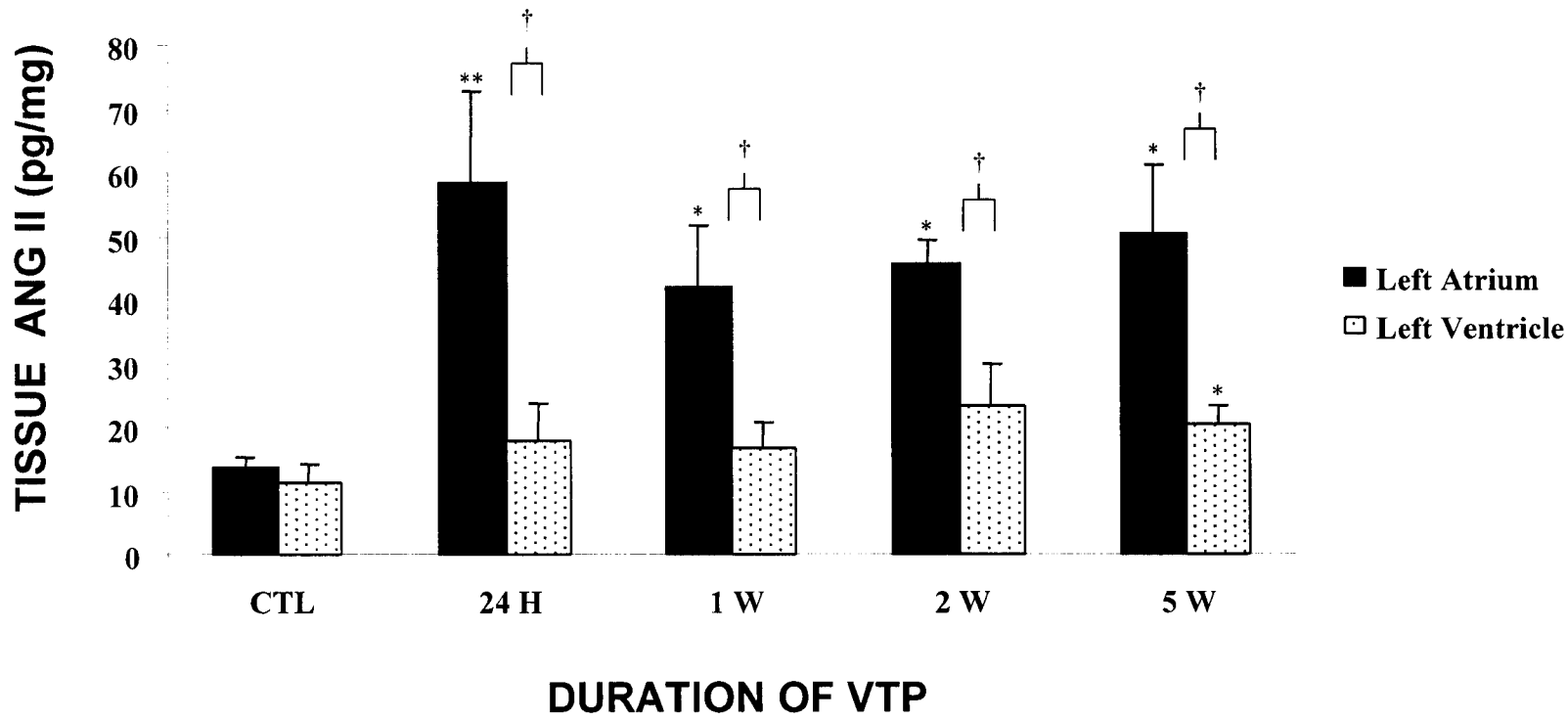

TISSUE ANG II (pg/mg)
80
70
60
50
40
30
20
10
0
CTL
24 H
1 W
2 W
5 W
DURATION OF VTP
Left Atrium
Left Ventricle

Figure 5

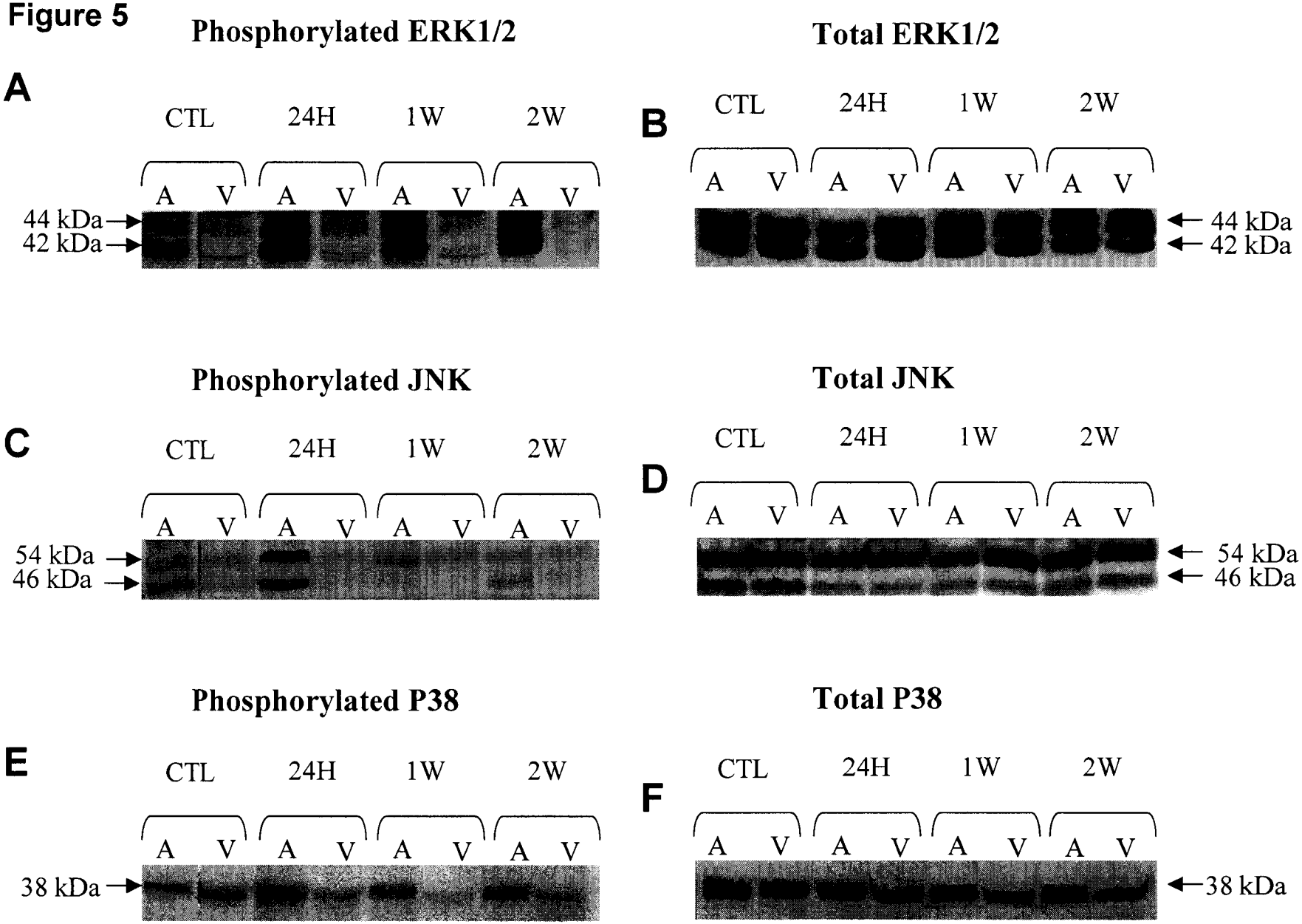

A
Phosphorylated ERK1/2
CTL
24H
1W
2W
A V A V A V A V
44 kDa
42 kDa
B
Total ERK1/2
CTL
24H
1W
2W
A V A V A V A V
44 kDa
42 kDa
C
Phosphorylated JNK
CTL
24H
1W
2W
A V A V A V A V
54 kDa
46 kDa
D
Total JNK
CTL
24H
1W
2W
A V A V A V A V
54 kDa
46 kDa
E
Phosphorylated P38
CTL
24H
1W
2W
A V A V A V A V
38 kDa
F
Total P38
CTL
24H
1W
2W
A V A V A V A V
38 kDa

Figure 6

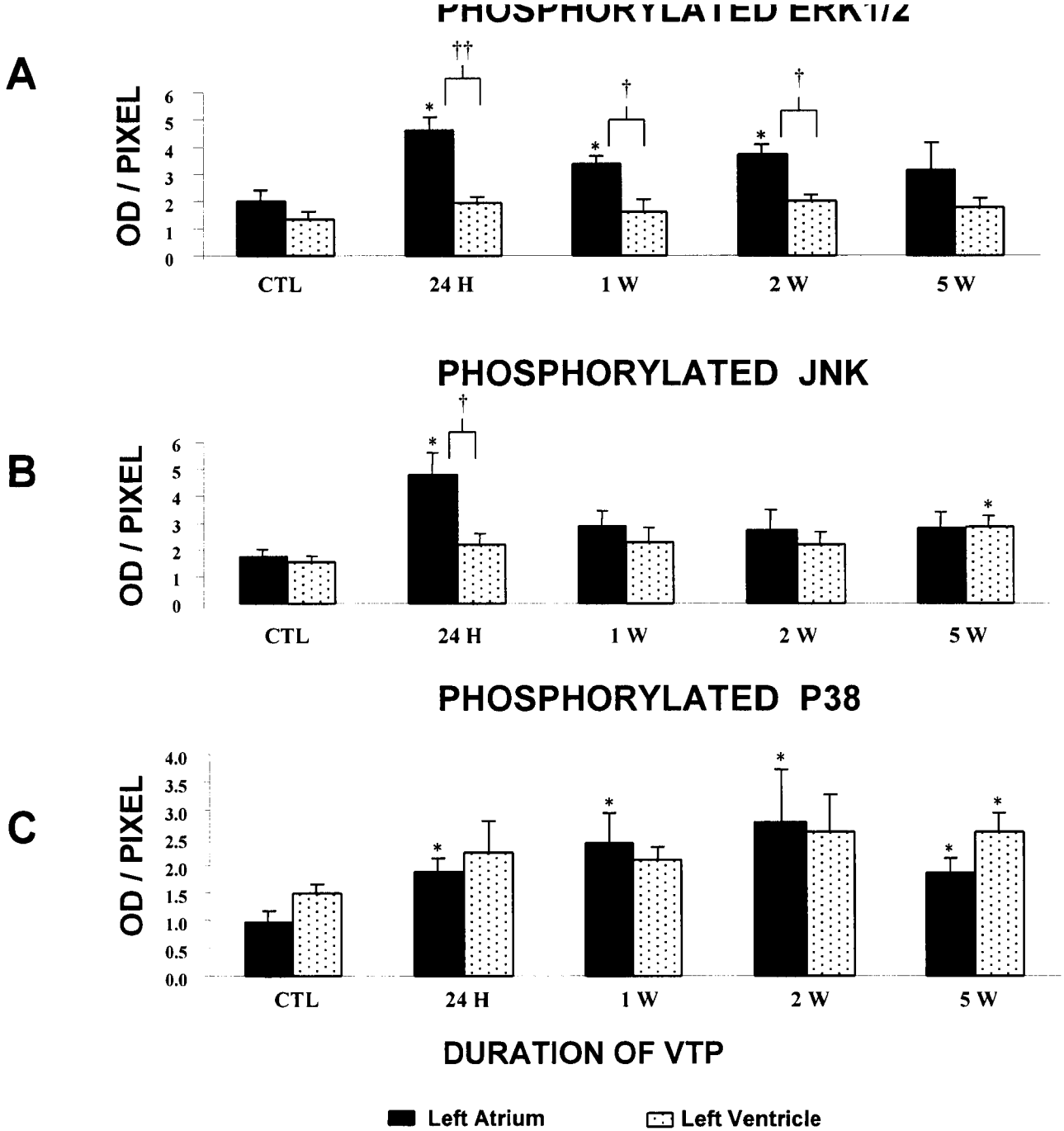

A
PHOSPHORYLATED ERK1/2
OD / PIXEL
0
1
2
3
4
5
6
CTL
24 H
1 W
2 W
5 W
B
PHOSPHORYLATED JNK
OD / PIXEL
0
1
2
3
4
5
6
CTL
24 H
1 W
2 W
5 W
C
PHOSPHORYLATED P38
OD / PIXEL
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
CTL
24 H
1 W
2 W
5 W
DURATION OF VTP
Left Atrium
Left Ventricle

Figure 7

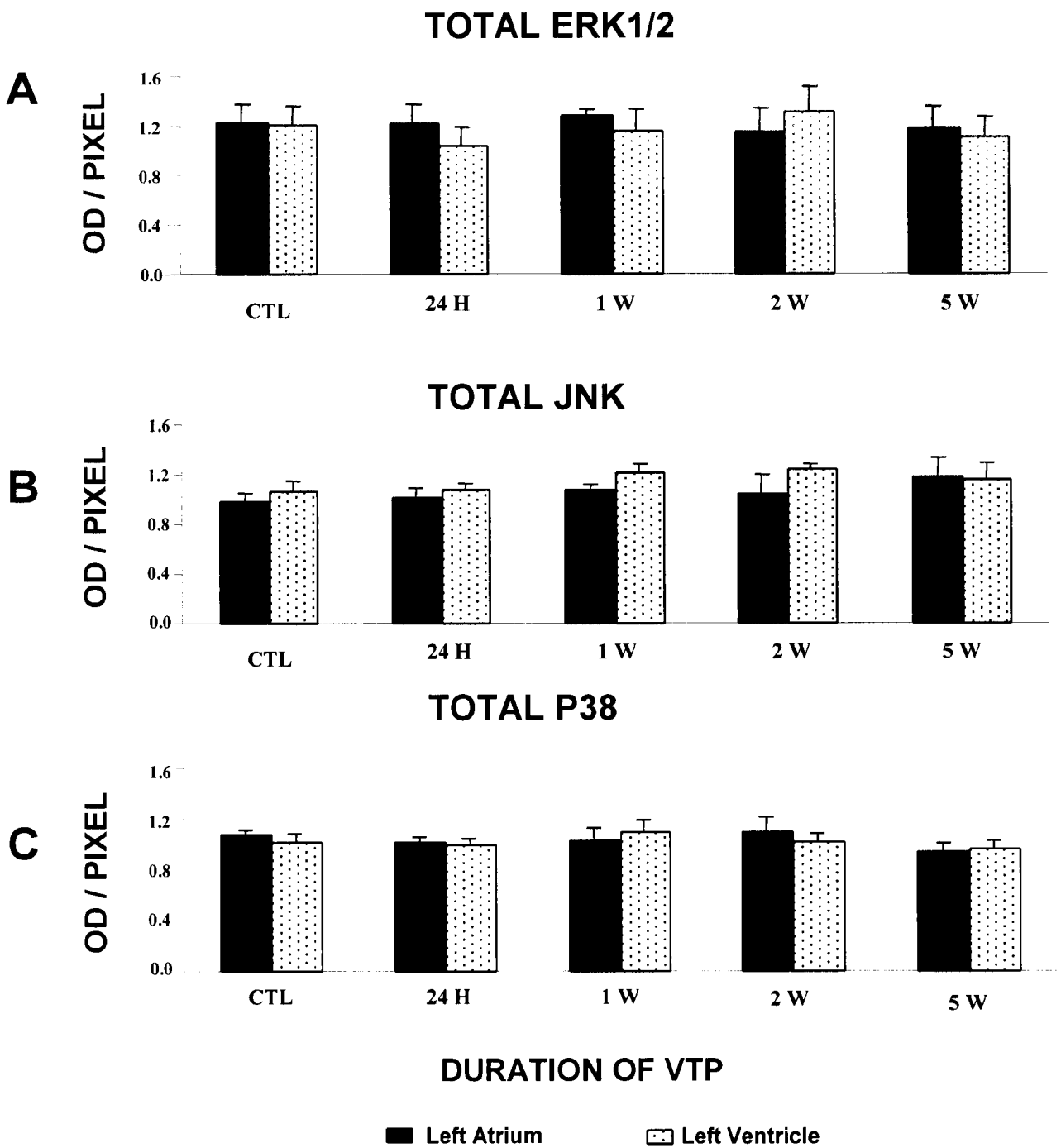
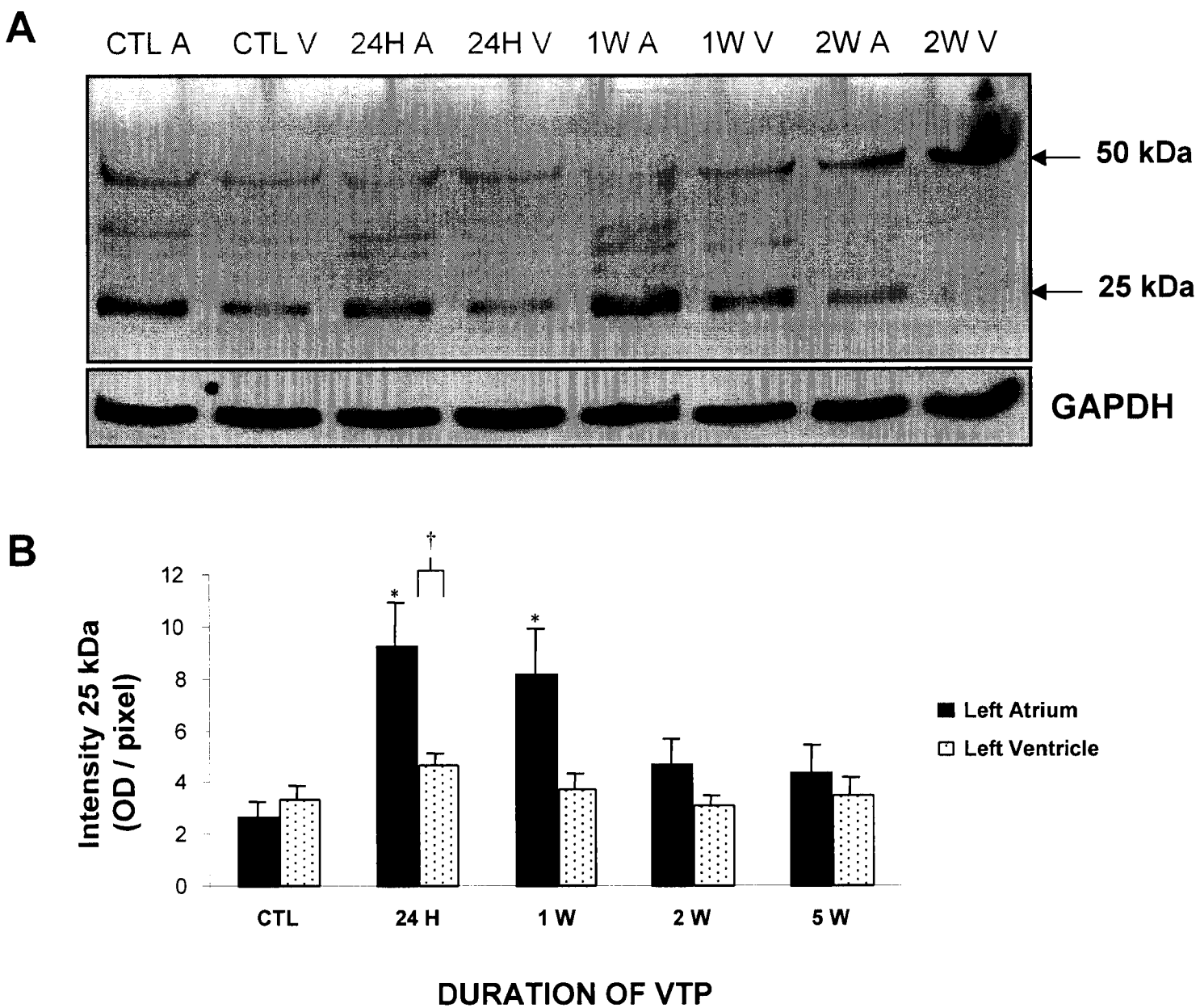

TOTAL ERK1/2
A
OD / PIXEL
1.6
1.2
0.8
0.4
0.0
CTL
24 H
1 W
2 W
5 W
TOTAL JNK
B
OD / PIXEL
1.6
1.2
0.8
0.4
0.0
CTL
24 H
1 W
2 W
5 W
TOTAL P38
C
OD / PIXEL
1.6
1.2
0.8
0.4
0.0
CTL
24 H
1 W
2 W
5 W
DURATION OF VTP
Left Atrium
Left Ventricle

Figure 8


A
CTL A
CTL V
24H A
24H V
1W A
1W V
2W A
2W V
50 kDa
25 kDa
GAPDH
B
Intensity 25 kDa
(OD / pixel)
12
10
8
6
4
2
0
*
†
*
CTL
24 H
1 W
2 W
5 W
Left Atrium
Left Ventricle
DURATION OF VTP

4. Discussion

4.1 New Findings

In our study, we have compared directly atrial versus ventricular remodeling as a function of time in dogs with CHF induced by ventricular tachypacing. There were clear and consistent differences between left atrial and left ventricular remodeling, with a variety of changes in left atrium (white cell infiltration, apoptosis, MAP kinase and TGFβ activation) being faster, quantitatively greater and more transient than in left ventricle, and with tissue fibrosis being much more intense in left atrial tissue.

CHF-related atrial remodeling has great significance for the pathophysiology of AF and appears to be a useful paradigm for a broad range of clinical conditions in which AF is associated with structural remodeling [82]; however, there is relatively little information available about the development of atrial structural remodeling in experimental models. In the present study, we report for the first time detailed comparisons of atrial versus ventricular remodeling in experimental CHF induced by ventricular tachypacing, based on the analysis of measurements on matched atrial and ventricular tissue samples from each dog. Our results show important differences between atrial and ventricular tissue in the time course and magnitude of a variety of important remodeling-induced changes. A wide range of alterations, including leukocyte infiltration, cell death, apoptosis, and ERK and JNK activation, were larger in atrium, occurred earlier and showed a more transient profile compared to ventricle. These observations suggest a more acute and severe stress on the left atrium compared to the left ventricle. One possible explanation is that the thin-walled atrium is more severely affected than the ventricle by the acute hemodynamic stress imposed by severe ventricular tachyarrhythmia. In addition, tachypacing-induced

CHF frequently causes at least moderate mitral regurgitation [82], which would cause an additional excess pressure load on the left atrium. The development of atrial fibrosis may help the atrium to adapt to these stresses by increasing atrial stiffness, possibly explaining the attenuation of changes in atrial cell death, leukocyte infiltration and apoptosis over time in parallel with the progressive development of atrial fibrosis. The relative lack of left ventricular fibrosis may be functionally important, because increased ventricular stiffness would be maladaptive in CHF. Although the maximum magnitude of a variety of responses were greater in the left atrium compared to the left ventricle, the transient nature of atrial changes contrasted with more progressive and persistent alterations in the left ventricle, so that statistically significant increases in white cell infiltration, apoptosis, cell-death and phosphorylated JNK and p38 expression were noted in left ventricular but not atrial tissues after 5 weeks of tachypacing. Such progressive changes may contribute to continuing left ventricular deterioration in CHF.

One important finding of our study was the observation of greater TGF-β1 activation in left atrial compared to left ventricular tissues. TGF-β is known to be an important profibrotic factor [42]. TGF-β1 mRNA concentrations are increased in human atrium by angiotensin II [52]. Furthermore, TGFβ overexpressing mice show an enhanced susceptibility to AF [88]. Thus, TGF-β1 activation may be an important contributor to the development of AF-promoting atrial fibrosis in CHF and signaling events leading to increased TGF-β1 activity may be interesting targets for therapy to prevent arrhythmogenic atrial structural remodeling.

4.2 Comparison of Results with Literature

Severe heart failure is accompanied by an increased amount of interstitial fibrous tissue in the walls of the heart, which is among the pathogenic mechanisms that contribute to maladaptive hypertrophy and arrhythmogenesis [2-4]. Angiotensin II can stimulate both inflammatory and fibrotic processes [19, 50, 51, and 65]. Many studies have demonstrated the role of Angiotensin II in the development of cardiac hypertrophy and cardiac fibrosis and modulation of cardiac fibroblast growth and collagen synthesis in human as well as in animal models [51, 54, 61, 67]. The accumulation of collagen in the extracellular matrix of ventricles has been observed in many pathological conditions [51, 52, and 54]. Chronic administration of ACE inhibitors, which prevents the conversion of angiotensin I to angiotensin II, or AT1 antagonists attenuates cardiac fibrosis, improves left ventricular function, prevents heart failure progression and improves survival in heart failure patients [19, 65, 66]. Paradis et al [67] have shown that overexpression of AT1 receptors in transgenic mice results in cardiac hypertrophy, interstitial fibrosis, and death due to progressive heart failure. Li et al [82] have suggested CHF induced by ventricular tachypacing leads to the development of fibrosis in the atria that was a substrate for promoting AF. In a subsequent study [19], they had shown that that ventricular tachypacing in a canine model leads to augmentation of local atrial angiotensin II, which peaked early, and activation of ERK1/2, so that a treatment with enalapril, an ACE inhibitor, attenuated these changes and reduced interstitial fibrosis and thus the incidence of atrial fibrillation [19]. A study by Barlucchi [86] et al have shown an upregulation of angiotensin II, renin and AT1 receptors in the ventricles of dogs with ventricular tachypacing induced heart failure. It was previously reported that concentration of angiotensin II increases in left atria [19, 50] and left ventricles in heart failure animal models [65, 86] and humans [63, 66]. In agreement with these

studies, our study shows an increase in the concentrations of angiotensin II in the left atria and left ventricles of dogs with ventricular tachypacing induced heart failure, with concentrations in left atria being greater than in left ventricles at all VTP intervals studied

TGFβ1, a particularly profibrotic mediator [42], plays a role in wound healing after a tissue injury, but sustained production leads to increase in the production of extracellular proteins and promote nonadaptive cardiac hypertrophy and myocardial fibrosis [41,42, 47, 48]. TGF-β1 mRNA concentrations are increased in human atrium by angiotensin-II [52]. Angiotensin receptor inhibition reduces TGF-β1 plasma concentrations and interstitial fibrosis in hypertensive kidney-transplant patients [89], and converting enzyme inhibition reduces TGF-β1 mRNA expression and ventricular fibrosis in a rat model of CHF [65]. A study by Nakajima et al has shown that targeted cardiac TGF-β1 overexpression in mice produces prominent atrial fibrosis but no significant ventricular fibrosis, despite similar degrees of TGF-β1 overexpression in atria and ventricles [74]. Another study by Koh et al [90] have observed no overt fibrosis in the myocardium of hearts engrafted with skeletal myoblasts expressing high levels of TGFβ1. Furthermore, TGFβ overexpressing mice show an enhanced susceptibility to AF [89]. These studies support our observation of greater TGF-β1 activation in left atrial compared to left ventricular tissues.

Apoptosis of cardiac myocytes represents an important cause of cell death and may contribute to progressive pump-failure, arrhythmias and cardiac remodeling [2,3, 10]. There had been discrepancies in reporting the frequency of apoptosis in the failing heart, some were as high as 0.2 %, 1 to12%, and even 35%. Such high rates of cell death would destroy the heart so rapidly as to be incompatible even with poor prognosis in heart

failure [20]. Explanations for high-published values include counting nonmyocyte cells undergoing programmed cell death and misidentifying necrosis as apoptosis. It was reported by Cesselli et al [87] that ventricular apoptosis in a dog model is increasing progressively. However, Cardin et al [50] showed a transient peak in atrial apoptosis in ventricular-tachypaced CHF dogs. We showed that the time course of apoptosis in left atria versus matched left ventricles was different in the same model, as it was transiently peaking after one day of pacing in left atria versus progressively increasing over five weeks of rapid pacing in left ventricles.

Several studies have shown that MAPKs play an important role in apoptosis [13, 21, 22, 24, 25] and that angiotensin II activates MAPKs [17, 59-61], particularly JNK [17] which is known to be a strong pro-apoptotic factor [25]. ERK pathway may be protective or neutral against apoptosis [13]. Insulin-like growth factor-1 (IGF-1), cardiotrophin-1 (CT-1), and catecholamines were each shown to exert their antiapoptotic effects, in part, by inducing ERK signaling [13]. An inhibitor of P38 MAPK protects neonatal cardiac myocytes from ischemia [24]. Another study showed a correlation between JNK activation and apoptosis induced by ischemia/reperfusion in the rabbit heart [25]. Other studies [19, 50] have shown that VTP increased phosphorylated ERK1/2, P38 and JNK transiently in the left atria after 24 hours of pacing. In a model of ischemia/reperfusion in the intact heart, ERK 1/2 activation was shown to attenuate the amount of apoptosis subsequent to reperfusion injury [21]. However, it was shown that phosphorylated ERK is increased in atrial tissue in patients with atrial fibrillation [85] and ERK activation promotes fibroblasts proliferation [60]. Recent work indicates that rabbits with 3-week tachypacing-induced CHF have increased ventricular expression of phosphorylated JNK and p38, but not of phosphorylated ERK

[91]. We similarly failed to observe changes in ventricular ERK phosphorylation with ventricular tachypacing-induced CHF, but noted statistically significant increases in phosphorylated JNK and p38 expression at 5 weeks. In contrast, atrial phosphorylated ERK expression was significantly increased at 24 hours to 2 weeks after tachypacing onset, consistent with previous evidence for ERK activation at the atrial level in AF patients [85] and in previous studies of atrial tissues from dogs with CHF [50]. These results suggest that ERK activation may play a more important role in atrial than ventricular remodeling associated with CHF.

Cesselli et al [87] observed progressive increases in ventricular apoptosis over 4 weeks of ventricular tachypacing in dogs. Heinke et al [73] also compared the number of apoptotic in the ventricular and atrial myocytes in pacing-induced canine heart failure and found the apoptotic ventricular myocytes nuclei in dogs subjected to 3-4 week ventricular-tachypacing is greater than apoptotic atrial myocytes, which is similar to our data. Taken together, our study suggests that there are differences between left atria versus left ventricles in the activation of MAPKs, and that apoptosis takes different time course in the atria (transient) vs. the ventricles (progressive) in our VTP-induced CHF model.

Local elevation of cytokines levels appears to be part of the initial response of the heart to hemodynamic overload [29, 38]. Locally produced cytokines can activate macrophages and monocytes, which then attract and activate fibroblasts leading to fibrosis [39]. Cytokines, as well, are responsible for production of free radicals that cause myocardial cell death and apoptosis [27, 31, 37]. Inflammatory cytokines have been implicated in the pathogenesis of several cardiovascular diseases such as CHF, and AF [39,

[92]. Nakamura et al [93] shows that in tachycardia-induced by ventricular pacing in dogs for 4W, there was an increase in the number of myocardial monocyte infiltration compared to control dogs. We studied the time-course of leukocyte infiltration and found that it was different in left atria versus left ventricles, with left atria showing a rapid and transient rise after one day of pacing, whereas in left ventricles the leukocyte count increased to a lesser extent but remained significantly increased over the entire 5-week VTP interval.

Fibrosis was previously reported in left atria [19, 50, 83] and left ventricles in experimental CHF and in humans [51,54, 63]. We studied the development of fibrosis in left atria and left ventricles in a dog model and found that there are differences in fibrous-tissue content in left atria versus left ventricles, with the fibrous-tissue content increasing progressively during VTP in both left atria and left ventricles, but the amount of fibrosis was substantially greater in left atria than left ventricles. Our observations are important in understanding the qualitative and quantitative differences in atrial and ventricular remodeling in experimental congestive heart failure, with important potential consequences for underlying mechanisms and therapeutic approaches.

4.3 Suggested Mechanisms

The processes responsible for remodeling are beneficial for the heart in the short term, however in the long term, they become decompensatory and aggravate the disease state (Figure 6).

The activation of the renin-angiotensin system is usually an adaptational mechanism important to maintain homeostasis in response to hemodynamic disequilibrium [56]. During pathological conditions such as heart failure, the renin-

angiotensin system is considered a defense mechanism against the hemodynamic imbalance [56]. But it leads to disastrous consequences in the long term. Several studies have shown that angiotensin II causes fibrosis [51, 53-56], stimulates cardiac myocyte hypertrophy [54, 61, 67] and promotes apoptosis [50, 59, 68] and necrosis [56]. Angiotensin II enhances the expression of TGFβ1 gene [52] and there is a cross talk between Angiotensin II receptors and SMAD proteins [64], effector proteins, which act down stream of TGFβ1 receptor, and generally inhibit cell proliferation and induce fibrosis [41, 42]. Blocking Angiotensin II type 1 receptor or using angiotensin converting enzyme (ACE) inhibitors prevents increase in the levels of TGFβ1and inhibits fibrosis [65]. It was shown that TGFβ1 gene expression as well as type I collagen gene expression is significantly decreased in both infracted and non-infarcted myocardium in rats treated with the angiotensin II receptor antagonist Losartan [95]. In our study, concentration of angiotensin II in left atria increased early after pacing and were greater than in left ventricles at all VTP intervals studied, and atrial TGFβ1 peaked early as well, whereas there was no change in ventricular TGFβ1. This suggests that probably in left atria, concentrations of angiotensin II were high enough to induce the expression of TGFβ1, but in left ventricles, angiotensin II was incapable of inducing TGFβ1, suggesting that maybe only after concentrations of angiotensin II reach a certain level, angiotensin II can induce expression of TGFβ1 or other pathways may be implicated in the activation of TGFβ1 in the ventricles (Figure 6).

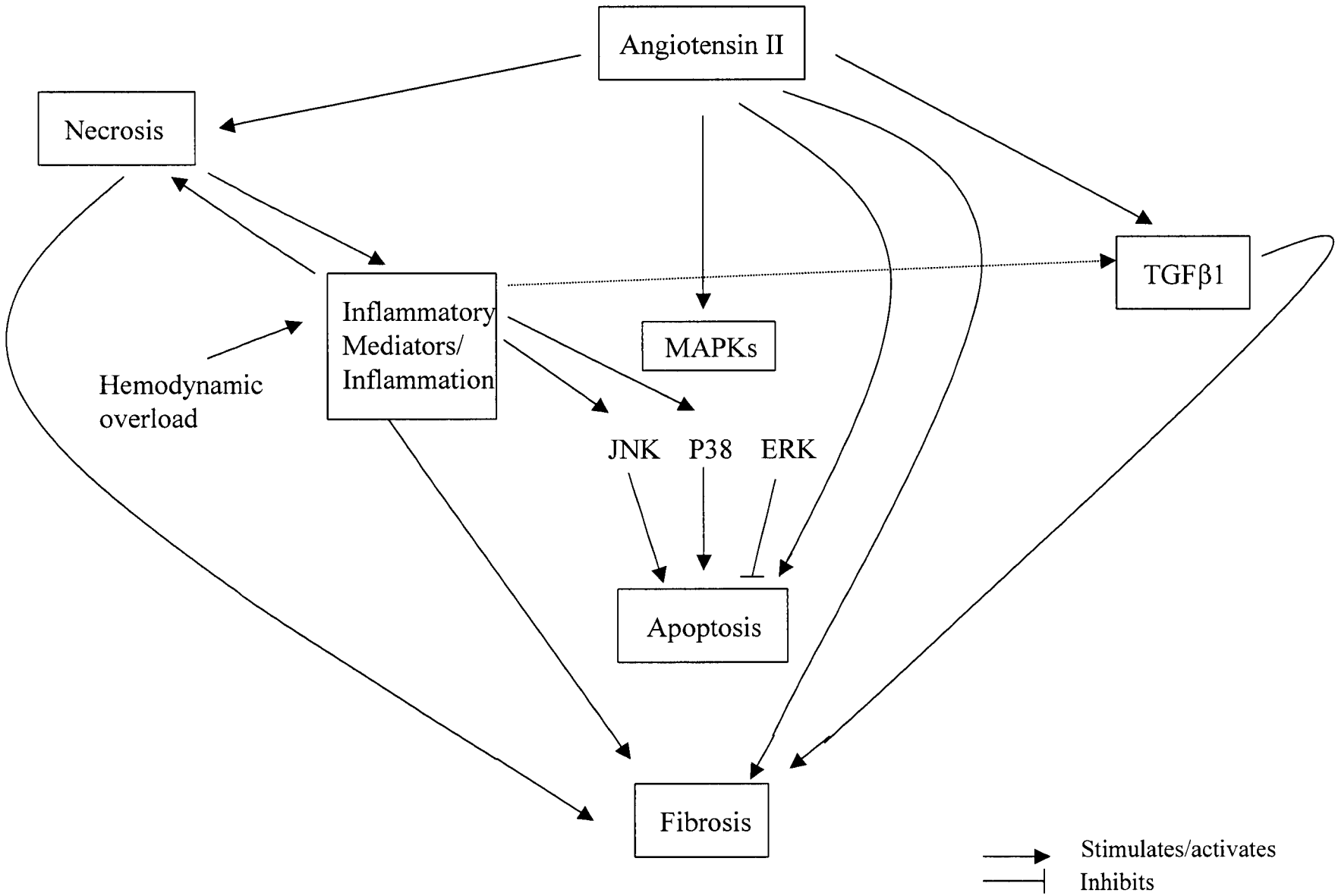
The implication of the three MAPKs (ERK1/2, P38, JNK) in cardiac remodeling has been the subject of numerous studies. It was shown that phosphorylated ERK is increased in atrial tissue in patients with atrial fibrillation [85] and that ERK is implicated in the mechanism of collagen synthesis by angiotensin II [19, 50, and 59]. Li et al [19] have shown

that inhibition of angiotensin II converting enzyme by enalapril significantly reduced P-ERK levels and attenuated interstitial fibrosis in the left atria in VTP-induced CHF dogs. Our study shows an increase in ERK activity after one day of pacing in the atria till after two weeks of VTP, while its activity did not change in the ventricles suggesting that ERK activation is implicated in atrial remodeling, but not in ventricular remodeling at least in this model. Interestingly, apoptosis and caspase activation were diminished by a non-specific p38 kinase inhibitor in an ischemia model in neonatal myocytes [24]. Moreover, a study showed a correlation between JNK activation and apoptosis induced by ischemia/reperfusion in the rabbit heart [25]. In our study, in the atria, JNK and P38 increased after one day of pacing and in the case of P38 remained elevated till one week and then returned to control levels. In the ventricles, JNK and P38 only became significant after five weeks of pacing. This suggests that P38 and JNK early peak in the atria may be implicated in the transient changes that occurred in the atria such as cell death and apoptosis, and their activation could be a response to the 24 hour-peak in leukocyte infiltration. In the ventricles, P38 and JNK are maybe contributing to the progressive increase in apoptosis that peaked at five weeks of pacing.

One of the major causes of damage due to ischemia/reperfusion is the production of free radicals, a major cytotoxic effect of cytokines [34, 37, 39]. Inflammatory mediators increase the expression of iNOS, the much more inducible form of nitric oxide synthase. The large amounts of NO generated by iNOS contribute to the vasodilation and depressed myocardial contractility, activate NF-κB to increase cytokine production, and damage cells and can cause myocardial cell death [30, 31, 36]. Proinflammatory cytokines could induce activation of JNK and P38 [25, 27, 30, 34] and it was shown that inhibition of P38 by

CSAIDS blocked the translation of the mRNA responsible for the expression of different cytokines including TNF-α [15]. Inflammatory cells such as macrophages release TGFβ1 that is strongly chemotactic to neutrophils, T-cells, monocytes, and fibroblasts, and it leads to synthesis of extracellular matrix proteins and production of tissue fibrosis [41,42,47]. Another major cause of infiltration of white blood cells in cardiac remodeling during heart failure, besides hemodynamic overload, is cell death by necrosis which evokes a robust inflammatory reaction in the failing heart [18,19] (figure 6). Our study shows that the time-course of leukocyte infiltration was different in left atria versus left ventricles, with left atria showing a rapid and transient rise after one day of pacing, whereas in left ventricles the leukocyte count increased to a lesser extent but remained significantly increased over the entire 5-week VTP interval. This suggests that there could be differences in sensitivity between atria and ventricles to initial hemodynamic overload. The pattern of inflammatory cells infiltration in the ventricles coincides with that of fibrosis and suggests that while blood cells infiltration could be a major factor implicated in the fibrosis seen in the ventricles, while other factors such as angiotensin II, TGFβ1 and ERK activation are more important in explaining the fibrosis in atria. One possible explanation for the changes observed in atria vs. ventricles is that the thin-walled atrium is more severely affected than the ventricle by the acute hemodynamic stress imposed by severe ventricular tachyarrhythmia. In addition, tachypacing-induced CHF frequently causes at least moderate mitral regurgitation [82], which would cause an additional excess pressure load on the left atrium. The development of atrial fibrosis may help the atrium to adapt to these stresses by increasing atrial stiffness, possibly explaining the attenuation of changes in atrial cell death, leukocyte infiltration and apoptosis over time in parallel with

Figure 6. Suggested Interactions between Remodeling Mechanisms



the progressive development of atrial fibrosis. The relative lack of left ventricular fibrosis may be functionally important, because increased ventricular stiffness would be maladaptive in CHF. Although the maximum magnitude of a variety of responses were greater in the left atrium compared to the left ventricle, the transient nature of atrial changes contrasted with more progressive and persistent alterations in the left ventricle, so that statistically significant increases in white cell infiltration, apoptosis, cell-death and phosphorylated JNK and P38 expression were noted in left ventricular but not atrial tissues after 5 weeks of tachypacing. Such progressive changes may contribute to continuing left ventricular deterioration in CHF.

4.4 Potential Significance

Our study is the first study investigating the differences between atrial and ventricular remodeling in dogs with ventricular tachypacing-induced congestive heart failure. Severe heart failure is accompanied by an increased amount of interstitial fibrosis in the heart, a late remodeling event that contributes to maladaptive hypertrophy and arrhythmogenesis [1-3, 10]. We determined the concentrations of angiotensin II, the expression TGFβ1 and MAPKs, the infiltration of white blood cells and the quantification of cell death and apoptosis in atrial and ventricular remodeling. Our results show that atrial remodeling is different from ventricular remodeling. Ventricular-tachypacing caused substantially more fibrosis in left atrium than in left ventricle. Tissue Angiotensin-II concentration increased early after one day of pacing in left atria, and was significantly higher in left atria than in left ventricles at all VTP intervals studied. Other changes peaked early (24hrs) and were transient in left atria, vs. progressive over five

weeks in left ventricles. Ventricular-tachypacing caused tissue apoptosis, inflammatory cell infiltration and cell-death, with maximum changes in left atria being much larger than maximum changes in left ventricles. MAP kinase activation was rapid (within 24 hrs) in left atria, but smaller and slower (P38, JNK) or non-significant (ERK) in left ventricles. The activated form of TGFβ1, a particularly important profibrotic mediator, increased significantly in left atria, but was not changed in Left ventricles. These observations indicate that progressive changes occur over five weeks of pacing in the ventricles suggesting that the earlier the intervention, the lesser the extent of remodeling, and the lesser the chance of ventricular arrhythmias. On the other hand, crucial remodeling events occur rapidly after the beginning of ventricular- tachypacing in the atria highlighting the importance of early intervention to prevent atrial remodeling in CHF and thus the development of a substrate for atrial fibrillation. In summary, this study shows that there are qualitative and quantitative differences in left atrial and left ventricular remodeling in experimental CHF, with important potential consequences for underlying mechanisms and therapeutic approaches.

4.5 Considerations of the Model

The pacing-tachycardia dog model seems very valuable for studying neurohumoral mechanisms and peripheral circulatory alterations, both of which closely resemble that observed in human heart failure [95]. Furthermore, alterations in myocardial function and molecular changes in calcium-handling proteins underlying altered myocardial function show considerable similarities to the failing human heart. This may allow the study of the transition from a compensated state of left ventricular dysfunction

to overt failure with respect to alterations in calcium homeostasis. The model also provides temporal and mechanistic information on left ventricular remodeling and allows the study of pharmacologic interventions to influence the remodeling process. The limitations of the rapid pacing model include an uncertain pathogenesis, and lack of long-term stability because heart failure is reversible when pacing is stopped [70, 84,95]. Our study evaluated the time course of changes involved in atrial and ventricular remodeling in a the dog model, which is a well-defined experimental model of CHF, but extrapolation to clinical CHF should be cautious. The pathophysiology of CHF differs for different experimental and clinical paradigms and, although sustained tachycardia can contribute to or cause clinical CHF [96], the pathophysiology of ventricular tachypacing-induced experimental CHF cannot be assumed to apply directly to clinical CHF [70].

4.5 Future Directions

It is of great interest to identify the exact underlying mechanisms of remodeling in the atria and the ventricles. Cell death is in itself a remodeling event that was the subject of numerous studies trying to precisely identify its mechanisms. Our study suggests that cell death and apoptosis were transit in left atria versus progressive in left ventricles and other studies had shown that angiotensin II and inflammatory cytokines, released from infiltrating white blood cells, by activating MAPKs, can induce apoptosis in both the atria and the ventricles [19, 27 50, 56, 59, 68, 92, 93]. However, according to Cesselli et al [87], oxidative stress is responsible for the ventricular apoptosis seen in experimental heart failure, and to Nickenig and Harrison [97], angiotensin II can, via its AT1 receptor, induce the production of free oxygen radicals. It is known as well that the inflammatory cells release

free radicals, which can induce cell death [27, 37]. This can be contradicted, as mentioned in the introduction, by the fact that inflammatory cells can activate the synthesis of enzymes that destroy free oxygen radicals, therefore their presence is expected to prevent massive cell death, resulting in an oxidative stress that is more elevated in their absence. It is therefore certainly interesting to verify if in our canine model, oxidative stress plays a role in atrial and ventricular remodeling and if it occurs as a consequence of augmentation in angiotensin II concentration or in the number of infiltrating inflammatory cells. To determine the role of each factor in remodeling and the mechanism responsible for cell death, dogs subjected to 24 hours of ventricular pacing can be treated with enalapril (an ACE inhibitor), ibuprofen (an anti-inflammatory), or vitamin C (an antioxidant), and then the level of cell death is determined with each treatment. This will provide an indication about the most important cause or mechanism of cell death in the atria. Major cause or mechanism of cell death implicated in the ventricles can be determined by the same treatments in dogs subjected to 2 weeks of rapid pacing. Using these treatments, we can also evaluate the role of inflammatory cells in cell death, and determine the importance of augmentation of tissue angiotensin II and its role in cell survival and death.

In our study we used TUNEL technique to determine the percentage of apoptotic cardiomyocytes. Other studies such as DNA laddering is measuring apoptosis in all types of cells in the heart, thus does not distinguish between apoptotic myocytes and apoptotic non-myocytes. Other types of cells such as fibroblasts, or endothelial cells could be implicated in the changes observed in different parameters. The implication of different

types of cells in atrial or ventricular remodeling is unknown, but can turn out to be of great interest in our understanding of the complicated mechanism of cell death.

In contrast to apoptosis, necrosis, another mechanism of cell death, is characterized by the rupture of intracellular and extracellular membranes that finally leads to the demise of the myocyte. As a result, cell death by necrosis evokes inflammatory responses, which perform the digestion of the debris of the cells died by necrosis. Moreover, it is known that necrosis leads to the formation of fibrosis that replaces the cardiomyocytes lost through necrosis [18, 19]. Consequently, necrosis could be a key in the remodeling process and a better understanding of the mechanisms that lead to it can direct the development of new therapeutic approaches.

Another subject of interest is determining the mechanisms responsible for interstitial fibrosis. Several studies have shown that angiotenisn II and TGFβ1 can induce the development of interstitial fibrosis [41, 42, 47, 51, 54, 64], however the exact mechanisms that lead to development of fibrosis remain poorly defined. Is fibrosis considered like a replacement tissue that provides structural support after cardiac myocytes are lost through necrosis? Or is it considered an independent process? The determination of mechanisms that induce fibrosis could permit development of new approaches to prevent atrial and ventricular remodeling. It will be interesting to determine if the changes that occur after 24 hours of pacing actually lead to the development of fibrosis in the atria and if the progressive changes seen in the ventricles are responsible for ventricular fibrosis. That can be verified by observing the atrial structural changes in animals after five weeks where they were subjected to rapid pacing only for one day, and then interrupted. In another set of animals, pacing is to be

stopped after two weeks and then we observe the ventricular structural changes after a period of five weeks.

Congestive heart failure is a complicated clinical condition as it affects a great diversity of systems, implicated in a variety of essential functions, therefore identification of "candidate genes" and molecular and biochemical mediators of myocardial hypertrophy and failure has been vigorously pursued to dissect the pathogenesis and signaling pathways of this disease. In our study, we investigated changes in some parameters that are implicated in the atrial and ventricular remodeling, but we did not study all the parameters that are involved in the remodeling process nor the interactions between them. Applying the latest genomic and proteomic technologies can help identify all the genes or proteins implicated in a certain pathological condition in one setting. For example, by using gene chip technique, it is easy to compare the genetic expression of 36 000 genes from healthy atria or ventricles and diseased atria or ventricles tissues [98]. This vigorous technique can help identify the major genes whose expression play an important role in atrial and ventricular remodeling in a complicated disease as heart failure. However, reliability of gene arrays are still of major concern, as they may contain inherent accuracies representing as much as 30% of genes on the arrays. This is largely caused by wrong sequence information in the databases as well as errors generated during manufacturing [98]. Where large number of comparisons between control and experimental samples are possible, the percentage of arrays that have the same sign of expression changes for a particular gene is more significant information than the quantitative expression difference of that gene. If one already has evidence implicating specific genes in an array, then think that a single gene array can provide information that will

inexorably lead to new insights that can then be pursued by more conventional assays such as Northern blots. Where the changes in genes involves a disease process, Proteomics or genomics can do more than simply report the changes. We must then speculate whether a particular gene (or its translation products) is causative in the development of a disease in the population [98].

Another interesting approach is utilization of genetically modified mice. The ability to engineer precise mutations in the heart—coupled with the technological sophistication to quantitate the effects of these mutations on cardiac function at cellular, organ, and intact animal levels—has provided novel insights into the molecular mechanisms of heart failure and led to the recognition of a wide array of previously unknown molecular initiators, transducers, and effectors for the development of cardiac hypertrophy and its transition to heart failure. Therefore mice genetically modified for different elements involved in the mechanisms that we think are responsible for atrial and ventricular remodeling [71, 88]. For example, studies had suggested that tissue angiotensin II and MAPKs, can induce apoptosis. A genetically modified mouse for the enzyme responsible for conversion of local angiotensin II or for MAPKs can help us evaluate the importance of these elements and their effects on cardiac apoptosis in heart failure. However, future refinement and generation of conditional gene targeted mouse model, which may allow for the temporal and spatial control of gene expression more precisely in restricted regions of the heart such as the atrium, or ventricle would be a tremendous advantage in exploring the genetic basis of cardiac remodeling, function and dysfunction [71]. In general, transgene expression results either in an increase or a decrease in the transcript levels of the gene of interest. Therefore, we can easily determine the role of a

certain protein assuming its inhibition, instead of using antagonists that can be general; non-specific, and do not totally inhibit a receptor. The genetically modified mice can develop a pathological condition without need of inducing it. For example, transgenic mice overexpressing AT1, as described by Paradis et al [67], develop cardiac hypertrophy and remodeling resulting in heart failure. Thus, it is tempting to conclude that angiotensin II can induce heart failure by the activation of its AT1 receptor, but this experiment can only allow us to conclude that angiotenisn II is generally implicated in the development process of heart failure.

In most instances, extreme caution should be exercised to establish the causality between a gene of interest and its role in the pathogenesis of cardiomyopathy or other cardiac phenotypes. The deficiency induced by a certain genetic mutation can induce the pathological condition by particular processes that are different from the usual mechanisms. In addition to the fact that there is growing body of evidence suggesting that mouse strain–specific genetic makeup and characteristics may have profound impact on the phenotype observed, gene redundancy and compensatory responses, which may well complicate the interpretation of the findings, and that the expression of a transgene in the heart may exert some nonspecific toxic effects.

In conclusion, differences in atrial and ventricular remodeling in CHF have not been studied previously. In this study, we compared atrial and ventricular remodeling in an experimental model of CHF, and found significant quantitative and qualitative differences. These differences may have important implications for understanding the mechanisms of cardiac remodeling in CHF and for the development of novel therapeutic

approaches to the prevention of the development of the AF substrate. In dogs with ventricular-tachypaced CHF, we found that fibrosis and tissue angiotensin II concentration were higher in atrium than in ventricle. Changes such as apoptosis, inflammatory-cell infiltration, and cell death peaked early and were transient in atrium, vs. progressive but quantitatively less in ventricle. MAP-kinase activation was rapid in atrium, but smaller and slower (p38, JNK) or non-significant (ERK) in ventricle. TGFβ1 increased significantly in atrium but was not significantly changed in ventricle. Thus, this is the first study to describe the temporal evolution of structural changes in atrium and ventricles of dogs with heart failure induced by ventricular tachypacing. We found that atrial and ventricular remodeling are different in experimental CHF, with potential consequences for understanding mechanisms and therapeutic approaches.

5. Bibliography

1. Katz AM. Heart Failure. Pathophysiology, molecular biology, and clinical management. Eds Lippincott Williams & Wilkins. 2000. 381p.
2. Francis GS, Tang W.H.W. Pathothophysiology of congestive heart failure. *Rev Cardiovasc Med.* 2003; 4 (suppl 2): S14-S20.
3. Francis GS. Pathophysiology of chronic heart failure. *Am J Med.* 2001; 110(7A): 37S-46S.
4. Francis GS, Gassler JP, sonnenblick EH. Pathophysiology and diagnosis of heart failure. In Fuster V, Alexander RW, O'Rourke RA. The Heart. Eds McGraw Hill 2001; Volume 1: 655-681.
5. Mancini DM, Henson D, La Manca J, et al. Benefit of selective respiratory muscle training on exercise capacity in patients with chronic heart failure. *Circulation.* 1995; 91: 320-329.
6. Poole-Wilson P, Ferrari R. Role of skeletal muscle in the syndrome of chronic heart failure. *J Mol Cell Cardiol.* 1996; 28: 2275-2285.
7. Simonini A, Massie BM, Long CS, et al. Alterations in skeletal muscle gene expression in the rat with chronic congestive heart failure. *J Mol Cell Cardiol.* 1996; 28: 1683-1691.
8. Johansen H, Strauss B, Arnold JM, et al. On the rise: The current and projected future burden of congestive heart failure hospitalization in Canada. *Can J Cardiol.* 2003; 19 (4): 430-435.

9. Bentkover JD, Stewart EJ, Ignaszewski A, et al. New technologies and potential cost savings related to morbidity and mortality reduction in class III/IV heart failure patients in Canada. *Int J Cardiol.* 2003; 88 (1): 33-41.

10. Cohn JN, Ferrari R, Sharpe N, et al. Cardiac Remodeling- concepts and clinical implications: A consensus paper from an international forum on cardiac remodeling. *J Am Coll Cardiol.* 2000; 35: 569-582.

11. Sugden PH, Clerk A. "Stress-Responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circ Res.* 1998; 83: 345-352.

12. Widmann C, Gibson S, Jarpe MB, et al. Mitogen-Activated Protein Kinase: Conservation of a three-kinase module from yeast to human. *Physiol. Rev.* 1999; 79: 143-180.

13. Beuno OF, Molkentin J. Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. *Circ Res.* 2002; 91: 776-781.

14. Clerk A, Fuller SJ, Michael A, et al. Stimulation of 'stress-regulated' mitogen-activated protein kinase subfamilies in perfused heart: p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *J Biol Chem.* 1998; 273: 7228-7234.

15. Lee JC, Laydon JT, McDonnell PC, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.* 1994; 372: 739-746.

16. Lazou A, Sugden PH, Clerk A. Activation of mitogen-activated protein kinases (P38 MAPKs, SAPKs/JNKs and ERKs) by the G-protein-coupled receptor agonist phenylephrine in perfused rat heart. *Biochem J.* 1998; 332: 459-465.

17. Izumi Y, Kim S, Zhan Y, et al. Important role of angiotensin II-mediated c-Jun NH2-Terminal Kinase Activation in Cardiac Hypertrophy in Hypertensive rats. *Hypertension*. 2000; 36: 511-516.

18. Swynghedauw B. Molecular mechanisms of myocardial remodeling. *Physiol Rev*. 1999; 79: 215-262.

19. Li D, Shinagawa K, Pang L et al. Effects of angiotensin converting enzyme inhibition on the development of the atrial fibrillation substrate in dogs with ventricular tachypacing induced congestive heart failure. *Circulation* 2001;104:2608-2614

20. Haunster A, Haass M, Izumo S. Cardiac myocytes. In: Schunkert H, Reigger GAJ. Apoptosis in cardiac biology. Eds Kluwer academic publishers. 2000: 175-188.

21. Yue TL, Wang C, Gu JL, et al. Inhibition of extracellular signal–regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ Res*. 2000; 86: 692-699.

22. Wang Y, Huang S, Sah VP, et al. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem*. 1998; 273: 2161-2168.

23. Zechner D, Craig R, Hanford DS, et al. MKK6 activates myocardial cell NF- κB and inhibits apoptosis in a p38 mitogen-activated protein kinase dependent manner. *J Biol Chem*. 1998; 273: 8232-8239.

24. Mackay K, Mochley-Rosen D. An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischemia. *J Biol Chem.* 1999; 274: 6272-6279.

25. Yue TL, Ma XL, Wang X, et al. Possible involvement of stress-activated protein kinase signaling pathway and Fas expression in prevention of ischemia/reperfusion-induced cardiomyocyte apoptosis by carvedilol. *Circ Res.* 1998; 82: 166-174.

26. Barns PJ, Karin M. Nuclear factor- κB: A pivotal transcription factor in chronic inflammatory diseases. *N Eng J Med.* 1997; 336:1066-1071.

27. Wallach D, Boldin M, Varfolmeev E, et al. Cell death induction by receptors of the TNF family: towards a molecular understanding. *FEBS Lett.* 1997; 410: 96-106.

28. Yokoyama T, Nakano M, Bednarczyk JL, et al. Tumor necrosis factor-α provokes a hypertrophic growth response in adult cardiac myocytes. *Circulation.* 1997; 95: 1247-1252.

29. Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insight. *Annu Rev Immunol.* 1996; 14: 649-681.

30. Pulkki KJ. Cytokines and cardiomyocyte death. *Ann Med.* 1997; 29(4):339-343.

31. Stein B, Frank P, Schmitz W, et al. Endotoxin and cytokines induce direct cardiodepressive effects in mammalian cardiomyocytes via induction of nitric oxide synthase. *J Moll Cell Cardiol.* 1996; 28:1631-1639.

32. DiDonato JA, Hayakawa M, Rothwarf DM. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature.* 1997; 388: 548-554.

33. Ye J, Young HA. Negative regulation of cytokine gene transcription. *FASEB J.* 1997; 11: 825-833.

34. Chandrasekar B, Colsten JT, Freeman JL. Induction of proinflammatory cytokine and antioxidant enzyme gene expression following brief myocardial ischemia. *Clin Exp Immunol.* 1997; 108: 346-351.

35. Nakano M, Knowlton AA, Yokoyama T, et al. Tumor necrosis factor-α induced expression of heat shock protein 72 in adult feline cardiac myocytes. *Am J Physio*l. 1996; 270: H1231-H1239.

36. Boxkurt B, Kribbs SB, Clubb FJ, et al. Pathophysiologically relevant concentrations of tumor necrosis factor-α promote progressive left ventricular dysfunction and remodeling in rats. *Circulation.* 1998; 97: 1382-1391.

37. Belch JJF, bridges AB, Scott N, et al. Oxygen free radicals and congestive heart failure. *Br Heart J.* 1991; 65: 245-248.

38. Kapadia S, Oral H, Lee J, et al. Hemodynamic regulation of tumor necrosis factor- α gene and protein expression in adult feline myocardium. *Circ Res.* 1997; 81: 187-195.

39. Shioi T, Matsumori A, Kihara Y, et al. Increased expression of interleukin-1β and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the hypertrophied and failing heart with pressure overload. *Circ Res.* 1997; 81: 664-671.

40. Bryant D, Becker L, Richardson J, et al. Cardiac failure in transgenic mice with myocardial expression of tumor necrosis factor α. *Circulation.*1998; 97: 1375-1381.

41. Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. *N Eng J Med.* 1994; 331: 1286-1292.

42. Lijnen PJ, Petrov VV, Fagard RH. Induction of cardiac fibrosis by transforming Growth factor β1. *Mol Genet Metab.* 2000; 71: 418-435.

43. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFβ activation. *J Cell Sci.* 2003; 116: 217-224.

44. Munger J.S., Harpl J.G, Gleizes P.E, et al. Latent transforming growth factor β: structural features and mechanisms of activation. *Kidney Int.* 1997; 51: 1376-1382.

45. Heldin C.H., Miyazono K, and ten Dijke P. TGFβ signaling from cell membrane to nucleus through SMAD proteins. *Nature (London).* 1997, 390: 465-471.

46. Massague J. TGFβ signal transduction. *Annu Rev Biochem.* 1998; 67: 753-791.

47. Postlethwaite AE, Keski-Oja J, Moses HL, et al. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. *J Exp Med.* 1987; 165: 251-256.

48. Border WA, Ruoslahti E. Transforming growth factor β in disease: the dark of tissue repair. *J Clin Invest.* 1992; 117:408-414.

49. Kawara T, Derksen R, de Groot JR, et al. Activation delay after premature stimulation in chronically diseased human myocardium relates to the architecture of interstitial fibrosis. *Circulation.* 2001; 104(25): 3069-75.

50. Cardin S, Li D, Thorin-Trescases N, et al. Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and independent pathways. *Cardiovasc Res.* 2003;60:315-325.

51. Crawford DC, Chobanian AV, Brecher P. Angiotensin II-induced fibronectin expression is associated with cardiac fibrosis in the rat. *Circulation.* 1994; 74: 727-739.

52. Kupfahl C et al. Angiotensin II directly increases transforming growth factor beta 1 and osteopontin and indirectly affects collagen mRNA expression in the human heart. *Cardiovasc Res.* 2000; 46:463-475.

53. Weber KT. Extracellular matrix remodeling in heart failure. A role for de novo angiotensin II generation. *Circulation.* 1997; 96: 4065-4082.

54. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. Fibrosis and the renin-angiotensin aldosterone system. *Circulation.* 1990; 83: 1849-1865.

55. Weber KT, Sun T, Tyagi SC, et al. Collagen network of the myocardium: function, structural remodeling and regulatory mechanisms. *J Mol Cell Cardiol.* 1994; 26: 279-292.

56. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev.* 2000; 52(1): 11-33.

57. Wei C, Lerman A, Rodeheffer R, et al. Endothelin in human congestive heart failure. *Circulation.* 1994; 89: 1580-1586.

58. Gasparo M, Catt KJ, Inagami T, et al. International union of pharmacology. XXIII. The angiotensin receptors. *Pharmacol Rev.* 2000; 415:472.

59. Yano M, Kim S, Izumo Y, et al. Differential activation of cardiac c-Jun amino-terminal kinase and extracellular signal-related kinase in angiotensin II-mediated hypertension, *Circ Res.* 1998; 83: 752-760.

60. Zou Y, Komuro I, Yamazaki T, et al. Cell type-specific angiotensin II-evoked signal transduction pathways: critical roles of Gbetagamma subunit, Src Family, and Ras in cardiac fibroblasts. *Circ Res*. 1998; 82: 337-345.

61. Sadoshima J, Qiu Z, Morgan JP, and Izumo S. Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kDS6 kinase in cardiac myocytes: The critical role of Ca^{2+}-dependent signaling. *Circ Res* .1995; 76: 1-15.

62. Sadoshima J and Izumo S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol*. 1997; 59**:** 551-571.

63. Serneri GGN, Boddi M, Cecioni I, et al. Cardiac angiotensin II formation in the clinical course of heart failure and its relationship with left ventricular function. *Circ Res*. 2001; 88: 961-968.

64. Hao J, Wang B, Jones SC, et al. Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and in vitro. *AJP*. 2000; 279(6): H3020-H3030.

65. Ma M, Watanable K, Wahed MI, et al. Inhibition of progression of heart failure and expression of TGF- beta 1 mRNA in rats with heart failure by the ACE inhibitor quinapril. *J Cardiovasc Pharmacol*. 2001; 38 (suppl 1): S1-S4.

66. Garg R, Yusuf S. Overview of randomized trials of angiotensin converting enzyme inhibitors on morbidity and mortality in patients with heart failure. Collaborative group on ACE-inhibitor trials. *JAMA*. 1995; 273: 1450-1456.

67. Paradis P, Dali-Yousef N, Paradis FW, et al. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *PNAS*. 2000; 97: 931-936.

68. Leri A, Claudio PP, Li Q, et al. Stretch-mediated release of angiotensin II induces myocytes apoptosis by activating P53 that enhances the local renin angiotensin system and decreases the Bcl-2-to-Bax protein ratio in the cell. *J Clin Invest.* 1998; 101(7): 1326-1342.

69. Smith HJ, Nuttal A. Experimental models of heart failure. *Cardiovasc Res.* 1985; 19: 181-186.

70. Yarbrough WM, Spinale FG. Large animal models of congestive heart failure: a critical step in translating basic observations into clinical applications. *J Nucl Cardiol.* 2003; 10 (1): 77-86.

71. Chu G, Haghighi K, Kranias EG. From mouse to man: understanding heart failure through genetically altered mouse models. *J Card Fail*. 2002; 8 (suppl 6): S432-S449.

72. Gronefeld GC, Hohnloser SH. Heart failure complicated by atrial fibrillation: mechanistic, prognostic, and therapeutic implications. *J Cardiovasc Pharmacol Ther*. 2003; 8(2): 107-113.

73. Heinke YM, Yao M, Chang D, et al. Apoptosis of ventricular and atrial myocytes from pacing-induced canine heart failure. *Cardiovasc Res*. 2001; 127-134.

74. Nakajima H, Nakajima HO, Salcher O, et al. Atrial but not ventricular fibrosis in mice expressing a mutant transforming growth factor-β1 transgene in the heart. *Circ Res*. 2000; 86:571-579.

75. Warnecke C, Surder D, Curth R. Analysis and functional characterization of alternatively spliced angiotensin II type 1 and 2 transcripts in the human heart. *J Mol Med.* 1999; 77: 718-727.

76. Khadour FH, O'Brien DW, Fu Y, et al. Endothelial nitric oxide synthase increases in left atria of dogs with pacing-induced heart failure. *Am J Physiol Heart Circ Physiol.* 1998; 275: H1971-H1978.

77. Vermeulen JT. Mechanisms of arrhythmias in heart failure. *J Cardiovasc Electrophysiol.* 1998; 9: 208-221.

78. Nattel S. Therapeutic implications of atrial fibrillation mechanisms: can mechanistic insights be used to improve AF management? *Cardiovasc Res.* 2002; 54: 347-360.

79. Nattel S, Li D, Yue L. Basic mechanisms of atrial fibrillation - very new insights into very old ideas. *Ann Rev Physiol.* 2000; 62: 51-77.

80. Maisel WH, Stevenson LW. Atrial fibrillation in heart failure: Epidemiology, pathophysiology, and rationale for therapy. *Am J Cardiol.* 2003; 91(suppl): 2D-8D.

81. Ehrlich JR, Nattel S, Hohnloser SH. Atrial fibrillation and congestive heart failure: Specific considerations at the intersection of two common and important cardiac disease sets. *J Cardiovasc Electrophysiol* 13: 399-405, 2002.

82. Li D, Fareh S, Leung TK, et al. Promotion of atrial fibrillation by heart failure in dogs: atrial remodeling of a different sort. *Circulation.* 1999; 100:87-95.

83. Boixel C, Fontaine V, Rucker-Martin C et al. Fibrosis of the left atria during progression of heart failure is associated with increased matrix metalloproteinases in the rat. *J Am Coll Cardiol.* 2003; 42:336-344.

84. Shinagawa K, Shi Y-F, Tardif JC, Leung T-K, Nattel S: Dynamic nature of the atrial fibrillation substrate during the development and reversal of heart failure in dogs. *Circulation.* 2002; 105: 2672-2678.

85. Goette A, Staack T, Rocken C et al. Increased expression of extracellular signal-regulated kinase and angiotensin-converting enzyme in human atria during atrial fibrillation. *J Am Coll Cardiol.* 2000;35:1669-1677.

86. Barlucchi L, Leri A, Dostal D, et al. Canine ventricular myocytes possess a renin-angiotensin system that is upregulated with heart failure. *Circ Res.* 2001; 88:298-304.

87. Cesselli D, Jakonuik I, Barlucchi L, et al. Oxidative stress-mediated cardiac cell death is a major determinant of ventricular dysfunction and failure in dog-dilated cardiomyopathy. *Circ Res.* 2001; 89(3): 279-286.

88. Olgin JE, Verheule S. Transgenic and knockout mouse models of atrial arrhythmias. *Cardiovasc Res.* 2002; 54(2): 280-6.

89. el-Agroudy AE, Hassan NA, Foda MA et al. Effect of angiotensin II receptor blocker on plasma levels of TGF-beta 1 and interstitial fibrosis in hypertensive kidney transplant patients. *Am J Nephrol.* 2003;23:300-306.

90. Koh GY, Kim SJ, Klug MG, et al. Targeted expression of transforming growth factor-β1 on intracardiac grafts promotes vascular endothelial cell DNA synthesis. *J Clin Invest.* 1995; 95: 114-121.

91. Schulz R, Aker S, Belosjorow S et al. Stress kinase phosphorylation is increased in pacing-induced heart failure in rabbits. *Am J Physiol (Heart Circ Physiol)* 2003; 285: H2084-H2090.

92. Chung MK, Martin DO, Sprecher D, et al. C-reactive protein elevation in patients with atrial arrhythmias: inflammatory mechanisms and persistence of atrial fibrillation. *Circulation.* 2001; 104(24): 2886-91.

93. Nakamura R, Egashira K, Machida Y, et al. Probucol attenuates left ventricular dysfunction and remodeling in tachycardia-induced heart failure: role of oxidative stress and inflammation. *Circulation.* 2002; 106(3): 362-367.

94. Brilla CG, Janiki JS, Weber KT. Impaired diastolic function and coronary reserve in genetic hypertension: role of interstitial fibrosis and medial thickening of intramyocardial coronary arteries. *Circ Res.* 1991; 69: 107-115.

95. Hasenfuss G. Animal models of human cardiovascular disease, heart failure and hypertrophy. *Cardiovasc Res.* 1998; 39(1):60-76.

96. Umana E, Solares CA, Alpert MA. Tachycardia-induced cardiomyopathy. *Am J Med.* 2003; 114(1): 51-5

97. Nickenig G, Harrison DG. The AT1-type angiotensin receptor in oxidative stress and atherogenesis. Part II: AT1 receptor regulation. *Circulation.* 2002; 105: 530-536.

98. Jiang L, Tsubakihara M, Heinke M.Y. Heart failure and apoptosis: Electrophoretic methods support data from micro- and macro-arrays. A critical review of genomics and proteomics. *Proteomics* 2001; 1: 1481–1488

6. Appendix