

**Reverse remodelling in a rat model of adrenergic-
induced cardiac dilatation and pump dysfunction.**

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ABSTRACT

In-part through a decrease in cardiac cavity dimensions (reverse remodelling), β -adrenergic receptor blockers have been demonstrated to produce marked benefits to morbidity and mortality in patients with chronic heart failure. However, maximum doses of these agents are often difficult to achieve in patients with chronic heart failure because of the negative inotropic, hypotensive and other side effects. Whether blockade of the excessive adrenergic effects achieves complete reverse remodelling in progressive heart failure is nevertheless uncertain. To test this hypothesis I simulated the adverse effects of chronic adrenergic stimulation on the heart by administering daily doses of the β -adrenergic receptor agonist, isoproterenol (ISO) (2.42×10^{-8} mmol.kg⁻¹) to rats for 6 months and compared left ventricular (LV) dimensions and systolic function to Saline-vehicle treated rats. To imitate the effects of complete adrenergic receptor blockade following the development of adrenergic-induced adverse cardiac changes, I similarly administered ISO for 6 months and then subsequently withdrew the daily ISO administration for a further 4 months (ISO+Recovery) before comparing left ventricular dimensions and function to Saline+Recovery treated rats.

In comparison to a Saline vehicle-treated group, after 6 months of ISO administration, LV end diastolic and systolic diameters, and the volume intercept of the left ventricular diastolic pressure-volume relationship (LV V_0), were markedly increased and LV endocardial fractional shortening (FS_{end}), LV end systolic chamber (slope of the systolic pressure-volume relationship- E_{es}) and myocardial (slope of the systolic stress-strain relationship- E_n) contractility were substantially decreased. The extent of the adverse remodelling produced by chronic ISO administration was exemplified by the 2.5 times increase in LV V_0 (ISO= 0.40 ± 0.04 vs Saline= 0.16 ± 0.01 , $p < 0.001$), a change proportionate to that noted in humans with chronic heart failure.

The proportion of ISO-treated rats with LV chamber diameters, and LV V_0 values above the 95% confidence interval for Saline-treated rats was markedly greater than the proportion of Saline-treated rats above their own 95% confidence intervals. Moreover, the proportion of ISO-treated rats with FS_{end}, LV Ees and LV En values below the 95% confidence interval for Saline-treated rats was markedly greater than the proportion of Saline-treated rats below their own 95% confidence intervals.

Following a 6 month period of ISO administration and a subsequent period of withdrawal of ISO administration for a further 4 months, LV chamber diameters, LV V_0 , FS_{end}, LV Ees and LV En were all noted to be similar to age-matched Saline+Recovery control rats. Indeed, the increases in LV V_0 observed after 6 months of ISO administration were completely reversed (ISO+Recovery=0.21±0.02 vs Saline=0.23±0.02, $p<0.001$). The proportion of ISO+Recovery rats with LV chamber diameters, and LV V_0 values above the 95% confidence interval for the Saline+Recovery rats was similar to the proportion of Saline+Recovery rats above their own 95% confidence intervals. Moreover, the proportion of ISO+Recovery rats with FS_{end}, LV Ees and LV En values below the 95% confidence interval for Saline+Recovery rats was similar to the proportion of Saline+Recovery rats below their own 95% confidence intervals. Chronic ISO administration and the withdrawal of ISO administration was not associated with changes in myocardial necrosis (pathological score and myocardial collagen concentrations).

In conclusion, marked cardiac dilatation and pump dysfunction produced by chronic β -adrenergic receptor activation can be completely reversed by withdrawal of the excessive adrenergic stimulus. These data highlight the importance in chronic heart failure of achieving complete blockade of the pathways activated by excessive β -adrenergic receptor stimulation even in individuals with advanced cardiac dilatation.

DECLARATION

I Hendrik Le Roux Booysen declare that the work contained in this dissertation is my own, unaided work. It is being submitted for the degree of Masters of Science in Medicine in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this university, or any other university.

.....

Hendrik Le Roux Booysen

Signed on.....day of, 2011

I certify that the studies contained in this thesis have the approval of the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg. The ethics clearance number is 2008/19/04.

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Hendrik Le Roux Booysen

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CONFERENCE PROCEEDINGS AND PRESENTATIONS

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LIST OF ABBREVIATIONS

AESC	=	Animal Ethics Screening Committee
α	=	Alpha
ANOVA	=	Analysis of variance
Bax protein	=	Bcl-2-associated X protein
Bcl-2 protein	=	B-cell lymphoma 2 protein
beats.min ⁻¹	=	beats per min
β	=	Beta
BW	=	Body weight
Ca ²⁺	=	Calcium
CaCl ₂	=	Calcium chloride
cAMP	=	adenosine 3',5'-cyclic monophosphate
CI	=	Confidence interval
CHF	=	Chronic heart failure
CNBr	=	Cyanogen bromide
CO ₂	=	Carbon dioxide
DAB	=	Diaminobenzidine
°C	=	Degrees centigrade
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
E	=	Slope of the linear portion of the LV peak systolic relation
En	=	Slope of the systolic (σ)-strain relation
FS	=	Fractional shortening
FS _{end}	=	Endocardial fractional shortening
FS _{mid}	=	Midwall fractional shortening

g	=	Grams
g.kg ⁻¹	=	Grams per kilogram
HCl	=	Hydrochloric acid
[HPRO]	=	Hydroxyproline concentration
HW	=	Heart weight
IDC	=	Idiopathic dilated cardiomyopathy
ISO	=	Isoproterenol
KCl	=	Potassium chloride
KH ₂ PO ₄	=	Potassium dihydrogen phosphate
LVAD	=	Left ventricular assist device
LV	=	Left ventricular
LVED	=	Left ventricular end diastole
LVEDD	=	Left ventricular end diastolic internal diameter
LVEDV	=	Left ventricular end diastolic volume
LV Ees	=	Left ventricular end systolic elastance
LVES	=	Left ventricular end systole
LVESD`	=	Left ventricular end systolic internal diameter
LVV	=	Left ventricular volume
LWW	=	Left ventricular weight
mg	=	Milligrams
µg.mg ⁻¹	=	Micrograms per milligram
mg.kg ⁻¹	=	Milligram per kilogram
µg.ml ⁻¹	=	Micrograms per milliliter
MgSO ₄	=	Magnesium sulfate
MHz	=	Megahertz
ml	=	Milliliter
µl	=	Microliter
ml.m ⁻²	=	milliliter per meter squared

ml.min ⁻¹ .g	=	Milliliter per minute per gram
mM	=	Millimolar
µm	=	Micrometer
mm Hg	=	millimeters of mercury
mmol.kg ⁻¹	=	millimoles per kilogram
MMP	=	Metalloproteinase
n	=	Sample size
NaCl	=	Sodium chloride
NaHCO ₃	=	Sodium bicarbonate
O ₂	=	Oxygen
OH	=	Hydroxide
p value	=	Probability value
PBS	=	Phosphate buffered saline
PWED	=	Posterior wall thickness at end diastole
PWES	=	Posterior wall thickness at end systole
PWT	=	Posterior wall thickness
P-V	=	Pressure-volume
r ²	=	Coefficient of determination
rTDT	=	Reverse terminal deoxynucleotidyl transferase
SD	=	Sprague-Dawely
SEM	=	Standard error of the mean
SSC	=	Saline-sodium citrate
Streptavidin HRP	=	Sterptavidin horseradish peroxidase
TdT	=	Terminal deoxynucleotidyl transferase
V	=	Volume
V _o	=	Unstressed left ventricular volume

PREFACE

Despite impressive advances over the past 10-20 years in the treatment of chronic heart failure, chronic heart failure still carries a considerable morbidity and mortality. Hence, an important aim in the care of patients with chronic heart failure is to achieve optimal therapy. One of the most important recently developed successful therapeutic approaches in chronic heart failure, an approach demonstrated in large scale clinical trials, has been the use of β -adrenergic receptor blocker (β -blocker) therapy. β -blockers, in-part by producing a decrease in cardiac cavity volumes or dimensions (i.e. attenuates cardiac dilatation) and hence improving pump function, have been shown to decrease morbidity and mortality in a number of forms of chronic heart failure including in patients with severe heart failure. However, because of the negative inotropic and hypotensive effects of β -blocker therapy, as well as additional side effect profiles produced by these agents, many patients with chronic heart failure, particularly those with advanced cardiac dilatation and pump dysfunction, cannot achieve maximal doses of these agents or may take a considerable period of time to achieve maximal doses of β -blocker therapy. Thus, these patients may be disadvantaged by low dose β -blocker therapy, a lack of β -blocker therapy or an extended period of time taken to achieve clinically important doses.

As adrenergic activation promotes cardiac dilatation and pump dysfunction in-part through myocardial changes which are associated with cardiac damage and cardiomyocyte cell slippage, whether β -blocker therapy is able to completely reverse adrenergic-induced cardiac dilatation is nevertheless still uncertain. No clinical or preclinical studies have demonstrated a capacity for complete reversal of adrenergic-mediated cardiac dilatation and pump dysfunction. If complete reversal is possible this should encourage further work in identifying downstream molecular and cellular targets from β -adrenergic receptors that are responsible for cardiac dilatation and pump dysfunction without mediating adrenergic-inotropic effects. In the present

dissertation I therefore explored whether marked cardiac dilatation and pump dysfunction mediated by chronic β -adrenergic receptor activation can be reversed by removal of the β -adrenergic receptor stimulus.

In the present dissertation in Chapter 1 I provide a review of the important scientific literature that describes the adverse effects of chronic adrenergic activation on the heart and argues in favour of performing the study described. In Chapters 2 and 3 I describe the methodology employed and the results obtained respectively. In Chapter 4 I discuss the results of the study in the context of the scientific literature described in Chapter 1; I highlight how the results of the dissertation extend our knowledge of the field; I underscore the strengths and limitations of the study and I suggest potential clinical and scientific implications of the study.

Chapter 1

Introduction

Adrenergic-induced cardiac chamber dilatation
in heart failure

1.1. Introduction

Data obtained from earlier studies conducted by the Framingham Heart Study indicated that ~1 % of 50 to 59 year olds in the United States of America (USA) may have had heart failure in the late 1980's and early 1990's and that the prevalence rate was noted to double with each decade of age thereafter (Armstrong & Moe, 1993). The incidence rate for heart failure in the USA has remained high and approximately 550 000 new cases of heart failure were reported on in 1999 (Levy *et al.*, 2002). Thus, in any one year, ~2 million people in the USA may be affected by heart failure, the consequence being an estimated \$9 billion in annual costs for hospitalisations, medical care and loss of skills (Armstrong & Moe, 1993). These figures are thought to be similar to a variety of nations with developed socio-economic infrastructures. However, the burden of heart failure is not restricted to developed countries. Indeed, in a recent clinical audit conducted in a hospital that services an urban developing community in South Africa, of the total cases reported on in a cardiology unit, 44% had heart failure (Stewart *et al.*, 2008).

Heart failure is a progressive condition that contributes to a considerable proportion of morbidity and mortality (Cowie *et al.*, 2000, Mosterd *et al.*, 2001). From the time of diagnosis, survival rates in people with heart failure are often comparable with malignancies with the worst possible outcomes (Lenfant, 1994, Stewart *et al.*, 2001, Hobbs, 2004) and it is estimated that approximately 287 200 deaths in the USA are attributed to heart failure every year (Levy *et al.*, 2002).

Heart failure represents the sum of multiple anatomical, physiological, cellular and molecular alterations that translate into a complex clinical syndrome. Heart failure may occur as a consequence of either an abrupt or acute event such as a myocardial

infarct following atheromatous plaque rupture and coronary artery occlusion, or myocarditis, or following an insidious process such as in conditions of pressure or volume overload of the heart, valvular disorders, or in hereditary or other forms of cardiomyopathies. Over the past three decades striking advances have been made in identifying the haemodynamic, neurohumoral, genetic, cell signalling and molecular pathways associated with cardiac abnormalities and disease progression in heart failure. A number of these discoveries have led to the development of novel therapeutic approaches to improving survival rates in heart failure, therapeutic approaches that are designed to target the mechanisms of disease progression, rather than the cause of the heart failure *per se* (Olson, 2004). In this regard, some of the most successful advances have been based on our understanding of the adrenergic mechanisms responsible for the progression of heart failure. Thus, many of the effective therapies in heart failure have been those that target adrenergic changes in heart failure (Packer *et al.*, 1996, 2001, MERIT-HF, 1999, Lechat *et al.*, 1997, CIBIS-II 1999, Domanski *et al.*, 2003, Poole-Wilson *et al.*, 2003, Flather *et al.*, 2005, Butler *et al.*, 2006, Hernandez *et al.*, 2009). Despite the success of adrenergic blockers, even with the best care the average 5-year survival rate of patients with heart failure is still only one quarter to one third of the survival rates of age-matched healthy counterparts from the time of discharge from hospital (Shahar *et al.*, 2004). What has not necessarily received careful consideration in the current scientific literature is the reasons for the inability to prevent mortality in a significant number of patients with heart failure.

In the present dissertation I pose the question that because many of the cardiac changes responsible for progressive heart failure are through advanced adrenergic-induced structural alterations in the heart, and that advanced structural alterations may be irreparable, that adrenergic-induced cardiac changes may therefore only be partially

reversed. Therefore in chapter 1 of the present dissertation, I will first describe the evidence to show that adrenergic activation contributes to progressive heart failure and consequently that adrenergic blockade is able to save lives in patients with heart failure. I will highlight the fact that the ability of adrenergic activation to increase mortality and the capacity of adrenergic blockade to save lives in heart failure may be attributed in-part to the ability to produce beneficial effects on cardiac structural remodelling associated with cardiac dilatation. Subsequently I will outline the potential cellular and molecular mechanisms involved in adrenergic-induced cardiac dilatation. I will then describe the evidence or the lack thereof to show reversibility of a number of these changes in clinical studies.

Essential to an understanding of the mechanisms of the adverse effects of adrenergic activation in heart failure and the benefits of adrenergic blockade is an understanding of the characteristic features of the different pathophysiological mechanisms responsible for heart failure. Thus, before addressing the aforementioned issues, I will first describe the general pathophysiological processes responsible for heart failure that are thought to be driven in-part by adrenergic activation.

1.2 Cardiac dysfunction in heart failure

Heart failure is a clinical syndrome which from a pathophysiological perspective may occur as a consequence of changes in systolic and/or diastolic function of the heart or in association with high or low output states. High output cardiac failure generally occurs as a result of a high venous return which produces an increase in cardiac filling and hence an enhanced cardiac output through the Frank-Starling effect. Even though the cardiac output is high, it is either inadequate for the bodies energy requirements

during exercise (the heart is performing at a peak level of performance even at rest) or, through increases in cardiac filling, results in excessive venous and capillary hydrostatic pressures in both the lungs and the systemic circulation. In contrast to high output heart failure where the heart muscle may have a normal or relatively normal function, there are two broad groups of pathophysiological abnormalities that affect the function of the heart muscle *per se*. In this regard, patients with chronic heart failure may develop diastolic (primary disorder of filling) or systolic (primary disorder of emptying) heart failure. As the characteristic pathophysiological mechanisms that distinguish diastolic from systolic heart failure have been employed to define cardiac changes in the present dissertation, these changes will be discussed in subsequent sections.

1.2.1 Diastolic dysfunction and diastolic heart failure

Diastolic heart failure is a previously unappreciated but nevertheless common cause of chronic heart failure (Vasan *et al.* 1999), accounting for a significant proportion of mortality and morbidity (Zile *et al.*, 2005). In 1988 Kessler first defined the term “diastolic” heart failure to identify a group of patients with chronic heart failure characterized by concentric cardiac remodelling (increased cardiac chamber wall thickness to radius ratio) with a normal or even reduced left ventricular filling volume and abnormal left ventricular diastolic features such as a slow or delayed relaxation with an increased cardiac stiffness (Kessler, 1988). Diastolic cardiac dysfunction refers to an abnormal mechanical property and not a clinical syndrome whereas diastolic heart failure is a clinical syndrome, identified by characteristic signs and symptoms of heart failure, but that is nevertheless associated with a relatively normal systolic function, but a reduced diastolic function (Vasan *et al.*, 1999, Zile *et al.*, 2004, 2005). Diastolic

dysfunction occurs when the ability of the ventricular myocardium to return to a relaxed unstressed length is prolonged, slowed or incomplete.

Measurements that reflect an abnormal diastolic function depend on the onset, rate, and extent of ventricular pressure decline and filling and the relationship between the pressure and volume or the stress and strain observed during diastole (Gilbert & Glantz, 1989). A characteristic feature of diastolic cardiac dysfunction (Gilbert & Glantz 1989) and diastolic heart failure (Zile *et al.*, 2004) is an increased filling pressure for a given filling volume subsequent to a reduced cardiac chamber compliance or an increased chamber stiffness. Indeed, patients with diastolic heart failure show a cardiac diastolic pressure-volume relationship that appears similar to that depicted in Figure 1.1 (Zile *et al.*, 2004). Figure 1.1 shows typical changes in left ventricular end diastolic pressure-volume relations in patients with diastolic heart failure, where ventricular cavity size and end-diastolic volumes (or filling volumes) may remain normal or even decrease, whilst ventricular filling pressures are elevated for a given filling volume (Zile *et al.*, 2004, Chatterjee & Massie, 2007). The mechanism responsible for the abnormal diastolic pressure-volume relationship is a decreased chamber compliance or an increased chamber stiffness as indicated by the steeper slope of the diastolic pressure-volume relationship (Figure 1.1) (Zile *et al.*, 2004). The increased filling pressures that occur at normal filling volumes are responsible for the development of pulmonary congestion (left heart failure) and thus ultimately the development of right-sided heart failure.

Although there are a number of causes of diastolic heart failure that are not necessarily associated with myocardial abnormalities (e.g. pericardial disease), diastolic heart failure is nevertheless most frequently associated with abnormalities of the myocardium. In patients with diastolic heart failure, cardiomyocyte sarcomeres may replicate in parallel, thus increasing myocyte cross-sectional area with no change in the

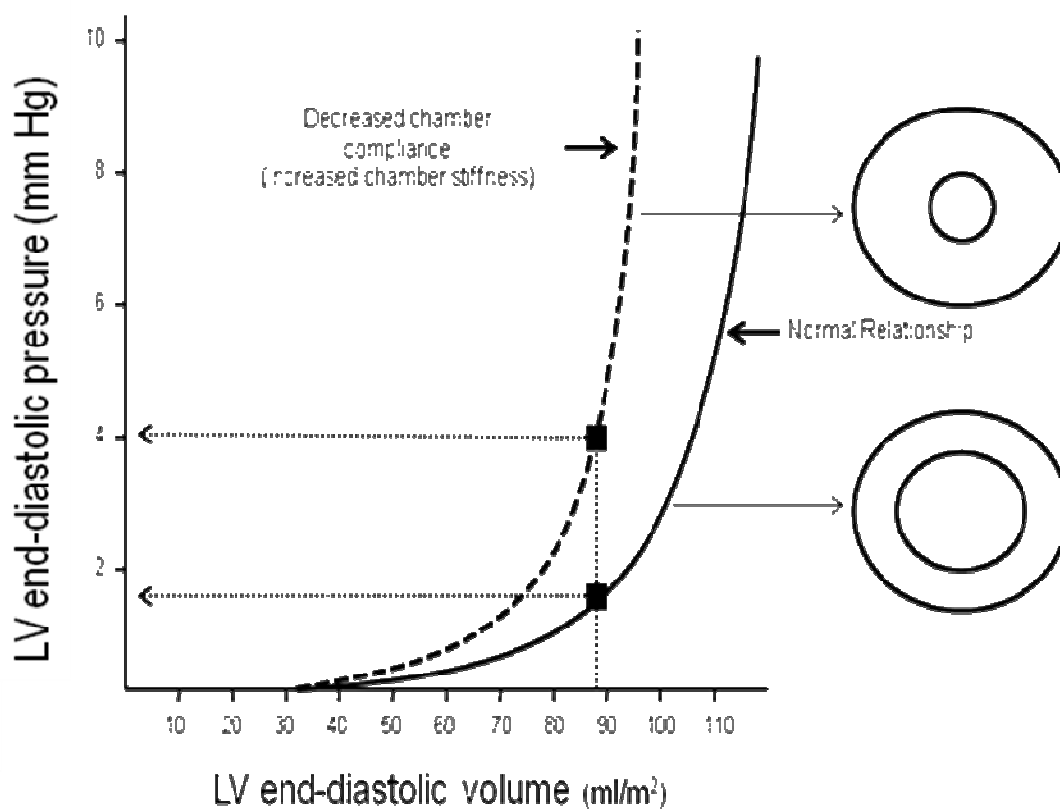


Figure 1.1 Left ventricular (LV) end diastolic pressure-volume relationships and cartoon of the geometry of the heart associated with these relationships (right side) in normal hearts and in hearts with diastolic heart failure. The figure shows a normal relationship (solid line) and the change in the relationship when chamber compliance decreases or stiffness increases (dashed line) in diastolic heart failure. The effect of a less compliant chamber on diastolic pressure is illustrated by the dashed arrow (left panel).

length-to-width ratio (Chatterjee & Massie, 2007). These changes in myocyte remodelling are fundamental to the marked increases in wall thickness that may occur (concentric cardiac remodelling) and this increased wall thickness may contribute toward the stiffer chamber (see Figure 1.1). Furthermore, alterations in the interstitial properties of the myocardium may occur in hearts with diastolic dysfunction, with increases in total collagen concentrations or in the concentrations of myocardial collagen with increased cross-linked properties (Norton *et al.*, 1996, Norton *et al.*, 1997, Badenhorst *et al.*, 2003a). The increased myocardial collagen with enhanced cross-linked properties determines the material properties of the heart (myocardial passive stiffness) and hence, also contributes toward an increased chamber stiffness or decreased chamber compliance (Norton *et al.*, 1996, Norton *et al.*, 1997, Badenhorst *et al.*, 2003a). Alternatively, in diastolic heart failure the capacity of the myocardium to actively relax may be impaired through a number of mechanisms including a reduced capacity of the sarcoplasmic reticulum to actively sequester Ca^{2+} ions (Sordahl *et al.*, 1973). An inability of the myocardium to relax will result in an attenuated ventricular filling and hence an enhanced filling pressure and subsequently the clinical signs of heart failure such as pulmonary congestion and peripheral oedema.

1.2.2 Systolic dysfunction and systolic heart failure

Systolic heart failure is a well-recognised cause of chronic heart failure also accounting for a significant proportion of mortality and morbidity (Zile *et al.*, 2005). Systolic heart failure is associated with a reduced ability of cardiac myofibrils to shorten and hence a decreased ability of the ventricle to eject blood despite a normal or even increased ventricular filling volume. The characteristic feature of systolic heart failure is

heart failure associated with a decreased systolic performance, most frequently determined as ventricular ejection fraction (stroke volume/end diastolic filling volume). The reduction in systolic performance may be attributed to an increased load on the heart, or a decreased myocardial or chamber contractility (defined in experimental studies as end systolic elastance or the slope of the end systolic pressure-volume relationship, which is a load-independent measure of systolic function).

In contrast to heart failure produced by diastolic dysfunction of the left ventricle, which is associated with a left shift in the left ventricular pressure-volume relationship and concentric cardiac remodelling (Figure 1.1), heart failure produced by systolic dysfunction of the left ventricle is associated with a right shift in the left ventricular diastolic pressure-volume relationship with ventricular eccentric chamber remodelling or cardiac chamber dilatation (Figure 1.2). In cardiac dilatation the ventricular cavity size is enlarged resulting in increases in both end-diastolic and end-systolic volumes. However, ventricular wall thickness may be unchanged or even decreased as a consequence of the chamber dilatation. Nevertheless, in contrast to diastolic heart failure where the cardiac chamber is stiff or non-compliant (compare slopes of relationships in Figure 1.1), in systolic heart failure cardiac passive stiffness and compliance may be unchanged (compare slopes of the relationships in Figure 1.2). Presumably the potential benefits of this process are to maintain normal filling pressures despite increases in filling volumes that occur as a consequence of a reduced ventricular ejection (note the greater filling volumes for the same filling pressure in Figure 1.2). However, as will be discussed in subsequent sections (section 1.3), cardiac dilatation is an advanced structural change that is well-recognised as being associated with morbidity and mortality in heart failure.

Importantly, from a structural perspective there are therefore distinct and characteristic differences in the ventricular chamber remodelling processes that

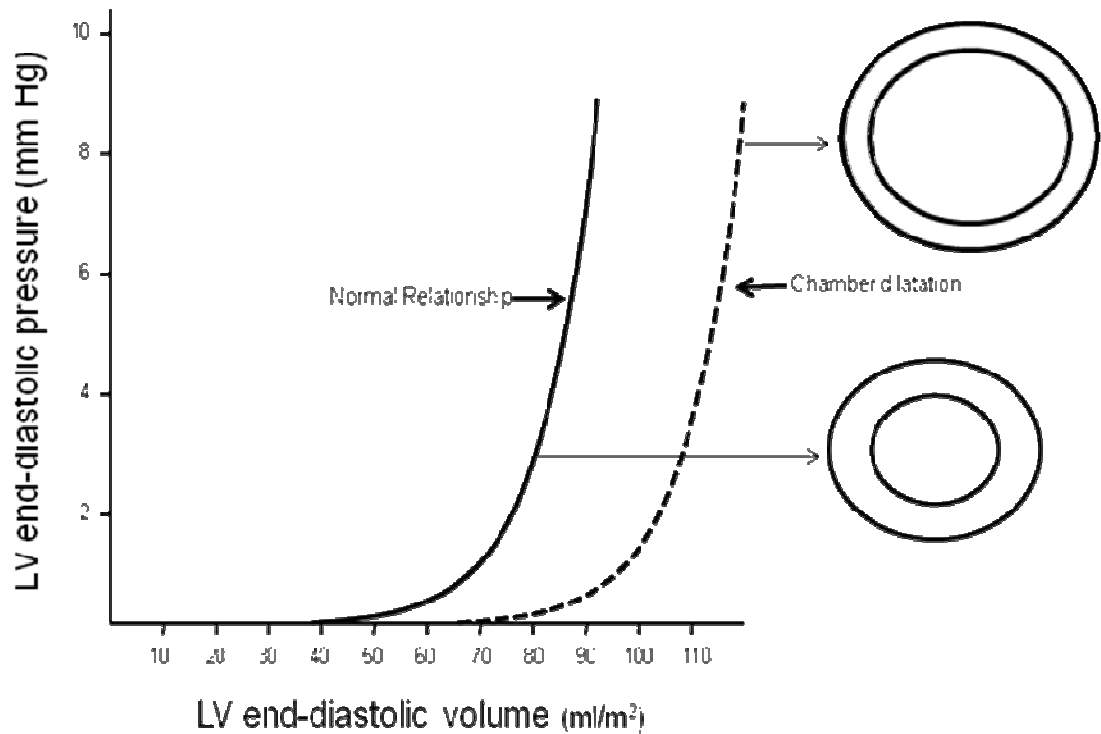


Figure 1.2 Left ventricular (LV) end diastolic pressure-volume relationships showing a normal relationship (continuous line) and the change in the relationship with cardiac chamber dilatation (dashed line). The change in left ventricular geometry associated with cardiac dilatation is depicted in the cartoon on the right.

accompany systolic as opposed to diastolic heart failure. These characteristic features are largely summarized in Table 1.1. What is important to note is that one of the primary distinguishing features that characterises systolic from diastolic heart failure is an increase in chamber dimensions in systolic heart failure.

1.3 Cardiac dilatation as a cause of cardiac dysfunction and failure.

As previously indicated, the potential benefits of cardiac dilatation is to maintain normal filling pressures despite increases in filling volumes that occur as a consequence of a reduced ventricular ejection. However, the question arises as to whether cardiac dilatation is indeed a change that benefits patients with heart failure? In this regard, in keeping with La Place's Law, where wall stress or tension is proportional to radius and inversely proportional to wall thickness, in systolic heart failure, the structural remodelling process (increased chamber volumes, but unchanged or decreased wall thickness) results in an increased wall stress. An increased cardiac wall stress is major determinant of a reduced pump function and indeed, in heart failure the lowest values for pump (systolic) function are associated with the cardiac highest cavity volumes (Norton *et al.*, 2002).

An alternative mechanism through which cardiac dilatation may contribute toward pump dysfunction is through alterations in chamber shape. With cardiac dilatation, the shape of the heart changes from a normal ellipsoid to a more spherical shape. As an elliptical shape is required to generate appropriate ventricular ejection during torsion (Sallin, 1969), cardiac dilatation may further reduce pump function through shape changes. Indeed, left ventricular shape changes predict mortality in dilated hearts (Douglas *et al.*, 1989). Thus, although cardiac dilatation may have benefits by

Table 1.1 Structural and functional changes in systolic versus diastolic heart failure.

Parameters	Systolic heart failure	Diastolic heart failure
Left ventricular Mass	Increased	Increased
Left ventricular cavity size	Increased	Increased or normal
Mass/Cavity	Decreased	Increased
Ejection fraction	Decreased	Normal
Wall thickness	Decreased	Increased
End-systolic stress	Increased	Normal
End-diastolic stress	Increased	Increased
End-systolic volume	Increased	Decreased or normal
End-diastolic volume	Increased	Normal
Left ventricular shape	Spherical	Unchanged

accommodating increased ventricular filling volumes for a given filling pressure; this benefit could be offset by the impact of cardiac dilatation on pump function. Importantly however, there are many that still hold the view that cardiac dilatation is a consequence, rather than a cause of pump dysfunction. This view is largely derived from the fact that myocardial contractile dysfunction frequently accompanies cardiac dilatation and a reduction in myocardial contractility results in increased chamber volumes. As indicated in the aforementioned discussion, chamber dilatation is thus viewed by many as a remodelling process that accommodates the increased chamber volumes rather than a change that promotes pump dysfunction and systolic heart failure. Is there evidence to indicate that cardiac dilatation can cause pump dysfunction? Moreover, is there evidence to indicate that cardiac dilatation is associated with worse outcomes in heart failure or heralds the onset of heart failure in otherwise well individuals?

1.3.1 Association between cardiac dilatation and pump dysfunction independent of myocardial systolic (contractile) dysfunction.

There is no clinical evidence that segregates the impact of cardiac dilatation from that of myocardial systolic dysfunction on cardiac pump function or the presence of heart failure. However, a preclinical study conducted by members of our group has demonstrated that the presence of heart failure (identified from the presence of pulmonary congestion) and pump dysfunction (a reduced endocardial fractional shortening) in marked pressure overload hypertrophy produced by abdominal aortic banding is associated with a combination of cardiac dilatation and myocardial contractile disturbances, whilst myocardial contractile disturbances alone were insufficient to account for the presence of heart failure (Norton *et al.*, 2002). In this regard, animals with

pressure overload hypertrophy and concentric left ventricular remodelling had a reduced myocardial contractility, but no evidence of pulmonary congestion or pump dysfunction (Norton *et al.*, 2002). Thus, in the absence of cardiac dilatation neither pump dysfunction nor pulmonary congestion (left heart failure) were noted despite the presence of myocardial contractile disturbances (Norton *et al.*, 2002). Although these data do not exclude a role for decreases in myocardial contractility in contributing toward heart failure in pressure overload states, this study certainly provides the evidence to indicate that pump dysfunction and heart failure in pressure overload states is to some extent dependent on the presence of cardiac dilatation (Norton *et al.*, 2002). Further studies from members of our group have provided additional support for a critical role of cardiac dilatation in mediating pump dysfunction independent of myocardial contractile disturbances. Indeed, our group has demonstrated that chronic adrenergic stimulation can promote the transition from compensated cardiac hypertrophy to pump dysfunction in association with cardiac dilatation, but not with decreases in intrinsic myocardial contractile disturbances (Veliotis *et al.*, 2005, Badenhorst *et al.*, 2003b, Gibbs *et al.*, 2004, Veliotis *et al.*, 2010). Thus, preclinical studies have provided the evidence to suggest that cardiac dilatation is a necessary prerequisite for the development of pump dysfunction and subsequent systolic heart failure at least in pressure overload states and following excessive adrenergic activation.

1.3.2 Association between cardiac dilatation and pump dysfunction in clinical studies.

A number of clinical studies have provided the evidence to indicate that cardiac dilatation is associated with a worse pump function and adverse clinical outcomes in

heart failure. In this regard there are presently innumerable clinical studies, too many to cite in the present dissertation, showing strong relationships between end diastolic diameters or volumes and pump dysfunction in patients with heart failure. However, it is well accepted that in patients with heart failure, a left ventricular end diastolic diameter \geq 65 mm is associated with impaired pump function as indexed by a reduced ejection fraction ($<40\%$) (Cohn *et al.*, 2000). A number of studies assessing the impact of the medical management of heart failure also show a close relationship between changes in left ventricular dimensions and pump function. For example, of 171 patients with heart failure, in the 38 patients who responded to β -adrenergic receptor blocker therapy, who had an average initial left ventricular end diastolic volume (LVEDV) of 175 ml/m² and a left ventricular ejection fraction of 20.2%, after β -adrenergic receptor blocker therapy LVEDV decreased to 113 ml/m² and ejection fraction increased to 43% (Metra *et al.*, 2003). However, it is perhaps unsurprising that a close relationship exists between cardiac chamber volumes and ejection fraction. After all ejection fraction is calculated as a fraction of filling volumes. Is there evidence from clinical studies to indicate that filling volumes are associated with outcomes in heart failure?

1.3.3 Association between cardiac dilatation and clinical outcomes in heart failure.

With respect to the relationship between cardiac dilatation and outcomes in cardiac failure, there is no question that cardiac dilatation is a major risk factor for mortality in advanced heart failure (Nestico *et al.*, 1985, Gadsboll *et al.*, 1990, Lee *et al.*, 1993, Foley *et al.*, 1995, Foley & Parfrey, 1998). Moreover, with the treatment of heart failure, reductions in cardiac chamber volumes and dimensions are associated with

better long-term outcomes, including survival (Doughty *et al.*, 1997, Sharpe & Doughty, 1998). Furthermore, patients predicted to be at risk for long-term left ventricular dilatation have an increased risk of mortality and heart failure at 6 months (de Kam *et al.*, 2002). This knowledge of the dependence of cardiac outcomes on chamber dimensions is now sufficiently well established that measurements of chamber dimensions have been incorporated as risk predictors into guidelines for the management of heart failure (Hunt *et al.*, 2001). However, this evidence still does not establish cause and effect relationships between cardiac dilatation and cardiac outcomes. Is there evidence to indicate that cardiac dilatation precedes the development of heart failure?

1.3.4 Association between chamber dimensions and the development of heart failure.

There is now substantial evidence to indicate that cardiac dilatation is a precursor of left ventricular dysfunction and clinical heart failure (Gaudron *et al.*, 1993, Pfeffer *et al.*, 1993, Vasan *et al.*, 1997). This may occur in individuals with cardiac pathology (Gaudron *et al.*, 1993, Pfeffer *et al.*, 1993) or without any evidence of pre-existing cardiac pathology (Vasan *et al.*, 1997). Moreover, medical therapy that prevents the development of cardiac dilatation prevents the development of cardiac dysfunction (Pfeffer *et al.*, 1993). Perhaps the most important evidence from the perspective of the present dissertation, is the evidence obtained from 4744 participants of the Framingham Offspring Study, during an 11 year follow-up period where 74 participants without myocardial infarction, or pre-existing heart failure developed congestive heart failure and adjusting for age, blood pressure, body-mass index, valve disease, diabetes, hypertension treatment and myocardial infarction, left ventricular internal dimensions

contributed significantly to the risk of developing congestive heart failure (Vasan *et al.*, 1997). This evidence provides strong support for a role of cardiac dilatation as a cause of heart failure. However, an intervention study specifically targeting chamber volumes is still required to establish this hypothesis.

1.4 The role of adrenergic activation in heart failure.

There is now considerable evidence to indicate that adrenergic activation occurs in heart failure and that the extent of adrenergic activation contributes to the progression and the outcomes in heart failure. Indeed, plasma noradrenaline and adrenaline concentrations are considerably increased in patients with heart failure (Kluger *et al.*, 1982, Cohn *et al.*, 1984, Hasking *et al.*, 1986, Swedberg *et al.*, 1990, Francis *et al.*, 1993, Sigurdsson *et al.*, 1994, Esler *et al.*, 1997, Anand *et al.*, 2003) and these concentrations are related to the severity of pump dysfunction (Kluger *et al.*, 1982), the functional class (Sigurdsson *et al.*, 1994) and mortality (Cohn *et al.*, 1984, Swedberg *et al.*, 1990, Francis *et al.*, 1993, Anand *et al.*, 2003) in heart failure.

The increased circulating concentrations of catecholamines in heart failure are attributed to both an increased sympathetic spillover and a reduced clearance (Hasking *et al.*, 1986). However, sympathetic over-activity in heart failure does not involve all organs equally with a more marked spillover occurring to the kidneys and the heart (Hasking *et al.*, 1986). Indeed, norepinephrine released from the myocardium of the failing heart may be ~50 times greater than that released from a normal heart (Esler *et al.*, 1997).

Irrespective of the source of the excess sympathetic activation in heart failure, based on the aforementioned lines of evidence, a number of intervention studies have

been performed to assess the impact of adrenergic blockade on pump dysfunction and cardiovascular outcomes in patients with heart failure.

1.4.1 Adrenergic blockade has beneficial effects on pump dysfunction and outcomes in heart failure.

Adrenergic receptor blockade with either carvedilol, a non-selective α - and β -adrenergic receptor blocking agent, or metoprolol, a selective β -adrenergic receptor blocking agent improves pump function in patients with heart failure (Waagstein *et al.*, 1993a, 1993b, Doughty *et al.*, 1997, Hall *et al.*, 1995, Olsen *et al.*, 1995, Quaife *et al.*, 1996, Lowes *et al.*, 1999, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Khattar *et al.*, 2001, Ramahi *et al.*, 2001, Gerson *et al.*, 2002, Toyama *et al.*, 2003, Waagstein *et al.*, 2003, Metra *et al.*, 2003, Doughty *et al.*, 2004, Rahko *et al.*, 2005, Paraskevaidis *et al.*, 2007, Gundogdu *et al.*, 2007, Malfatto *et al.*, 2007, Lotze *et al.*, 2001). Furthermore, clinical studies have demonstrated improved survival benefits and reduced hospitalizations in patients with heart failure in response to a variety of β -adrenergic receptor blockers including carvedilol (Packer *et al.*, 1996, 2001, Poole-Wilson *et al.*, 2003), metoprolol (MERIT-HF, 1999), bisoprolol (Lechat *et al.*, 1997, CIBIS-II, 1999), and nebivolol (Flather *et al.*, 2005). In addition, in patients with heart failure, withdrawal from beta-blocker therapy after admission to hospital is associated with a marked increase in mortality as compared to those patients who continue with beta-blocker therapy (Fonarow *et al.*, 2008). The benefits of heart failure therapy on survival and hospitalisations have been demonstrated in both moderate-to-severe heart failure (Packer *et al.*, 1996, CIBIS-II, 1999) as well as mild-to-severe heart failure (MERIT-HF, 1999). These data therefore provide a high level of evidence in support of a role for

adrenergic activation in the progression of heart failure. How does adrenergic over-activation promote progressive heart failure?

1.4.2 Mechanisms of the deleterious effect of adrenergic activation: Potential role of cardiac dilatation.

Although an increased sympathetic drive in heart failure is likely to increase myocardial contractility through direct effects on myocardial adrenergic receptors, and thus act as a potential compensatory change in a failing myocardium with systolic dysfunction, as discussed in the aforementioned sections, it is now well-recognized that this change promotes progressive heart failure and mortality in heart failure. What are the potential mechanisms of these adverse effects?

From a perspective of the haemodynamic view of progressive heart failure, an increased sympathetic activation to the peripheral circulation and kidneys causes widespread vasoconstriction via α -adrenergic receptor-mediated effects. The consequence is an increase in blood pressure and fluid retention with subsequent increases in preload and afterload and an enhanced workload on the heart. Moreover, via β -adrenergic receptor-mediated effects, adrenergic activation will increase myocardial contractility and heart rate, which in the presence of associated coronary vascular impairment, is likely to result in a myocardial oxygen demand-to-supply ratio that favors oxygen demand and subsequently promotes myocardial ischaemia and further myocardial damage. However, notwithstanding the importance of the haemodynamic hypothesis of progressive heart failure, there is also substantial evidence to indicate that sympathetic activation promotes a number of deleterious effects on the myocardium which are mediated through direct neurohumoral actions on heart muscle

rather than haemodynamic effects. The neurohumoral hypothesis is in-part explained by an effect on chamber dimensions or cardiac dilatation. What is the evidence that adrenergic activation promotes progressive heart failure through cardiac dilatation?

As indicated in previous sections there is significant evidence to suggest that cardiac dilatation may contribute toward pump dysfunction. The clinical evidence to indicate that adrenergic activation may mediate cardiac dilatation and hence pump dysfunction comes from intervention studies with adrenergic blocker therapy. A number of studies have demonstrated that β -adrenergic receptor blocking agents reduce cardiac cavity dimensions (Hall *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007, Lotze *et al.*, 2001). However, decreases in cardiac cavity dimensions with adrenergic blockers may be as a consequence of an improved myocardial contractility increasing ejection volumes and thus decreasing filling volumes, rather than through direct benefits on the myocardium. Is there more direct evidence to indicate that adrenergic-induced cardiac dilatation could contribute toward pump dysfunction?

As previously indicated, our group has demonstrated that chronic β -adrenergic receptor stimulation can promote the transition from compensated cardiac hypertrophy to pump dysfunction in association with cardiac dilatation, but not with decreases in intrinsic myocardial contractile disturbances (Veliotes *et al.*, 2005, 2010, Badenhorst *et al.*, 2003b, Gibbs *et al.*, 2004). This evidence is the only direct evidence to my knowledge to indicate that adrenergic-induced cardiac dilatation is a critical mediator of the development of pump dysfunction associated with excessive sympathetic activation. What are the potential mechanisms through which adrenergic activation could promote cardiac dilatation?

1.5 Mechanisms of cardiac dilatation and a potential role for adrenergic activation in mediating these mechanisms.

Three hypotheses, illustrated in Figure 1.3, have been developed over the years that could explain the mechanism of cardiac dilatation. First, cardiomyocyte cell death (Figure 1.3) mediated either through necrosis or apoptosis may reduce the capacity to tether cardiomyocytes and hence promote side-to-side slippage. Second, cellular hypertrophy (Figure 1.3) especially cell lengthening could account for a dilated chamber. Third, alterations in myocardial collagen structure (Figure 1.3) may reduce the capacity for side-to-side cell tethering and hence could encourage cell slippage and cardiac dilatation. In the following section I will discuss the evidence to suggest a role of each of these potential mechanisms, and the evidence to support a role of adrenergic activation as a potential contributing factor toward each of these cellular changes.

1.5.1 Cellular hypertrophy

A major cardiomyocyte hypertrophic change that may explain cardiac dilatation is lengthening of cells that lie in parallel to the endocardium. In contrast to the adaptive phase of cardiomyocyte hypertrophy where concentric remodelling is the consequence of increases in cardiomyocyte cross-sectional area and diameter in proportion to increases in cell length, in the maladaptive and failing heart with cardiac dilatation,

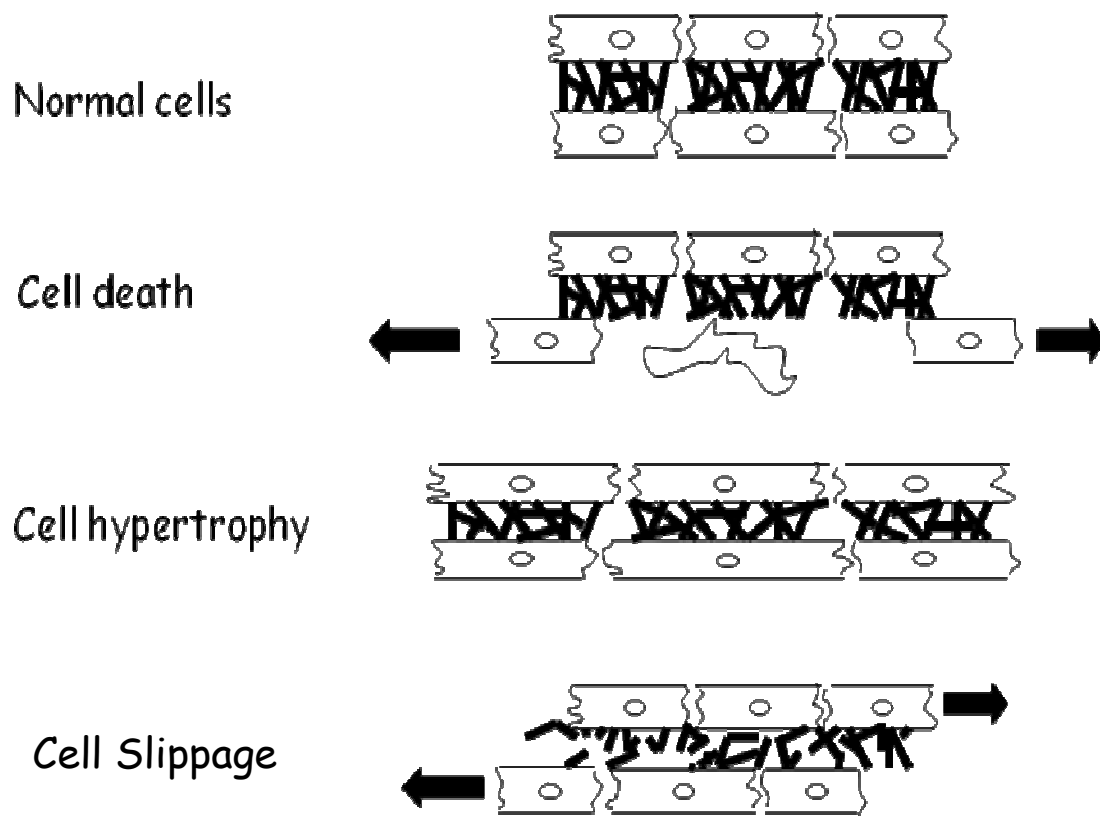


Figure 1.3 Cellular mechanisms responsible for cardiac dilatation. See text for explanation.

myocyte length may exceed width. Indeed, studies have provided the evidence to indicate that in the dilated heart, cardiomyocyte lengthening may exceed increases in cell width (Zimmer *et al.*, 1990, Spinale *et al.*, 1991, Gerdes *et al.*, 1992, Gerdes & Capasso, 1995, Tamura *et al.*, 1998). Although it is well recognised that the drive behind the cardiomyocyte hypertrophic process is through a combination of haemodynamic overload, together with neurohumoral (including adrenergic) activation and inflammatory cytokines (Tarone & Lembo, 2003), the stimulus for the transition from adaptive to maladaptive hypertrophy is nevertheless still unclear. Is there evidence to implicate adrenergic activation in promoting cell lengthening that is out of proportion to widening of cells?

There is no direct evidence to my knowledge to indicate that adrenergic stimulation could preferentially lengthen as opposed to widen cells. Moreover, there are no studies to my knowledge that have addressed the issue of whether adrenergic blockade could attenuate maladaptive hypertrophy of cardiomyocytes and convert them into concentrically remodelled cells. However, there is some clinical evidence that may cast light on whether adrenergic-induced cardiac dilatation can be explained by cardiac hypertrophy. Indeed, in patients with heart failure receiving the β -adrenergic receptor blocker metoprolol, decreases in left ventricular volumes were noted after three months of metoprolol therapy, whilst left ventricular mass only decreased in this study by 18 months of metoprolol therapy (Hall *et al.*, 1995). These data suggest that the beneficial effects of adrenergic blockade on cardiac cavity volumes in heart failure can precede alterations in left ventricular mass. Therefore, it is possible that adrenergic activation promotes cardiac dilatation and mediates pump dysfunction independent of cellular hypertrophy.

1.5.2 Cellular side-to-side slippage produced by collagen changes

Cardiomyocytes are connected in parallel at the level of the Z lines by collagen struts which insert into the sarcolemma (Robinson *et al.*, 1987). Destruction of these struts may decrease the capacity of the fibrillar collagen matrix to connect cardiomyocytes, the consequence being side-to-side slippage and hence cardiac chamber dilatation. The integrity of the collagen matrix is determined by two key features of the interstitium. Degradation of myocardial collagen may occur as a consequence of activation of collagenases or matrix metalloproteinases (MMPs), which are an endogenous family of proteolytic enzymes that degrade all components of the myocardial extracellular matrix (Gunasinghe *et al.*, 2001). A number of lines of evidence support a key role for MMPs in mediating cardiac dilatation. Indeed, an increased myocardial expression and activation of MMPs has been demonstrated in patients with congestive heart failure or in patients with a reduced systolic function and cardiac dilatation (Spinale *et al.*, 2000, Li *et al.*, 2001, Spinale, 2002, Reddy *et al.*, 2004, Polyakova *et al.*, 2004). Furthermore, an increased myocardial expression and activation of MMPs has been demonstrated in animal models of pump dysfunction and cardiac dilatation (Spinale *et al.*, 1998, Rohde *et al.*, 1999, Peterson *et al.*, 2001, Mukherjee *et al.*, 2003, King *et al.*, 2003, Sakata *et al.*, 2004). However, associations between MMP activation and cardiac dilatation do not necessarily indicate cause and effect. More direct evidence in favour of a role for myocardial MMPs in mediating cardiac dilatation is that MMP inhibition attenuates left ventricular dilatation in animal models of pacing-induced heart failure (Spinale *et al.*, 1999), myocardial infarction (Rohde *et al.*, 1999, Mukherjee *et al.*, 2003) and heart failure in the spontaneously hypertensive rat (Peterson *et al.*, 2001). Moreover, a loss of MMP inhibitory control of MMPs, through a gene deletion of

the tissue inhibitor of the matrix metalloproteinase-type 1 (TIMP-1), has been demonstrated to lead to ventricular dilatation in mice (Roten *et al.*, 2000).

Although there is substantial evidence to support a role for collagenases in contributing to cardiac dilatation, there is nevertheless still debate as to the role of changes in myocardial collagen concentrations in contributing to side-to-side cardiomyocyte slippage and hence cardiac dilatation. Although collagenase activation may mediate tears in collagen struts, this may not be accompanied by decreases in collagen concentrations. Indeed, many forms of cardiac dilatation are associated with increases in myocardial collagen concentrations, and reductions in cardiac cavity dimensions following the use of left ventricular assist devices are generally accompanied by increases and not decreases in myocardial collagen concentrations (Scheinin *et al.*, 1992, Li *et al.*, 2001). However, pacing-induced cardiac dilatation (Spinale *et al.*, 1991) and adrenergic-induced cardiac dilatation (Woodiwiss *et al.*, 2001) may be accompanied by decreases rather than increases in myocardial collagen concentrations. Nevertheless, a more recent view of how increases in myocardial collagen synthesis could contribute toward chamber dilatation is through the production of collagen that is susceptible to degradation (Woodiwiss *et al.*, 2001, Badenhorst *et al.*, 2003a). In this regard, collagen of the non-cross-linked phenotype is associated with systolic dysfunction and cardiac dilatation (Capasso *et al.*, 1989, Gunja-Smith *et al.*, 1996, Spinale *et al.*, 1991, Woodiwiss *et al.*, 2001). It is possible that non-cross-linked collagen may be more susceptible to degradation by collagenases and thus to cardiac dilatation. There is direct evidence in support of this theory. In this regard, by genetically decreasing the susceptibility of collagen to degradation, a reduced degree of dilatation accompanies pressure-overload states (Lindsey *et al.*, 2003).

What is the evidence that adrenergic activation could contribute toward changes in either myocardial collagenases or to changes in the cross-linked properties of myocardial collagen. In this regard, there is presently considerable evidence to indicate that adrenergic activation can modify the interstitium in a manner that can promote cardiac dilatation. Indeed, the β -adrenergic receptor agonist, isoproterenol, has been shown to stimulate cardiomyocyte MMP activity in isolated cardiomyocytes (Coker *et al.*, 2001). Moreover, chronic isoproterenol administration to intact animals for 5-6 months either increases the relationship between myocardial non-cross-linked and cross-linked collagen or increases the non-cross-linked collagen content of the myocardium in association with cardiac dilatation (Woodiwiss *et al.*, 2001). However, the change in the phenotypic properties of myocardial collagen in response to chronic adrenergic stimulation is unlikely to occur through direct myocardial β -adrenergic receptor-mediated effects, as these changes could be prevented by both angiotensin-converting enzyme inhibitor administration (Woodiwiss *et al.*, 2001) as well as aldosterone receptor blockade (Veliotes *et al.*, 2005, Veliotes *et al.*, 2010).

1.5.3 Apoptosis and necrosis

Cardiomyocyte cell death mediated either by tissue apoptosis or necrosis may also promote the development of cardiac dilatation (Yussman *et al.*, 2002). Although apoptosis describes an active, regulated, energy demanding process controlled by an inherited genetic program (Sabbah & Sharov, 1998), whilst necrosis is an unregulated process (Kang & Izumo, 2000), both processes ultimately result in cell death. An excessive loss of viable myocardium through apoptosis has been reported to occur as a consequence of sustained pressure overload from hypertension or aortic valvular

stenosis or from volume overload (Sabbah & Sharov, 1998). Following the initiation of the apoptotic pathway, cytochrome *c* is released from mitochondria (Narula *et al.*, 1999), which forms a cocktail with protein-activating factor-1 and caspase-9 resulting in the activation of downstream caspases (mainly caspase 9) that cause the morphological and biochemical alterations responsible for apoptosis (Li *et al.*, 1997). The multigene family of proteins that control apoptosis include the antiapoptotic Bcl-2 proteins and the proapoptotic Bax proteins (Sabbah & Sharov, 1998). The cellular alterations that accompany apoptosis include the loss of surface contact with bordering cells, cell shrinkage, and condensation of chromatin leading to fragmentation of chromosomal deoxyribonucleic acid (DNA) (Arends *et al.*, 1990).

The role of sympathetic activation in mediating cardiomyocyte cell death is well-established. Adrenergic agonists, in excessive concentrations promote both necrosis (Benjamin *et al.*, 1989, Mann *et al.*, 1992, Teerlink *et al.*, 1994) and apoptosis (Communal *et al.*, 1998, Singh *et al.*, 2001) in cardiomyocyte cell cultures and β -adrenoreceptor blockade attenuates the apoptotic effects (Communal *et al.*, 1998, Singh *et al.*, 2001). Adrenergic stimuli induce cardiomyocyte apoptosis via activation of β_1 -adrenoreceptors, cAMP-dependent pathways, protein kinase A, by increasing the ratio of Bax to Bcl-2 expression and by stimulating voltage-dependent calcium influx channels (Zaugg *et al.*, 2000). The mitogen-activated protein kinase's (MAPK) involved in the β -adrenergic signalling pathway responsible for cardiomyocyte apoptosis include the c-Jun NH₂-terminal kinase (JNK), p38 kinases, extracellular signal related kinase 1/2 (ERK 1/2) and apoptosis signal-regulating kinase 1 (ASK1) (Fan *et al.*, 2006).

Adrenergic activation may also promote cardiomyocyte apoptosis through indirect mechanisms by stimulating the renin-angiotensin-aldosterone system which is well recognised as a trigger for cardiomyocyte apoptosis (De Angelis *et al.*, 2002). Indeed,

both angiotensin II and aldosterone have the capacity to promote cardiomyocyte apoptosis (De Angelis *et al.*, 2002, Garg *et al.*, 2005). In addition, adrenergic activation may promote cardiomyocyte apoptosis through increases in myocardial cytokine expression (Baumgarten *et al.*, 2000).

1.6 Can cardiac dilatation be reversed?

As argued in previous sections, cardiac dilatation may be a fundamental change that mediates pump dysfunction. Therefore, a major goal of therapy in heart failure is to return cardiac cavity dimensions back to normal values. However, if one reviews all of the aforementioned mechanisms that could promote cardiac dilatation, it is difficult to conceive of the possibility that complete reversal could occur. Indeed, if cell death is a major mechanism responsible for cardiac dilatation, the possibility of complete reversal of cardiac dilatation under these conditions may be unrealistic. Furthermore, once side-to-side slippage has occurred, the question arises as to the chances that realignment of cells may occur. Furthermore, as little is known of what switches the cardiomyocyte hypertrophic process from a process that produces increases in both length and width, to one which promotes increases in predominantly length, the possibility of reversal of this process is similarly difficult to conceive of. The question therefore arises as to whether increases in cardiac cavity dimensions (cardiac dilatation) in patients with systolic heart failure can be returned to normal values? In the following section I will therefore review the evidence to suggest the extent to which reverse remodelling can be achieved with current heart failure therapy.

1.6.1 Impact of medical therapy on cardiac cavity dimensions in heart failure.

As described in aforementioned sections there is substantial evidence to indicate that medical therapy can reduce cardiac cavity dimensions in patients with systolic heart failure. This has been mentioned in section 1.3.2 and the evidence to show a beneficial effect of medical therapy on cardiac cavity dimensions may be found in excellent reviews on this topic including a consensus document (Cohn *et al.*, 2000). However, some noteworthy studies will be discussed.

The Studies of Left Ventricular Dysfunction (SOLVD) trial demonstrated that angiotensin-converting enzyme inhibitor therapy given to patients with symptomatic and asymptomatic heart failure produced reductions in left ventricular cavity diameters and an increase in pump function (Konstam *et al.*, 1992, Konstam *et al.*, 1993, Greenberg *et al.*, 1995). Moreover, in the Survival and Ventricular Enlargement (SAVE) trial, angiotensin-converting enzyme inhibitor therapy attenuated increases in left ventricular cavity diameters and volumes within the first year after myocardial infarction (St. John Sutton *et al.*, 1997). Despite the aforementioned benefits of angiotensin-converting enzyme inhibitors on cardiac cavity size in heart failure, no study has demonstrated that cavity size can be normalized with these agents. Indeed, angiotensin-converting enzyme inhibitors may only produce benefits on cardiac cavity size within the first year of therapy (St John Sutton *et al.*, 1997). Consequently, the impact of angiotensin II receptor blocker therapy combined with angiotensin-converting enzyme inhibitor therapy on cardiac cavity size was evaluated and this therapeutic approach was demonstrated to produce greater benefits on cardiac cavity dimensions than angiotensin-converting enzyme inhibitor therapy alone (Wong *et al.*, 2002). Despite the accrued benefits of combined

angiotensin-converting enzyme inhibitor and angiotensin II receptor blocker therapy however, cavity size was again by no means normalized (Wong *et al.*, 2002).

A number of studies have reported on the beneficial effects of β -adrenergic receptor blockers on cardiac cavity dimensions in heart failure (Waagstein *et al.*, 1989, Eichhorn *et al.*, 1990, Gilbert *et al.*, 1990, Woodley *et al.*, 1991, Hall *et al.*, 1995, Heesch *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2000, Lotze *et al.*, 2001, Bello *et al.*, 2003, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007) and in those studies that provided mean data in the whole group, Table 1.2 summarises some characteristics and the size effect of β -adrenergic receptor blockade on left ventricular cavity size or volumes. As compared to therapeutic agents that inhibit the effects of the renin-angiotensin system, β -adrenergic receptor blockers may have more pronounced beneficial effects on cardiac cavity dimensions. Indeed, as compared to the angiotensin-converting enzyme inhibitor capropril, the non-selective α and β -adrenoreceptor blocker, carvedilol has a substantially greater beneficial effect on cardiac cavity dimensions in heart failure (Khattar *et al.*, 2001). It is not only the non-selective α and β -adrenoreceptor blocker, carvedilol that is capable of reducing cardiac cavity dimensions or volumes in heart failure (Table 1.2), but also the selective β -adrenoreceptor blocker, metoprolol has similarly been demonstrated to have a striking ability to reduce cardiac cavity dimensions or volumes in heart failure (Table 1.2).

What is important to note is that of the aforementioned clinical studies assessing the impact of adrenergic blockade on cardiac cavity dimensions (See references Table 1.2).

Table 1.2. Summary of important characteristics of studies assessing the impact of adrenergic receptor blockers on left ventricular end diastolic (LVED) cavity dimensions or volumes in patients with chronic heart failure (CHF).

Reference	Disease	Adrenergic receptor blocker employed	Duration of treatment	Measurement	Baseline value	Final value
Waagstein <i>et al.</i> , 1989	CHF	Metoprolol	16 months	LVED diameter	7.26 mm	6.44 mm
Eichhorn <i>et al.</i> , 1990	CHF	Bucindolol	3 months	LVED volume	257 ml	228 ml
Gilbert <i>et al.</i> , 1990	IDC	Bucindolol	3 months	LVED diameter	66 mm	63 mm
Woodley <i>et al.</i> , 1991	IDC	Bucindolol	3 months	LVED diameter	66.5 mm	62.9 mm
Heesch <i>et al.</i> , 1995	CHF	Metoprolol	3 months	LVED volume	137 ml/m ²	116 ml/m ²
		Bucindolol	3 months	LVED volume	127 ml/m ²	114 ml/m ²
Hall <i>et al.</i> , 1995	CHF	Metoprolol	18 months	LVED volume	252 mls	177 mls
Quaife <i>et al.</i> , 1996	CHF	Carvedilol	4 months	LVED volume	209 mls	178 mls
Doughty <i>et al.</i> , 1997	CHF	Carvedilol	12 months	LVED volume	100 mls/m ²	95.6 mls/m ²
Metra <i>et al.</i> , 2000	CHF	Metoprolol	12 months	LVED volume	175 ml/m ²	160 ml/m ²
		Carvedilol	12 months	LVED volume	167 ml/m ²	147 ml/m ²
Capomolla <i>et al.</i> , 2000	CHF	Carvedilol	6 months	LVED volume	142 mls/m ²	135 mls/m ²
Groenning <i>et al.</i> , 2000	CHF	Metoprolol	6 months	LVED volume	150 mls/m ²	126 mls/m ²
Lotze <i>et al.</i> , 2001	CHF	Carvedilol	12 months	LVED diameter	6.7 cm	6.2 cm
Waagstein <i>et al.</i> , 2003	CHF	Metoprolol	6 months	LVED volume	~200 mls	~178 mls
Toyama <i>et al.</i> , 2003	CHF	Carvedilol	12 months	LVED diameter	6.4 cm	5.5 cm
	CHF	Metoprolol	12 months	LVED diameter	6.5 cm	5.8 cm
Rahko <i>et al.</i> , 2005	CHF	Carvedilol	36 months	LVED volume	204 mls	182 mls
Bello <i>et al.</i> , 2003	CHF	Carvedilol	6 months	LVED volume	124mls/m ²	112 mls/m ²
Gundogdu <i>et al.</i> , 2007	CHF	Carvedilol	3 months	LVED diameter	6.45 cm	6.23 cm
Malfatto <i>et al.</i> , 2007	Hypertensive CHF	Carvedilol	6 months	LVED volume	176mls	133 mls
	Ischaemic CHF	Carvedilol	6 months	LVED volume	200mls	185 mls
	Idiopathic CHF	Carvedilol	6 months	LVED volume	187ml	154 mls

there is little evidence to suggest that adrenergic receptor blockade has the capacity to return cardiac cavity dimensions or volumes to normal values. Indeed, assuming that a normal left ventricular end diastolic diameter is <5.5 cm, and a normal left ventricular end diastolic volume is between 130-140 mls or 70-80 ml/m², as indicated in Table 1.2 only one study (Malfatto et al., 2007) has demonstrated a capacity of adrenergic receptor blockade to decrease cardiac cavity dimensions or volumes to mean values that can be considered to lie within a normal range. In that study (Malfatto et al., 2007), a return to normal cavity volumes was only noted in patients with hypertensive heart failure, and this may not have been achieved through direct effects of adrenergic receptor blockade on the heart, but through the antihypertensive properties of these agents. Furthermore, in one study (not listed in Table 1.2), 12 months of high-dose metoprolol therapy failed to produce significant decreases in left ventricular end diastolic volumes (109 ml/m² at baseline and 96.8 ml/m² after therapy) (Colucci et al., 2007). The inability of adrenergic receptor blockers to normalise cardiac cavity dimensions or volumes is entirely consistent with the findings that pharmacological therapy in general is only ever able to return cardiac cavity dimensions or volumes back to normal values in a few patients (Murphy et al., 2007).

An important omission from clinical studies that have assessed pharmacological reverse remodelling in the treatment of heart failure, including those assessing the effects of adrenergic receptor blockade (Table 1.2) is that none of these studies have evaluated whether diastolic pressure-volume relationship return back to normal values (see Figure 1.2). Indeed, all clinical studies (Table 1.2) have relied on measurements of filling volumes or cavity dimensions without simultaneously assessing filling pressures over a range of filling volumes. As depicted in Figure 2, decreases in filling volumes may simply reflect a downward shift along the diastolic pressure-volume relationship

produced by blood volume reduction, rather than a left shift of the pressure-volume relationship back toward the normal relationship. As in the treatment of heart failure, a diuresis is an important pathophysiological change that accompanies the improvement of symptoms, without the evidence to indicate that medical therapy returns the diastolic pressure-volume relationship toward normal values, it is difficult to determine whether medical therapy does indeed have the capacity to moderate true adverse structural remodelling, or whether it is simply moving filling pressures and volumes down the right shifted curve.

A third important limitation of clinical studies that have assessed pharmacological reverse remodelling in the treatment of heart failure, including those assessing the effects of adrenergic receptor blockade (Table 1.2) is that none of these studies have evaluated whether the myocardial cellular changes that mediate adverse structural remodelling are reversed or abolished with medical therapy.

An obvious solution to the limitations of clinical studies where the effect of pharmacological therapies on cardiac diastolic pressure-volume relations or myocardial structural alterations generally cannot be measured, is to assess these changes in appropriate animal models. In this regard, there are some preclinical studies (animal studies) that have demonstrated an ability of adrenergic blocking therapy to prevent or reverse cardiac dilatation and the associated adverse cellular structural changes (Hu *et al.*, 1998, Chan *et al.* 2004, Gan *et al.*, 2007a, 2007b, Li *et al.*, 2007). In two of these studies the investigators evaluated the effect of adrenergic blockade initiated at a time when animals had already developed established pump dysfunction and adverse remodelling (Hu *et al.*, 1998, Gan *et al.*, 2007b). Importantly, in these studies adrenergic receptor blockade produced either only modest changes in cardiac cavity dimensions (Gan *et al.*, 2007b) or an attenuated capacity to produce left shifts in ventricular diastolic

pressure-volume relations when initiated later in the progression of the disease as opposed to earlier (Hu *et al.*, 1998). Thus, even preclinical studies suggest that adrenergic blocker therapy cannot reverse cardiac dilatation or if they are able to do so, the mechanisms thereof. Furthermore, in one of these studies, changes in cardiac cavity dimensions, but not in diastolic pressure-volume relations was assessed (Gan *et al.*, 2007b). Are there other forms of therapy which lend insights into whether cardiac dilatation and the associated cellular changes can be completely reversed?

1.6.2 Impact of left ventricular assist devices on cardiac cavity dimensions in heart failure.

Left ventricular assist devices (LVAD), are employed to provide haemodynamic support for patients with end-stage heart failure awaiting transplantation. These devices divert blood from the left atrium to the aorta and hence remove the preload and afterload to the left ventricle. The use of LVADs has been demonstrated to produce marked reverse cardiac remodelling and decreases in cardiac chamber dimensions, often to values that may be considered to be normal (McCarthy *et al.*, 1995, Madigan *et al.*, 2001, Barbone *et al.*, 2001, Margulies, 2002, Sabbah, 2004, Wohlschlaeger *et al.*, 2005, Klotz *et al.*, 2008). Importantly, LVAD support has been shown not only to decrease chamber dimensions, but also to shift the diastolic pressure-volume relationship to the left (Heerdt *et al.*, 2000, Barbone *et al.*, 2001) and this effect may be sufficiently profound as to normalise this relationship (Barbone *et al.*, 2001). Moreover, the decreases in chamber diameters produced by LVAD support are sustained once the assist device is removed from patients (Patterson *et al.*, 2010). Hence, the use of LVADs has provided substantial evidence to support the view that cardiac dilatation is a

reversible structural change. However, some time after LVADs are removed from patients with end-stage heart failure, cardiac dimensions may gradually return to pathological levels again (Scheinin *et al.*, 1992, Mancini *et al.*, 1998). Nevertheless there is some evidence that combined LVAD support together with medical therapy may produce even better outcomes than LVAD support alone (Birks *et al.*, 2006). Is there evidence to indicate that LVADs are capable of reversing the structural changes that occur at a cellular level in patients with cardiac dilatation and end-stage heart failure?

Myocardial collagen concentrations may increase after the use of an LVAD (Li *et al.*, 2001). Although this has been interpreted as indicating a deleterious change, the phenotypic characteristic of the myocardial collagen change may nevertheless be beneficial. Indeed, after LVAD support, the ratio of insoluble (cross-linked) to total soluble (non-cross-linked) collagen concentrations increases (Li *et al.*, 2001). As the cross-linked collagen phenotype is more likely to resist collagen degradation by collagenases, an increased myocardial collagen in this context could prevent cardiomyocyte side-to-side slippage. Not only does LVAD support potentially improve the myocardial collagen characteristics, but it also decreases the myocardial expression and activity of the MMPs that may be responsible for myocardial collagen degradation (Li *et al.*, 2001). Thus, the interstitial changes that may be responsible for cardiomyocyte side-to-side slippage are indeed reversed by LVAD support. Despite this evidence however, the question of whether cardiomyocyte side-to-side slippage is reversible has not been evaluated.

Is there evidence in favour of LVAD support influencing cardiomyocyte length-to-width ratios? Indeed, although LVAD support reduces both cardiomyocyte width and length, cardiomyocyte length-to-width ratio is also reduced by approximately 32% (Zafeiridis *et al.*, 1998). Thus, it is possible that maladaptive cellular hypertrophy is

important in contributing towards reverse remodelling after LVAD support and unloading of the heart.

Does LVAD support prevent ongoing cardiomyocyte apoptosis or necrosis? A decrease in cardiomyocyte apoptosis has indeed been observed in patients receiving LVAD support as compared to patients in whom a LVAD had recently been inserted (Patten *et al.*, 2005). However, this does not indicate that apoptotic cells have necessarily been replaced after LVAD support or that after significant cell loss a normally functioning heart may occur once the process of cell death is halted. Is there evidence that normalisation of cardiac size and function can be achieved when unloading the heart, even when significant cardiomyocyte apoptosis has preceded the event? In this regard, after unloading the heart, reversal of cardiac structure and function has been noted in rats (Tsuneyoshi *et al.*, 2005) despite the possibility that cardiomyocyte apoptosis and myocardial atrophy had occurred (Schena *et al.*, 2004).

In summary, data obtained from the use of LVAD support suggest that cardiac dilatation and the cellular mechanisms responsible for cardiac dilatation can be reversed. However, the conundrum of why removal of LVAD support subsequently results in the return of cardiac dimensions to pathological levels again still requires resolution. This could indicate that not all the essential cellular mechanisms responsible for cardiac dilatation are reversible and hence that further work is required to determine whether sustained reverse remodelling is indeed a possibility.

1.7 Problem statement and aims of the dissertation

As indicated in the aforementioned discussion, although there is significant evidence to indicate that adrenergic activation is an important mediator of heart failure, and that adrenergic receptor blockade saves lives in patients with heart failure, there is little evidence to indicate whether adrenergic-induced cardiac dysfunction is completely reversible. In this regard, as outlined, adrenergic activation produces cardiac dilatation and there is still question as to whether reversal of cardiac dilatation in patients with heart failure can be sustained. Moreover, there is little evidence to indicate whether adrenergic-induced cardiac dilatation can be completely reversed. Thus, further studies are necessary to evaluate whether adrenergic-induced cardiac dilatation can be completely reversed. These studies would provide further evidence either in favour or against identifying therapeutic targets downstream from β -adrenergic receptors that mediate cardiac dilatation and pump dysfunction, but do not influence myocardial inotropy. In this regard, the use of β -adrenergic receptor blocker therapy in chronic heart failure is presently far from ideal (Cleland *et al.*, 2002, Pont *et al.*, 2003, Rutten *et al.*, 2003, Komajda *et al.*, 2003, Murphy *et al.*, 2004, Fernandes *et al.*, 2005, Lenzen *et al.*, 2005, Bongers *et al.*, 2006, Fowler *et al.*, 2007, Sturm *et al.*, 2007, Kavookjian & Mamidi, 2008) possibly because of the negative inotropic and hence hypotensive effects of these agents in many patients, an effect that could be more pronounced in those with advanced cardiac dilatation and pump dysfunction.

Our laboratory is ideally positioned to study reverse remodelling produced by adrenergic over-activation. As indicated in the aforementioned, we have repeatedly demonstrated that chronic administration of isoproterenol, a β -adrenoreceptor agonist, promotes the development of cardiac dilatation and pump dysfunction, even though

intrinsic myocardial function is preserved (Woodiwiss *et al.*, 2001, Badenhorst *et al.*, 2003b, Veliotes *et al.*, 2005, Osadchii *et al.*, 2007). Unlike other animal models of heart failure, such as pressure overload states or myocardial infarction, where extensive tissue necrosis accompanies pump dysfunction, the model of cardiac dilatation and pump dysfunction mediated by chronic β -adrenoreceptor activation is not necessarily accompanied by tissue necrosis (Woodiwiss *et al.*, 2001, Badenhorst *et al.*, 2003b, Veliotes *et al.*, 2005, Osadchii *et al.*, 2007). Thus, to remove the stimulus for pump dysfunction mediated by chronic β -adrenoreceptor activation, one simply stops the daily injections of the isoproterenol, whereas in other animal models of pump dysfunction, such as pressure overload states or myocardial infarction, the stimulus cannot be removed as large portions of the myocardium are necrotic. Thus, in the present dissertation I aimed to evaluate whether adrenergic-induced cardiac dilatation and cardiac structural remodelling can be completely reversed once the adrenergic (neurohumoral) stimulus for the adverse remodelling is removed. I also aimed to identify the cellular mechanisms associated with reverse remodelling and the cellular changes that persist if residual cardiac dilatation is noted. Any residual changes may be important for developing new therapeutic strategies for treating heart failure.

Chapter 2

Materials and Methods

2.1 Groups and treatment regimen

The rodent model of pump dysfunction studied in the present dissertation was that induced by chronic administration of the β -adrenoreceptor agonist, isoproterenol (ISO) to male Sprague-Dawley (SD) rats. Rats received daily subcutaneous injections of either ISO at a dose of 0.006 mg.kg^{-1} ($2.42 \times 10^{-8} \text{ mmol.kg}^{-1}$), or the vehicle control (0.9% saline). Although the target daily ISO dose was 0.02 mg.kg^{-1} as given in prior studies (Badenhorst et al 2003, Veliotis et al 2005), in the present study when gradually increasing the dose of ISO given in daily injections over a 2 week period (starting from $0.0001 \text{ mg.kg}^{-1}$), when achieving a dose of 0.02 mg.kg^{-1} one rat died suddenly. Hence, the dose selected for use was 0.006 mg.kg^{-1} , a dose which did not produce further deaths. Each injection consisted of a total volume of 0.2 ml. Forty eight SD rats weighing between 250-to-300g (obtained from Central Animal Services of the University of Witwatersrand) were randomly assigned to four groups. Two groups of rats were used to assess the impact of adrenergic stimulation on cardiac structure and function after six months of adrenergic stimulation. One group of 10 rats received daily injections of ISO and the other group of 10 rats received daily injections of the saline vehicle for six months. The remaining two groups of rats were employed to assess the extent to which adrenergic-induced cardiac dilation, structural remodelling and pump dysfunction could be reversed. One group of 14 rats received daily injections of ISO for six months and then subsequently received no injections for four months. The other group of 14 rats received daily injections of the saline vehicle for six months and then subsequently received no injections for four months. An ISO withdrawal period of four months was employed as an initial stage of the study. Longer periods of ISO withdrawal would have been evaluated in subsequent studies if reverse remodelling had been incomplete.

2.2 Echocardiography

At least 24 hours after the last dose of ISO or saline injection, two-dimensional targeted M-mode echocardiography was performed using a 7.0 MHz transducer and a ACUSON CYPRESS portable ultrasound machine system (Figure 2.1), as previously outlined (Woodiwiss *et al.*, 2001, Norton *et al.*, 2002). Rats were anaesthetized with intraperitoneal injections of ketamine ($75\text{mg}\cdot\text{kg}^{-1}$) and xylazine ($15\text{mg}\cdot\text{kg}^{-1}$). The rat's chest was shaved of hair and the rat was placed in the prone position in a container with an open window over which the chest area was positioned. The high resolution ultrasonic probe was inserted through the window and placed on the rat chest wall to obtain echocardiographic images.

A two dimensional image was obtained in the short axis of the left ventricle at the level of the papillary muscle. The transducer was positioned to obtain clear images across the maximal diameter of the short axis of the left ventricle. An M-mode image was obtained which was considered to be of high quality if the endocardial surface of both the anterior (septal) wall and the posterior wall were clearly visible throughout systole and diastole (Figure 2.2). Care was taken not to include the endocardial surface of the papillary muscle in the posterior wall measurements. On-line recordings were obtained and recordings were also obtained for later off-line analysis to ensure quality control.

Left ventricular internal dimensions and posterior wall thickness were measured at the point at which internal diameters were at the smallest value (end systole) and at the point at which internal diameters were at the maximum value (end diastole) according to the American Society for Echocardiography's leading edge method (Sahn *et al.*, 1978) (Figure 2.2). Left ventricular anterior wall thickness was not determined as



Figure 2.1 ACUSON CYPRESS portable echocardiograph used to obtain in-vivo measures of left ventricular structure and function in anaesthetised rats.

A - LV end diastolic internal diameter

B - LV end systolic internal diameter

C - LV end diastolic posterior wall thickness

D - LV end systolic posterior wall thickness

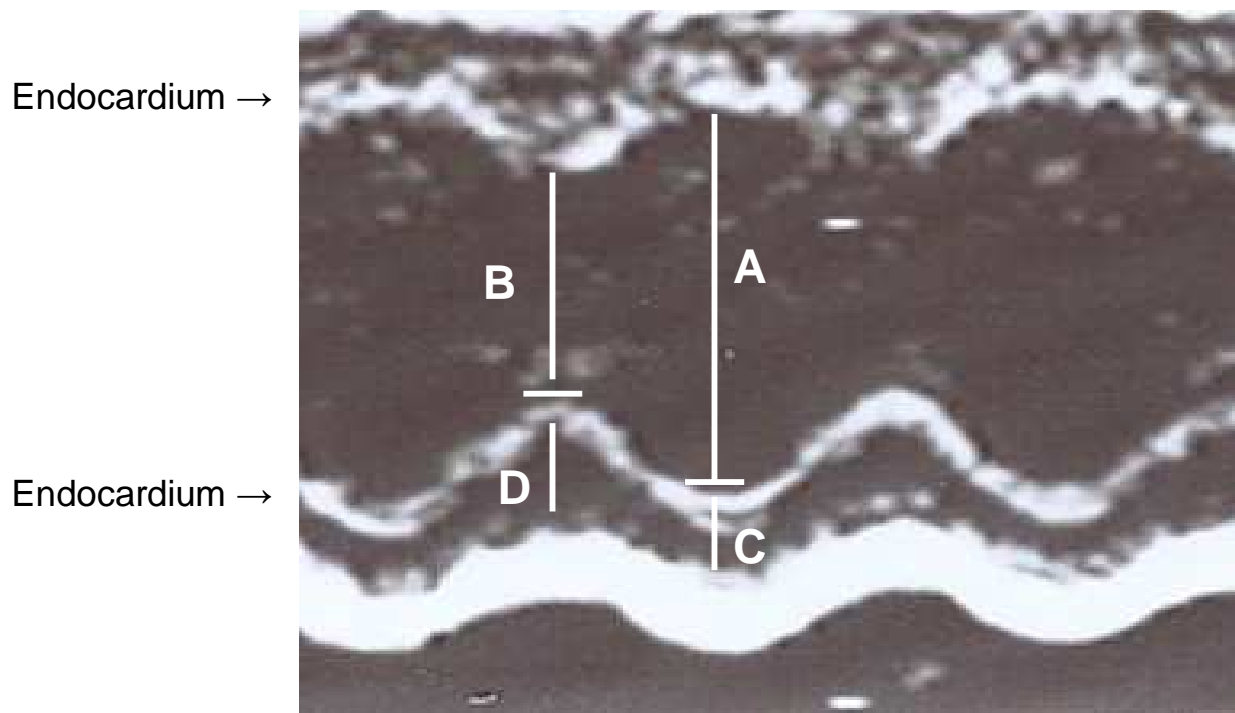


Figure 2.2 Typical recordings obtained to determine left ventricular dimensions using two-dimensional guided M-mode echocardiography.

the right ventricular surface of the septal wall was seldom clearly evident. Anterior wall thickness was therefore assumed to be equivalent to posterior wall thickness for all calculations. An example of measurements made from actual recordings is provided in Figure 2.2. Measurements were made from at least 3 consecutive beats and then averaged.

Left ventricular endocardial and midwall fractional shortening were utilized as indices of chamber and myocardial function respectively (Norton *et al.*, 2002), (Chung *et al.*, 1998). Left ventricular endocardial (FS_{end}) and midwall (FS_{mid}) fractional shortening were determined from the following equations. For the calculations of FS_{mid} , anterior wall thickness was assumed to be equivalent to posterior wall thickness. Hence $\frac{1}{2}$ LV PWT + $\frac{1}{2}$ LV anterior wall thickness was assumed to be equivalent to LV PWT.

$$FS_{end} = \frac{LV\ EDD - LV\ ESD}{LVEDD} \times 100$$

Where

LV EDD = left ventricular end diastolic internal diameter

LV ESD = left ventricular end systolic internal diameter

$$FS_{mid} = \frac{(LVEDD + LVED\ PWT) - (LVESD + LVES\ PWT)}{(LVEDD + LVED\ PWT)} \times 100$$

Where

LVED PWT = left ventricular end diastolic posterior wall thickness

LVES PWT = left ventricular end systolic posterior wall thickness

Importantly, left ventricular ejection fraction was not used as an index of left ventricular chamber systolic function as clear endocardial surface images were not always available throughout the whole circumference of the heart on two-dimensional imaging. Diastolic function (transmitral early-to-late velocity ratios) was not assessed using echocardiography as the reproducibility of this measurement in our hands is poor. Neither endocardial nor midwall fractional shortening are independent of afterload or heart rate, but like left ventricular ejection fraction, they do account for preload-dependent Frank-Starling effects.

2.3 Isolated perfused heart preparations

As LV dimensions and systolic function are influenced by loading conditions, LV remodelling and function was also determined *ex vivo* under controlled conditions as previously described (Weber *et al.*, 1988, Norton *et al.*, 2002, Woodiwiss *et al.*, 2001). A midline thoracotomy was performed in anaesthetised rats and the hearts were immediately excised and placed in an ice-cold perfusion solution (see description of solution in subsequent discussion) to maintain their viability prior to perfusion. Hearts were subsequently placed on a Langendorff apparatus and retrogradely perfused via the aorta. Using this approach, perfusion fluid is pumped down the aorta toward the heart using a peristaltic pump, the aortic valve stays in a closed position because of the pressure gradient produced across the aortic valve, and perfusion fluid flows down the coronary arteries only. The perfusion solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 2.5 CaCl₂, 25.0 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄ and 10.0 glucose with a pH of 7.4. The solution was saturated with 95% O₂ and 5% CO₂ gas and carefully filtered through a size 0.45µm Millipore Durapore membrane filter before assessing the pH.

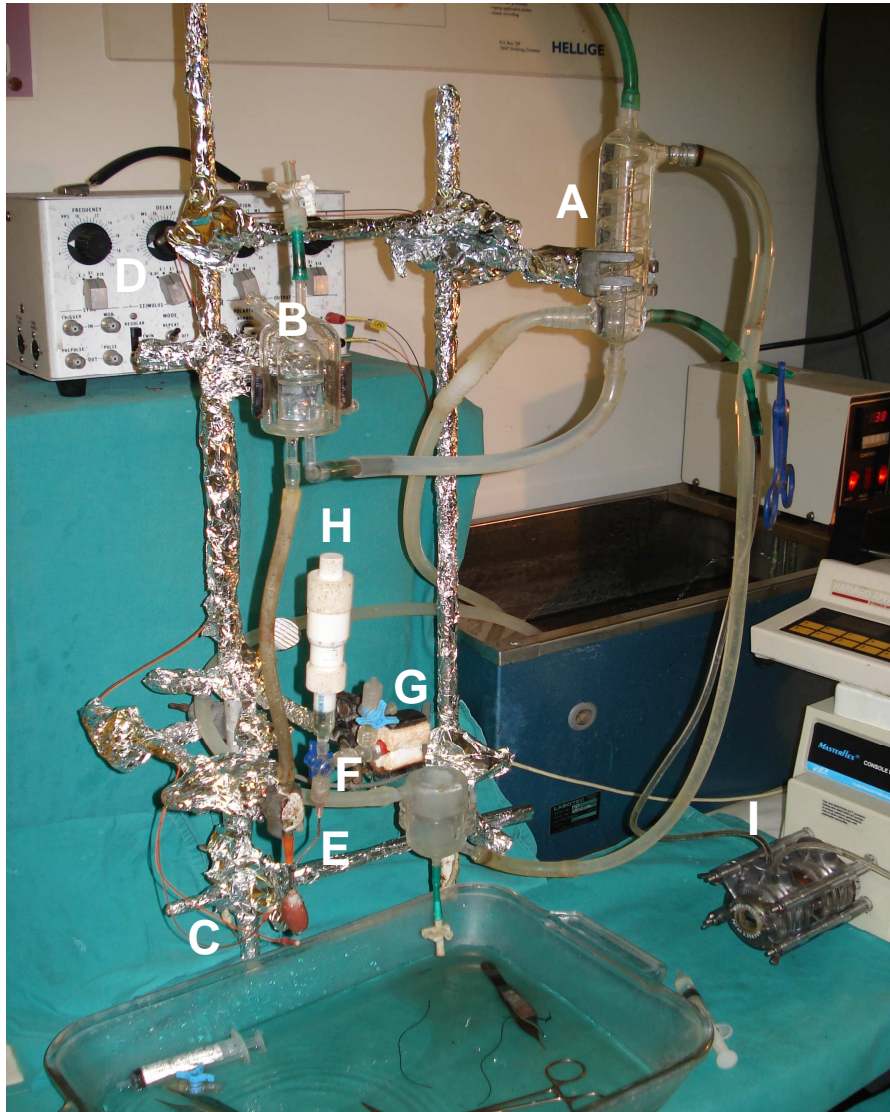


Figure 2.3 Isolated, perfused heart apparatus. A, water jacket; B, bubble trap; C, platinum electrodes attached to isolated heart; D, pacing device; E, fluid filled catheter with balloon attached which has been inserted into the left ventricular lumen; F, three way tap open to E, G and H; G, pressure transducer; H, micromanipulator; I, peristaltic pump.

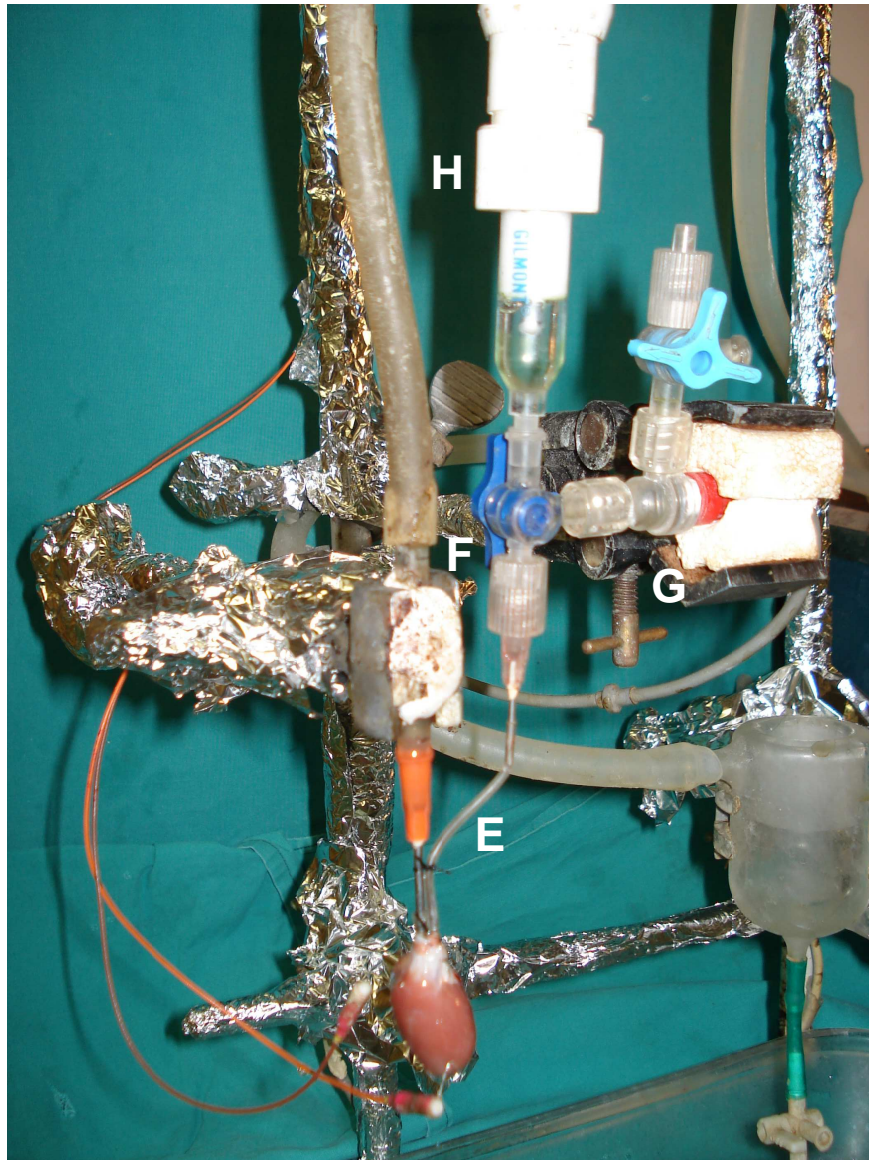


Figure 2.4 Enlargement of a portion of the isolated perfused heart apparatus shown in figure 2.8. This figure better shows E, the fluid filled catheter with balloon attached which has been inserted into the left ventricular lumen; F, the three way tap; G, the pressure transducer; and H, the micromanipulator.

The isolated, perfused organ system is depicted in Figures 2.3 and 2.4. The perfusion solution was constantly gassed with 95% O₂ and 5% CO₂ for the duration of each study. Hearts were perfused retrogradely at a constant flow of 12 ml.min⁻¹.g wet heart weight. This was achieved by first obtaining a crude heart weight (heart weight with left ventricle, right ventricle, atria and large vessels) before mounting the heart on the perfusion apparatus and then setting the speed on a peristaltic pump to achieve the appropriate coronary flow. Coronary flow rate was assessed from timed samples obtained of the coronary effluent. A standard proportion of left ventricular weight to crude heart weight was assumed for each preparation and then checked at the end of the study. Using this approach myocardial viability is maintained via constant coronary perfusion without relying on the function of the left ventricular chamber to determine coronary flow and myocardial tissue viability.

The perfusion apparatus maintained a constant temperature of the perfusion solution by passing the perfusion solution through a tube surrounded by a water jacket through which heated water flowed (Figure 2.3). The temperature of the coronary effluent was maintained at 37°C by controlling the temperature of the water flowing through the water jacket. To avoid air bubbles entering the coronary arteries, a bubble trap was placed proximal to the heart (Figure 2.3). Once the heart was mounted on the perfusion apparatus, platinum electrodes were attached to the right atrium and the apex of the heart and the heart was paced at 360 beats.min⁻¹ using a Grass (Astro Med Inc.) model SD9 stimulator (Figure 2.3). All hearts were paced at the same rates in order to avoid having to account for an impact of heart rate on functional measurements. The heart rate selected for these studies was much lower than what we have measured in conscious restrained rats *in vivo* (400-500 beats.min⁻¹). This lower heart rate was employed as this is a crystalloid perfused preparation rather than a blood perfused

preparation. It is only in blood perfused preparations in which physiological heart rates can be employed because of the presence of a greater arterial oxygen content. The heart rate selected for the study was one aimed to achieve maximal systolic function through the “Treppe” and other effects without producing demand-induced ischaemia. To ensure that rat hearts are not ischaemic at 360 beats.min⁻¹ previous studies have been performed to assess the pacing rate at which diastolic pressures in the rat heart begin to increase. Demand-induced ischaemia characteristically results in an increase in diastolic left ventricular pressures, whereas low-flow ischaemia decreases contractile function (Umeda *et al.*, 2003). In our hands, we have found that 360 beats.min⁻¹ is well below the rate at which left ventricular diastolic pressures first begin to increase in crystalloid perfused rat hearts. Hearts were paced at a voltage that was estimated to be 10% above threshold for spontaneous excitation (Norton *et al.*, 2002).

In order to measure left ventricular developed pressures and diastolic pressures a latex balloon was placed through the mitral valve into the left ventricular chamber (Figure 2.3 and 2.4). The latex balloon was coupled via fluid-filled catheters and a three way tap to both a pressure transducer and a micromanipulator (Figure 2.4). The lumen of the latex balloon was sufficiently large to accommodate volumes well beyond the maximal left ventricular volume of a normal rat or a rat with a dilated ventricle. Indeed, the balloon had a pressure-volume relationship where the pressure in the balloon only started to increase well beyond that of the maximal left ventricular volume of a normal rat or a rat with a dilated ventricle. In the assessment of intraventricular volumes, the balloon material was included as part of volume. The volume of the balloon material was calculated using a volume displacement technique employing larger quantities of the same material and calibrating against the weight of the material. Before inserting the balloon into the left ventricular cavity, the balloon was emptied by removing the

micromanipulator, opening the three way tap to atmosphere, squeezing the balloon and allowing fluid to move from the balloon, up the catheter and out of the tap, and then closing the three way tap to the balloon (Figure 2.4).

Left ventricular pressures were determined at as many multiple small increments in volume as were practically possible. The micromanipulator has a Vernier scale (Figure 2.4) that allows for 0.005-0.01 ml increments in volume to be injected into the balloon. The micromanipulator was regularly calibrated by weighing 0.005-to-0.01 ml increments of volumes of fluid. Left ventricular developed pressures and diastolic pressures were measured and recorded on a Hellige recorder (Figure 2.5). Left ventricular developed pressures were recorded on a different channel to left ventricular diastolic pressures (Figure 2.5). An amplified calibration scale was used to assess left ventricular diastolic pressure to ensure the accuracy of recordings (Figure 2.5). The channel used to record left ventricular developed pressures was calibrated using a mercury manometer. The channel used to record left ventricular diastolic pressures was calibrated with a water-filled U-tube system designed to calibrate low pressure systems (Norton *et al.*, 1996). Calibrations for both left ventricular developed pressure and diastolic pressure recordings were performed both before and after each heart preparation. Left ventricular pressures were determined over a range of volumes measured in the absence of an inotropic stimulus. As the isolated, perfused heart preparation used in this dissertation is isovolumic, left ventricular minimum pressures (diastolic pressures) were assumed to be the equivalent of left ventricular end diastolic pressure in a heart with volume changes that correspond to a normal cardiac cycle.

Left ventricular diastolic pressure-volume relations were constructed to assess the degree of left ventricular dilatation. For statistical comparisons, left ventricular

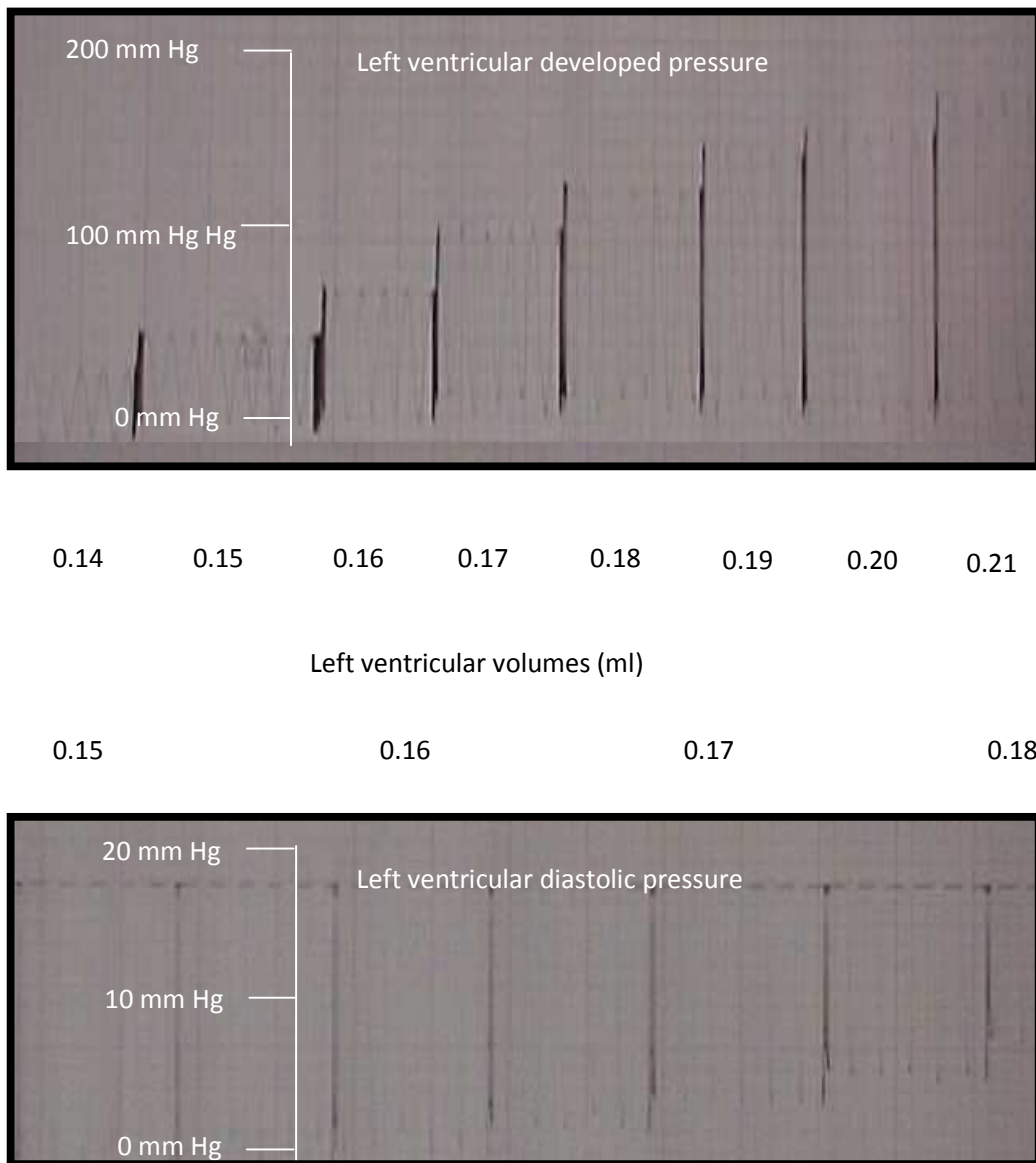


Figure 2.5 Typical recordings obtained of left ventricular developed pressures and left ventricular diastolic pressures in isolated, perfused heart preparations.

dilatation (remodeling) was assessed by comparing left ventricular volumes obtained at a left ventricular diastolic pressure of 0 mm Hg (volume intercept of the left ventricular diastolic pressure-volume relationship-LV V_0) (Badenhorst *et al.*, 2003a, Badenhorst *et al.*, 2003b, Woodiwiss *et al.*, 2001, Norton *et al.*, 2002). To determine systolic chamber function, the slope of the linear portion of the systolic developed pressure-volume relationship was calculated (systolic chamber elastance-E). This is the equivalent of left ventricular end systolic elastance in an ejecting and filling left ventricle. Left ventricular end systolic elastance is the slope of the end systolic pressure-volume relationship. Left ventricular end systolic elastance has been well established as being afterload and preload-independent (Sagawa *et al.*, 1981, Sugawa *et al.*, 1988). The linear portion of the systolic developed pressure-volume relationship in an isovolumic preparation is considered to be the equivalent of end systolic elastance in a heart that ejects and fills during the cardiac cycle, as peak systolic pressures in an isovolumic preparation are the same as end systole pressures. Data points were included in the peak systolic pressure-volume relationship if on linear regression analysis for individual rats, the r^2 value with each point included was 0.95 or more. Using this approach I could include the first five left ventricular developed pressures in the left ventricular developed pressure-volume relationship for all rats for baseline measurements.

To determine intrinsic systolic myocardial function, the slope of the systolic developed stress-strain relationship was calculated (myocardial systolic elastance- E_n) (Norton *et al.*, 2002, Badenhorst *et al.*, 2003b, Veliotes *et al.*, 2005). By converting pressures and volumes into stress and strain data, the impact of alterations in left ventricular chamber geometry on systolic function are eliminated (Weber *et al.*, 1988). Thus, E_n is the myocardial equivalent of E and hence is also afterload and preload-independent. E_n is a linear relationship and hence substantially more data points were

included in the analysis than five data points. However, to ensure that calculations of E_n were representative of data obtained for E , E_n was also recalculated from only the first five left ventricular developed pressure and volume data and provided essentially the same outcomes. This is not surprising as the relationship is linear. Left ventricular developed stress and strain values were calculated from previously described formulae (Weber *et al.*, 1988, Norton *et al.*, 2002, Badenhorst *et al.*, 2003b, Veliotes *et al.*, 2005) assuming a thick-walled spherical geometry of the left ventricle as follows:

$$\text{Left ventricular systolic stress} = \frac{1.36 \times \text{LV developed pressure} \times (\text{LVV})^{2/3}}{[\text{LVV} + (0.943 \times \text{LV mass})]^{2/3} - \text{LVV}^{2/3}}$$

$$\text{Left ventricular systolic strain} = \frac{\text{LVV}^{1/3} + [\text{LVV} + (0.943 \times \text{LV mass})]^{1/3} - 1}{\text{LV } V_0^{1/3} + [\text{LV } V_0 + (0.943 \times \text{LV mass})]^{1/3}}$$

Where LVV is left ventricular volume and LV V_0 is the volume intercept of the LV developed pressure- volume relationship, i.e. LV volume when LV developed pressure = 0 mm Hg.

2.4 Cardiac fibrosis and apoptosis

After weighing heart tissue, a longitudinal slice of the left ventricle from the apex to the base through the left ventricular free wall was obtained from all rats for histology. Left ventricular tissue was stored in 10% buffered formaldehyde for subsequent histology. Myocardial tissue was subsequently processed, embedded in paraffin wax and

cut into sections. Left ventricular tissue was processed routinely for light microscopy and 50 µm-thick sections of the long axis circumference were cut through the full thickness of the left ventricular wall. Ten slices were obtained at 1-mm intervals and stained with van Gieson's stain. After staining a pathological grade was assigned, where 0 indicates no damage; 1 and 2, patchy fibrosis in less than or more than 20% of the field respectively; 3 and 4, diffuse contiguous subendocardial fibrosis in less than or more than 50% of the field respectively and 5 and 6, full thickness fibrosis in less than or more than 50% of the field respectively (Teerlink *et al.*, 1994, Woodiwiss *et al.*, 2001). Representative slides stained with van Gieson's stain are shown in Figure 2.6.

The degree of apoptosis was quantified on myocardial tissue sections obtained from the same tissue blocks used to assess the pathological score. For each tissue block, 50 µm thick sections were stained and evaluated. Nuclear deoxyribonucleic acid (DNA) fragments in the tissue sections were detected using a non-radioactive *in situ* apoptotic cell death detection kit (DeadEnd™ Colorimetric TUNEL system, Promega, Madison, WI, USA), where terminal deoxynucleotidyl transferase (TdT) was used to incorporate biotinylated nucleotide at the 3'-OH DNA ends. Horseradish-peroxidase-labeled streptavidin binds to biotinylated nucleotides, which subsequently stain dark brown in response to hydrogen peroxide and diaminobenzidine (Agarwala & Kalil, 1998). Both positive (DNase treated) and negative (no addition of TdT) control tissue sections were incorporated into each assay. A separate Coplin jar was used for the positive slide due to DNase I activity from the positive control which may affect the experimental slides by staining non-apoptotic cells.

To identify apoptotic nuclei, all procedures were carried out at room temperature except where otherwise stated. Paraffin embedded sections were first immersed in

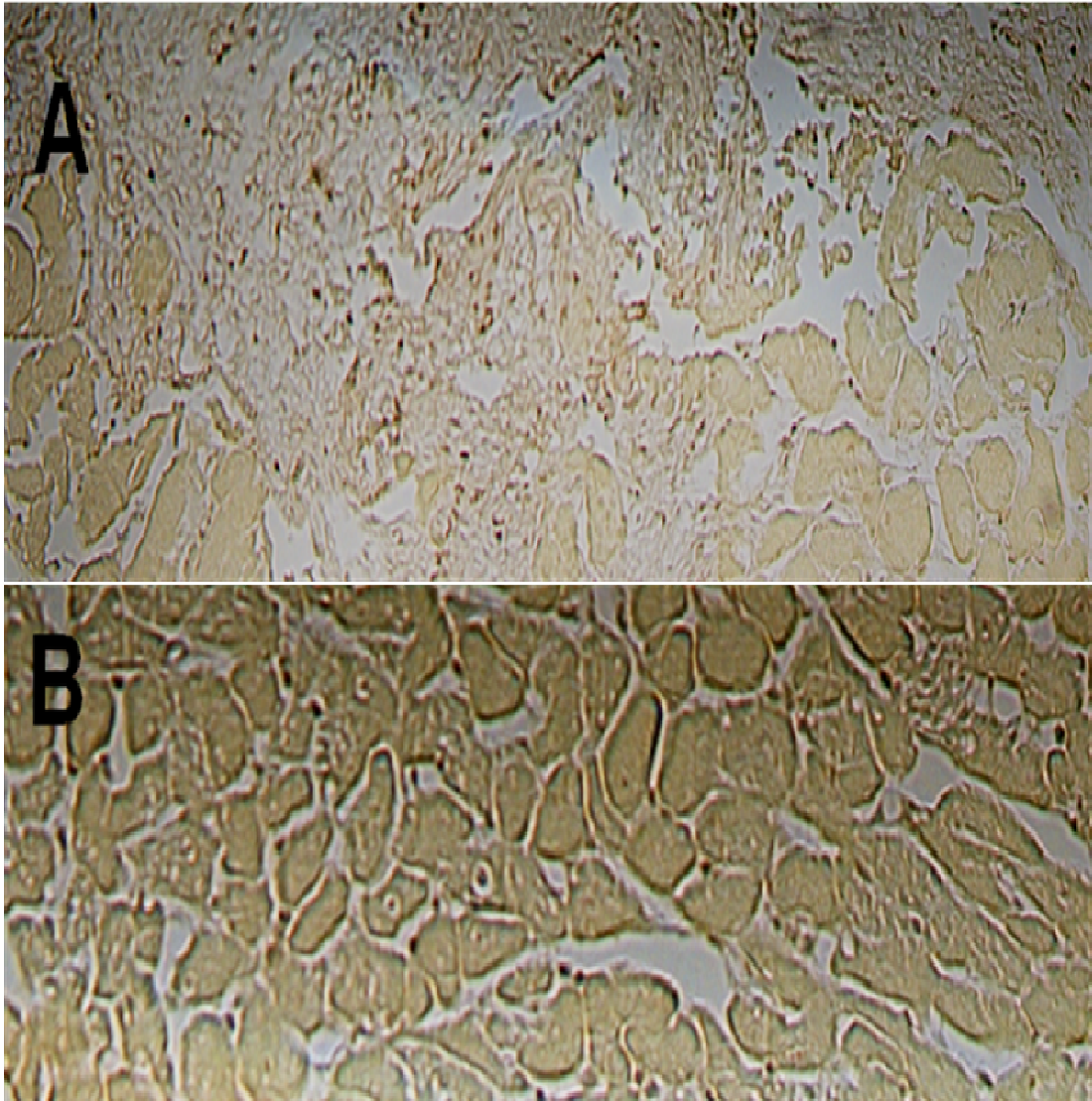


Figure 2.6 Typical images obtained of sections of the left ventricle of rats stained with Van Giesson's stain. Image A shows areas of tissue fibrosis. Image B depicts normal cardiac tissue with no fibrosis.

xylene for 5 minutes to de-paraffinize the tissue sections. The tissue sections were then washed by immersing the slides in 100% ethanol for 5 minutes and again for 3 minutes. The sections were then rehydrated by immersing the slides through graded ethanol washes (95%, 85%, 70% and 50%) for 3 minutes each. The slides were then washed in 0.85% NaCl solution for 5 minutes and in PBS for 5 minutes. The tissue sections were then fixed by immersing the slides in 4% paraformaldehyde solution for 15 minutes. The slides were then immersed in PBS for 5 minutes. The liquid was then dried from the tissue sections and the slides were placed on a flat surface. A 20µg/ml protein kinase K solution was prepared from the 10µg/ml Proteinase K stock solution by diluting it with PBS. 100µl of the proteinase K solution was then added to the slides to cover each tissue section. The slides were then incubated for 30 minutes at room temperature to allow the proteinase K to increase the permeability of the cells. The tissue sections were then washed by immersing the slides in PBS for 5 minutes and re-fixed by immersing in 4% paraformaldehyde solution and washed again in PBS for 5 minutes. At this point the positive control slide was treated with DNase I to cause DNA fragmentation whilst the experimental slides remained in a PBS solution. 100µl of DNase I buffer was added to the positive control slide to cover the tissue sections and incubated at room temperature for 5 minutes. The DNase I buffer liquid was then tapped off the tissue sections and DNase I buffer containing DNase was added to cover the tissue sections. The slides were then incubated for 10 minutes at room temperature. The excess liquid was removed by tapping the slides. The positive control slide was then washed 4 times in distilled water and in PBS for 5 minutes. After DNase treatment the positive control slide was again processed with the experimental slides. The excess liquid was removed by tapping the slides and the tissue sections were then covered with Equilibration Buffer for 8 minutes. Whilst the sections were equilibrating, 10µl of Biotinylated Nucleotide Mix and

10µl of rTDT Enzyme were added to 980µl of Equilibration Buffer for the reaction mix. A control incubation buffer was prepared for the negative control slide by adding 1µl of Biotinylated Nucleotide Mix and 1µl of distilled water to 98µl of Equilibration buffer. After equilibration the slides were blotted with tissue paper to remove excess liquid and 100µl of the rTDT reaction mix was then added to each tissue section. The sections were then covered with plastic cover slips and incubated at 37°C for 60 minutes inside a humidified chamber to allow the end-labelling reaction to occur. After 60 minutes the slides were removed from the incubator and the plastic cover slips were removed. 20X saline-sodium citrate (SSC) was diluted with distilled water. The rTDT reaction was terminated by immersing the slides in 20 x SSC solution for 15 minutes. This procedure was repeated. The tissue sections were subsequently washed in PBS twice for 5 minutes each to remove unincorporated biotinylated nucleotides. The slides were then immersed in 0.3% hydrogen peroxide for 5 minutes to block the endogenous peroxidases and washed with PBS for 5 minutes. Streptavidin HRP was diluted in PBS. 100µl was added to each slide to cover the tissue sections and the slides were incubated at room temperature for 30 minutes. The slides were then washed with PBS for 5 minutes. 50µl of DAB Substrate 20X Buffer, 50µl of DAB 20X Chromogen and 50µl of Hydrogen Peroxide 20X were added to 950µl of distilled water. 100µl of the DAB solution was then added to each slide to cover the tissue sections for 8 minutes at room temperature. The slides were then rinsed 4 times with distilled water, dehydrated by immersing the slides in graded ethanol washes (50%, 70%, 85% and 95%) and immersed in xylene. The slides were subsequently mounted using permanent mounting medium (Entellan, Merck KGaA, Germany).

The number of apoptotic cardiomyocyte nuclei and the total number of cardiomyocyte nuclei (haematoxylin and eosin stain) in each slide were counted on ten

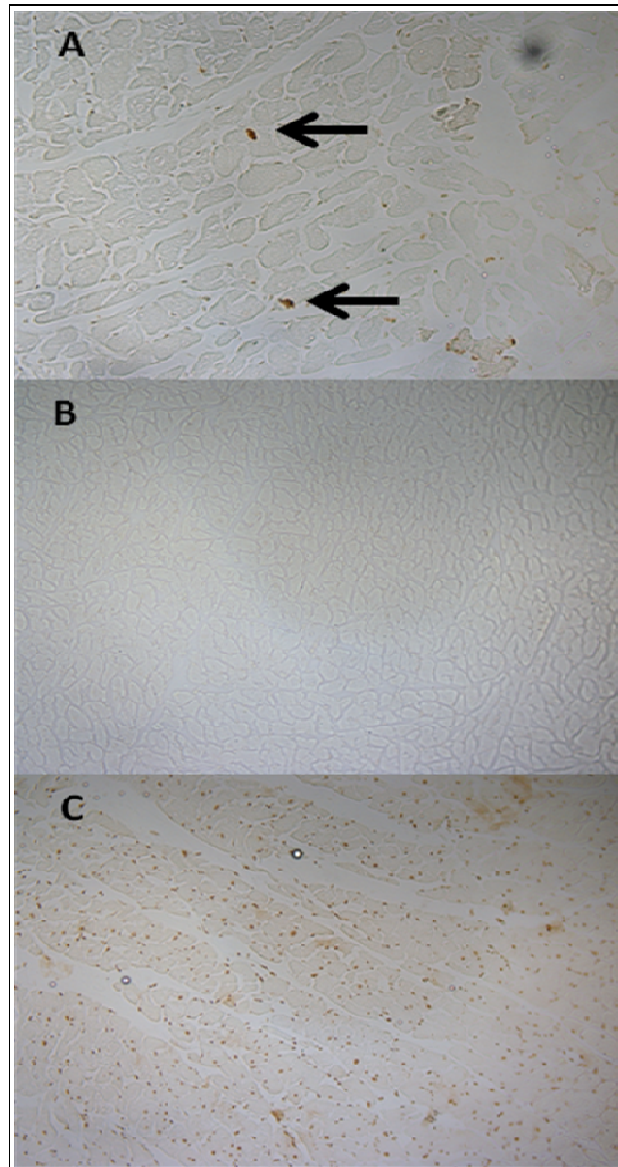


Figure 2.7 Typical images obtained of sections of the left ventricle of rats stained for apoptotic nuclei. Image A shows apoptotic nuclei (arrows). Image B shows the negative control slide showing a lack of apoptotic nuclei and image C shows a positive control slide showing many apoptotic nuclei.

evenly spaced fields from the apex to the base using a computer-based image acquisition and analysis system at 400 times magnification (Axiovision 3, Carl Zeiss, Gottingen, Germany). Typical images of apoptotic nuclei from cardiomyocytes are shown in Figure 2.7. Cardiomyocyte apoptotic nuclei were expressed as a percentage of the total number of cardiomyocyte nuclei. All sections were coded and a single observer “blinded” to the identity of the rat from which the section was obtained recorded the number of apoptotic nuclei, and counted the total number of cardiac myocyte nuclei from slides stained with haematoxylin and eosin (H & E).

2.5 Myocardial collagen

Samples of LV tissue were weighed and stored at -70°C prior to tissue analysis. Myocardial hydroxyproline concentration ([HPRO]) was determined after acid (HCL) hydrolysis using the method of Stegeman and Stalder (1967) and previously described by members of our lab (Norton *et al.*, 1997, Woodiwiss *et al.*, 2001). Myocardial collagen was also extracted by means of tissue homogenation and then digested with cyanogen bromide (CNBr) overnight in water-bath ($\pm 25^{\circ}\text{C}$ at slight angle) (Norton *et al.*, 1997, Woodiwiss *et al.*, 2001). A portion of the CNBr digested collagen sample was vacuum sealed and subjected to acid hydrolysis (18 hours at 108°C in 6N HCl) and [HPRO] determination followed. The amounts of non-cross-linked (soluble) and cross-linked (insoluble) collagen in the myocardium were ascertained based on the solubility of myocardial collagen to CNBr digestion (Norton *et al.*, 1997, Woodiwiss *et al.*, 2001).

2.6 Data analysis

All values in text are represented as mean \pm SEM. Regression analysis was used to determine the lines of best fit for cardiac function. To compare cardiac weights, left ventricular internal dimensions and wall thickness values, left ventricular V_0 (volume intercept of the diastolic pressure-volume relationship), left ventricular systolic chamber or myocardial function (FS_{end} , FS_{mid} , Ees and En), left ventricular cardiomyocyte apoptosis and myocardial collagen concentrations between groups, a one-factor ANOVA followed by a Newman-Keuls *post hoc* test was employed. To compare pathological scores between groups, a one-factor ANOVA followed by a Kruskal-Wallis *post hoc* test was employed. To assess whether complete reversal of the adverse effects of 6 months of ISO administration had occurred after 4 months of cessation of ISO administration, I not only compared mean values, but also the proportion of rats with data either greater than or equal to, or less than or equal to the 95% confidence intervals for controls. Proportions were compared using a Fishers Exact test.

Chapter 3

Results

3.1 Impact of cessation of chronic isoproterenol administration on body and heart weights.

Table 3.1 and Figure 3.1 show the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on body and heart weights of rats. Chronic ISO administration produced no statistically significant effect on body weights (Table 3.1). However, 6 months of daily ISO administration increased heart weight (Table 3.1), left ventricular weight (Figure 3.1) and right ventricular weight (Table 3.1) in rats, an effect that was completely reversed 4 months after cessation of ISO. After 4 months of recovery, heart weight (Table 3.1), right ventricular weight (Table 3.1), left ventricular weight (Figure 3.1), heart weight-to-body weight ratio (Table 3.1) and left ventricular-to-body weight ratio (Table 3.1) decreased to values that were lower than age-matched control rat values.

Although 90% of rats receiving ISO for 6 months had a left ventricular weight that was greater than or equal to the upper 95% confidence interval for control rats at 6 months ($p < 0.01$ for comparison of proportion of rats within the control group that had a left ventricular weight that was greater than or equal to the upper 95% confidence interval for control rats) (Figure 3.1), only 14.3% of rats 4 months after cessation of ISO administration had a left ventricular weight that was greater than or equal to the upper 95% confidence interval for age-matched control rats ($p = 0.39$ for comparison of proportion of rats within the control group that had a left ventricular weight that was greater than or equal to the upper 95% confidence interval for age-matched control rats) (Figure 3.1). Thus, withdrawal of ISO administration resulted in complete reversal of cardiac hypertrophy.

Table 3.1 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on body and heart weights in rats. Left ventricular weights are shown in Figure 3.1.

	ISO (6 months) (n = 10)	Control (6 months) (n= 10)	ISO (6 months) + Recovery (n= 14)	Control (6 months) + Recovery (n= 14)
Body weight (g)	599 ± 15.1	612 ± 19.5	584 ± 11.5	610 ± 16.3
Heart weight (g)	1.84 ± 0.102*	1.61 ± 0.066	1.29 ± 0.036†	1.53 ± 0.073
Right ventricular weight (g)	0.392 ± 0.016*	0.331 ± 0.015	0.284 ± 0.007†	0.333 ± 0.018
HW/BW (g.kg⁻¹) x 10³	3.1 ± 0.13*	2.6 ± 0.06	2.2 ± 0.05†	2.5 ± 0.09
LVW/BW (g.kg⁻¹) x 10³	2.4 ± 0.11*	2.0 ± 0.04	1.7 ± 0.04†	1.9 ± 0.07

HW, Heart weight; BW, Body weight; LVW, Left ventricular weight. * p<0.05 vs other groups. † p<0.05 vs control (6 months) and Control (6 months) + Recovery.

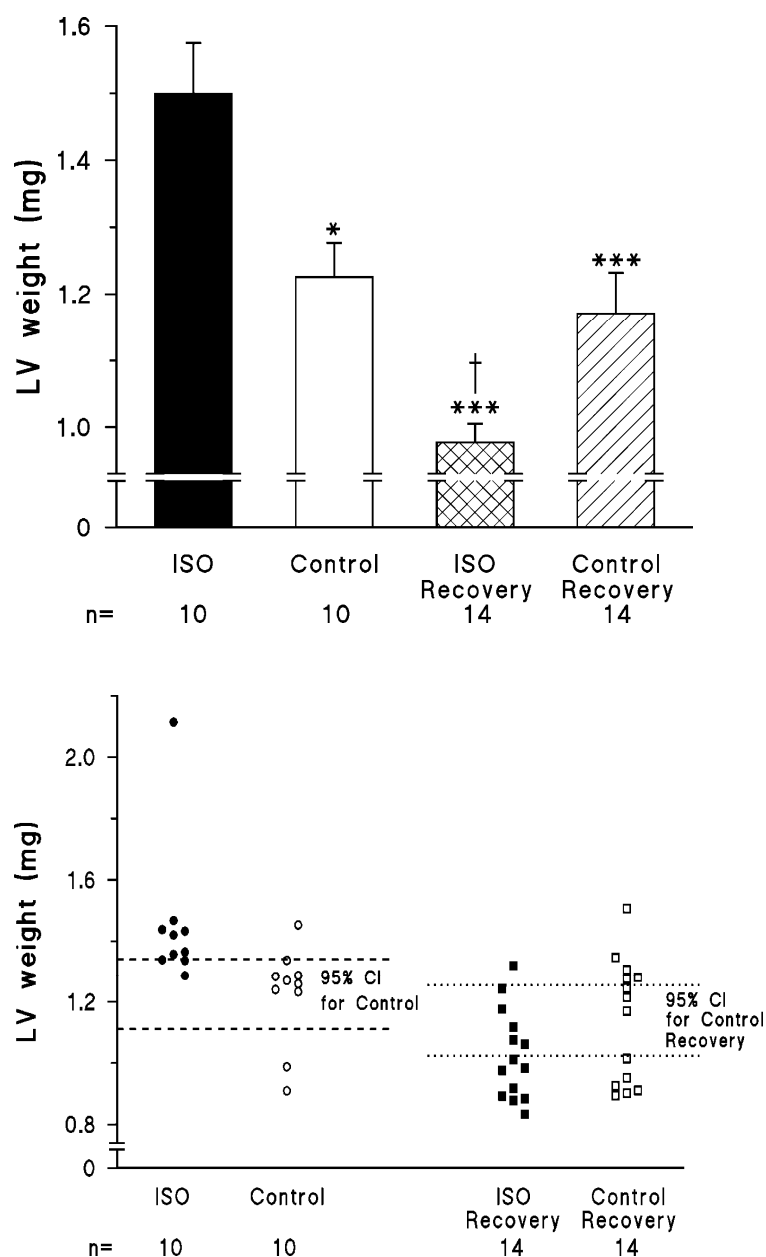


Figure 3.1 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on left ventricular (LV) weight in rats. The upper panel shows means and standard errors and the lower panel shows individual data and 95% confidence intervals for control rat data. The lower panel therefore illustrates the proportion of experimental animals whose data lies at or outside of the 95% intervals for controls. * $p < 0.05$, *** $p < 0.001$ vs ISO, † $p < 0.05$ vs Control and Control recovery. Statistical comparisons of proportions of ISO or ISO Recovery rats that are above or equal to the upper 95% confidence intervals for controls are given in the text.

3.2 Impact of cessation of chronic isoproterenol administration on echocardiographic parameters.

Table 3.2 and Figures 3.2 and 3.3 show the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on echocardiographic parameters. Six months of daily ISO administration resulted in left ventricular dilatation as indexed by an increased left ventricular end diastolic (Figure 3.2) and end systolic diameters (Table 3.2), and a decreased left ventricular relative wall thickness (Table 3.2), an effect that was completely reversed 4 months after cessation of ISO.

Although 100% of rats had a left ventricular end diastolic diameter that was noted to lie beyond the upper 95% confidence interval for control rats at 6 months ($p < 0.02$ for comparison of proportion of rats within the control group that had a left ventricular end diastolic diameter that was noted to lie beyond the upper 95% confidence interval for control rats) (Figure 3.2), only 21.4% of rats 4 months after cessation of ISO administration had a left ventricular end diastolic diameter that was noted to lie beyond the upper 95% confidence interval for age-matched control rats ($p = 0.42$ for comparison of proportion of rats within the control group that had a left ventricular end diastolic diameter that was noted to lie beyond the upper 95% confidence interval for age-matched control rats) (Figure 3.2). Thus, withdrawal of ISO administration resulted in complete reversal of cardiac dilatation as determined by *in vivo* measurements assessed at uncontrolled heart rates and loading conditions.

Six months of daily ISO administration also resulted in a reduced left ventricular pump function as indexed by a decrease in left ventricular endocardial fractional shortening (LV FS_{end}), an effect that was completely reversed 4 months after cessation

Table 3.2 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on echocardiographic parameters. Other echocardiographic data are shown in Figures 3.2 and 3.3

	ISO (6 months) (n=10)	Control (6 months) (n=10)	ISO (6 months) + Recovery (n=14)	Control (6 months) + Recovery (n =14)
LV ESD (mm)	5.7 ± 0.19*	4.0 ± 0.25	3.5 ± 0.14	3.8 ± 0.14
LV PWED (mm)	1.8 ± 0.07	2.1 ± 0.11	1.8 ± 0.07	2.0 ± 0.08
LV PWES (mm)	2.9 ± 0.16	3.4 ± 0.12	3.1 ± 0.07	3.3 ± 0.07
LV relative wall thickness (mm)	0.39±0.012*	0.55 ± 0.037	0.49 ± 0.022	0.52 ± 0.019
Heart rate (beats.min⁻¹)	220 ± 9.5	239 ± 7	222 ± 8.3	234 ± 7.8

LV, left ventricle; ESD, end systolic diameter; PWED, posterior wall thickness at end diastole; PWES: posterior wall thickness at end systole. *p<0.05 vs the other three groups.

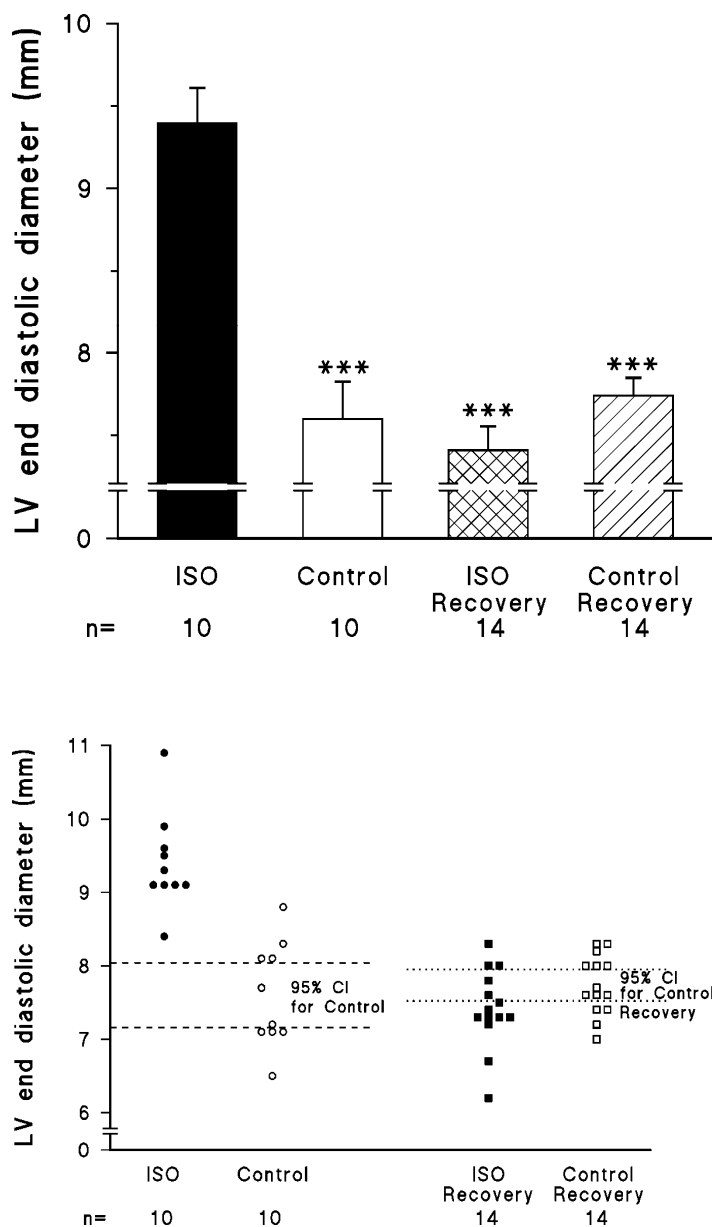


Figure 3.2 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on left ventricular (LV) end diastolic diameter in rats. The upper panel shows means and standard errors and the lower panel shows individual data and 95% confidence intervals for control rat data. The lower panel therefore illustrates the proportion of experimental animals whose data lies at or outside of the 95% intervals for controls.*** $p < 0.001$ vs ISO. Statistical comparisons of proportions ISO or ISO Recovery rats that are above or equal to the upper 95% confidence intervals for controls are given in the text.

of ISO (Figure 3.3).

Although 80% of rats had a LV FS_{end} that was below or equal to the lower 95% confidence interval for control rats at 6 months ($p < 0.05$ for comparison of proportion of rats within the control group that had a LV FS_{end} that was below or equal to the lower 95% confidence interval for control rats) (Figure 3.3), only 28.6% of rats 4 months after cessation of ISO administration had a LV FS_{end} that was below or equal to the lower 95% confidence interval for age-matched control rats ($p = 0.70$ for comparison of proportion of rats within the control group that had a LV FS_{end} that was below or equal to the lower 95% confidence interval for age-matched control rats) (Figure 3.3). Thus, withdrawal of ISO administration resulted in complete reversal of a reduced pump function as determined by *in vivo* measurements assessed at uncontrolled heart rates and loading conditions. Differences in LV endocardial fractional shortening could not be attributed to differences in heart rate (Table 3.2).

3.3 Impact of cessation of chronic isoproterenol administration on left ventricular chamber dimensions assessed *ex vivo* under controlled conditions.

Figure 3.4 shows the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on left ventricular diastolic pressure-volume relations as determined in isolated perfused heart preparations under controlled conditions. Figure 3.5 shows the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on the volume intercept (LV V_0) of these relations. Six months of daily ISO administration resulted in left ventricular dilatation as indexed by a right shift in

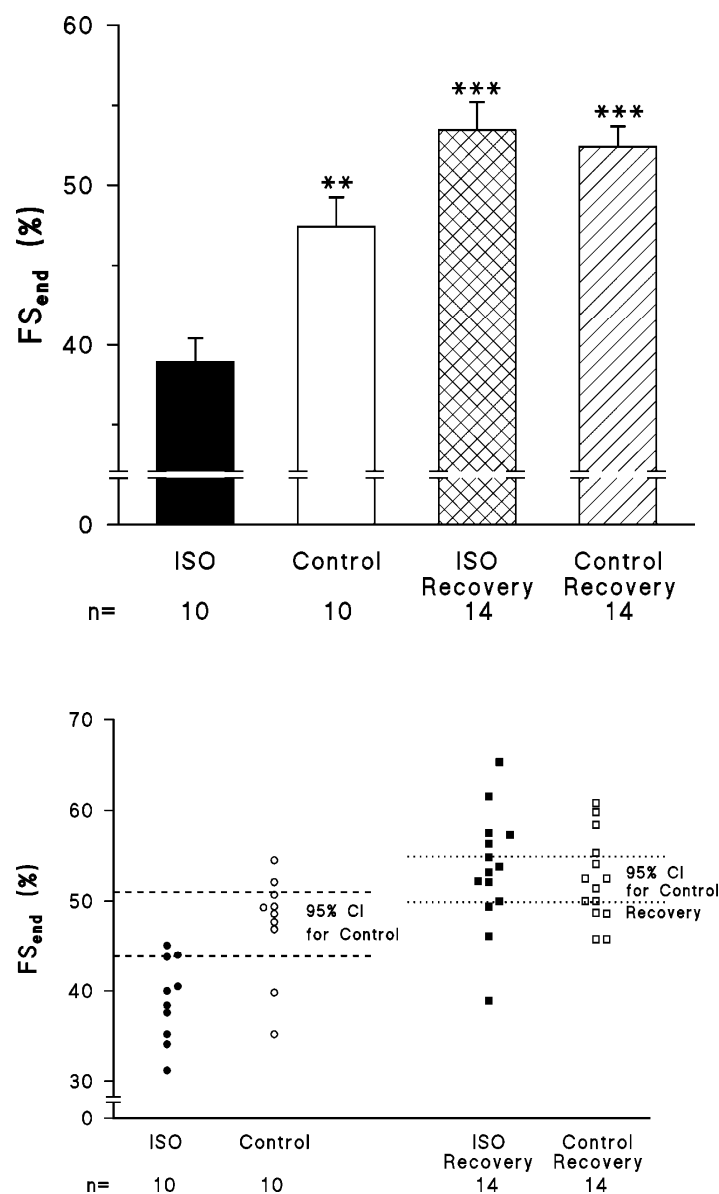


Figure 3.3 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on left ventricular endocardial fractional shortening (FS_{end}) (pump function) in rats. The upper panel shows means and standard errors and the lower panel shows individual data and 95% confidence intervals for control rat data. The lower panel therefore illustrates the proportion of experimental animals whose data lies at or outside of the 95% intervals for controls. ** $p < 0.01$, *** $p < 0.001$ vs ISO. Statistical comparisons of proportions ISO or ISO Recovery rats that are below or equal to the lower 95% confidence intervals for controls are given in the text.

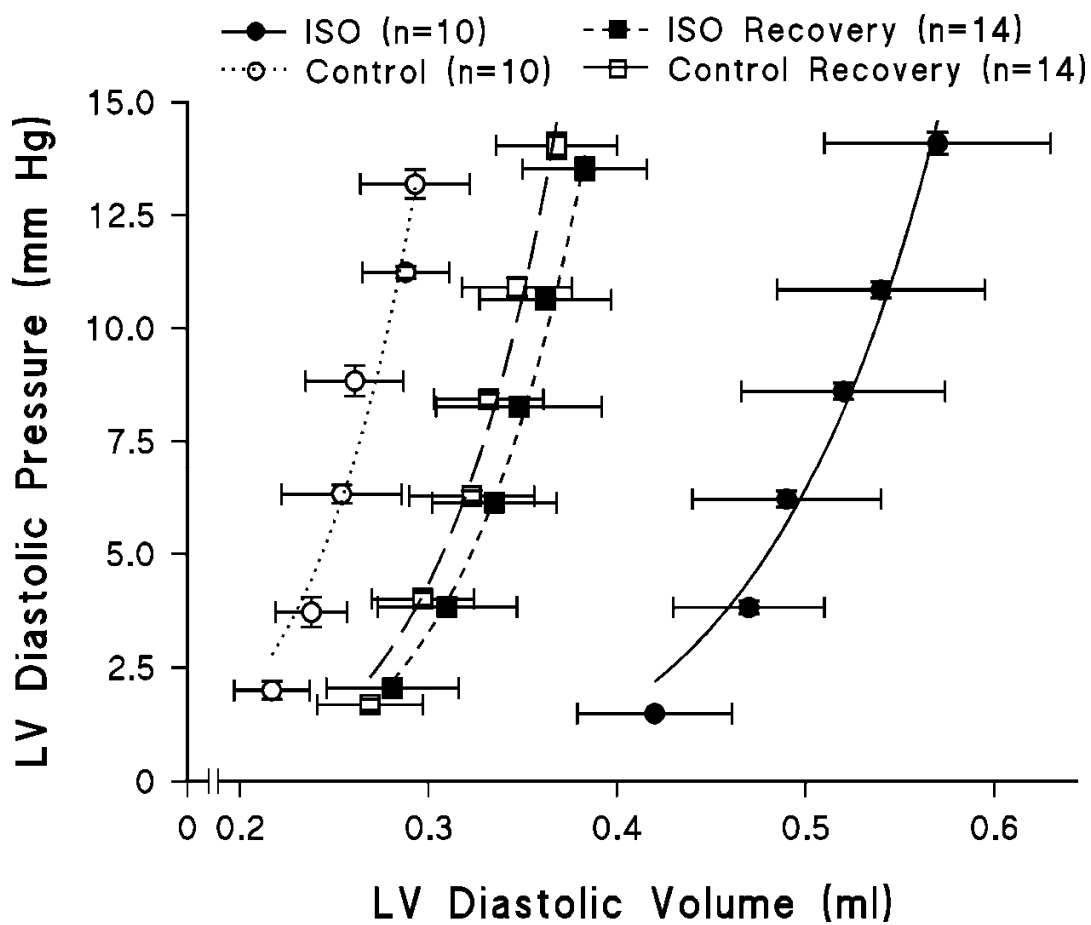


Figure 3.4 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on left ventricular (LV) diastolic pressure-volume relations in rats. Statistical comparisons of the volume intercepts of these relations are given in Figure 3.5.

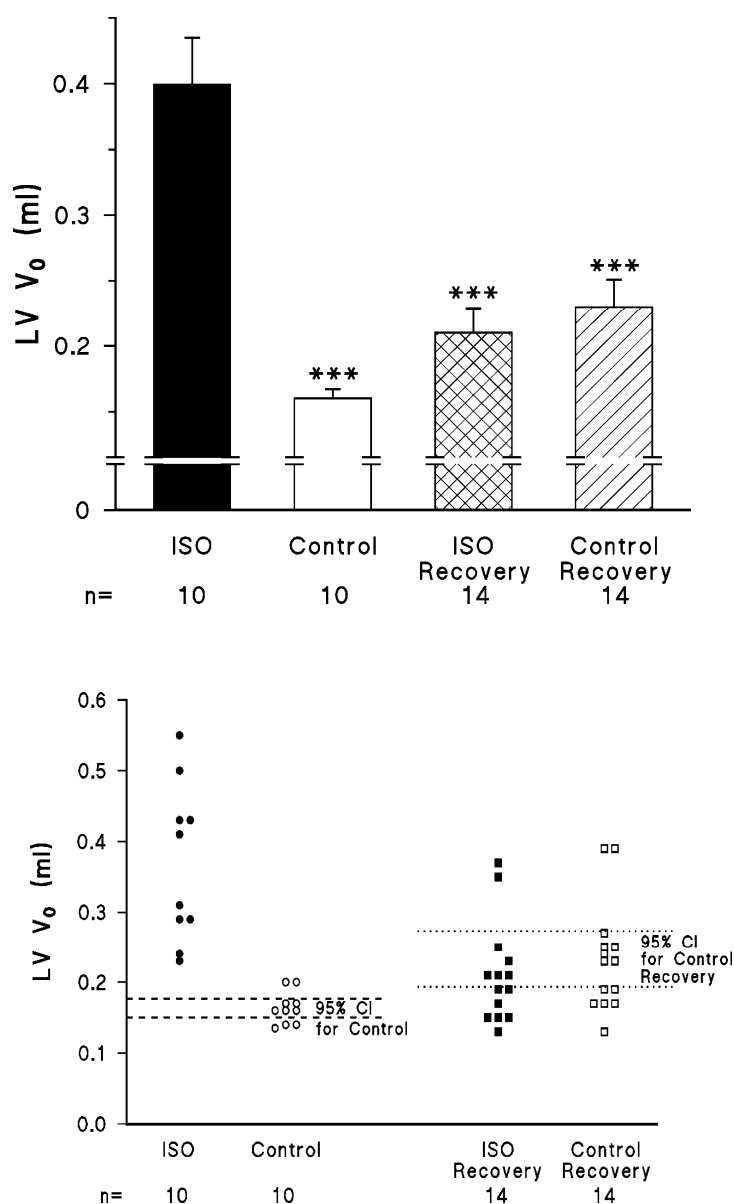


Figure 3.5 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on the volume intercept at 0 mm Hg ($LV\ volume_0$) of the left ventricular diastolic pressure-volume relations shown in Figure 3.4 in rats. The upper panel shows means and standard errors and the lower panel shows individual data and 95% confidence intervals for control rat data. The lower panel therefore illustrates the proportion of experimental animals whose data lies at or outside of the 95% intervals for controls. *** $p < 0.001$ vs ISO. Statistical comparisons of proportions ISO or ISO Recovery rats that are above the upper 95% confidence intervals for controls are given in the text.

the left ventricular diastolic pressure-volume relationship (Figure 3.4) and an increase in LV V_0 (Figure 3.5), an effect that was completely reversed 4 months after cessation of ISO (Figures 3.4 and 3.5).

Although 100% of rats had LV V_0 values that lay beyond the upper 95% confidence interval for control rats at 6 months ($p < 0.001$ for comparison of proportion of rats within the control group that had LV V_0 values that were noted to lie beyond the upper 95% confidence interval for control rats) (Figure 3.5), only 14.3% of rats 4 months after cessation of ISO administration had LV V_0 values that were noted to lie at or beyond the upper 95% confidence interval for age-matched control rats ($p = 1.40$ for comparison of proportion of rats within the control group that had LV V_0 values that were noted to lie at or beyond the upper 95% confidence interval for age-matched control rats) (Figure 3.5). Thus, withdrawal of ISO administration resulted in complete reversal of cardiac dilatation as determined by *ex vivo* measurements assessed at controlled heart rates, under controlled loading conditions and at matched coronary flows.

3.4 Impact of cessation of chronic isoproterenol administration on left ventricular systolic chamber contractility assessed *ex vivo*.

Figure 3.6 shows the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on left ventricular systolic pressure-volume relations as determined in isolated perfused heart preparations. Figure 3.7 shows the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on the slope (LV E_{es}) of these relations. Six months of daily ISO administration resulted in a

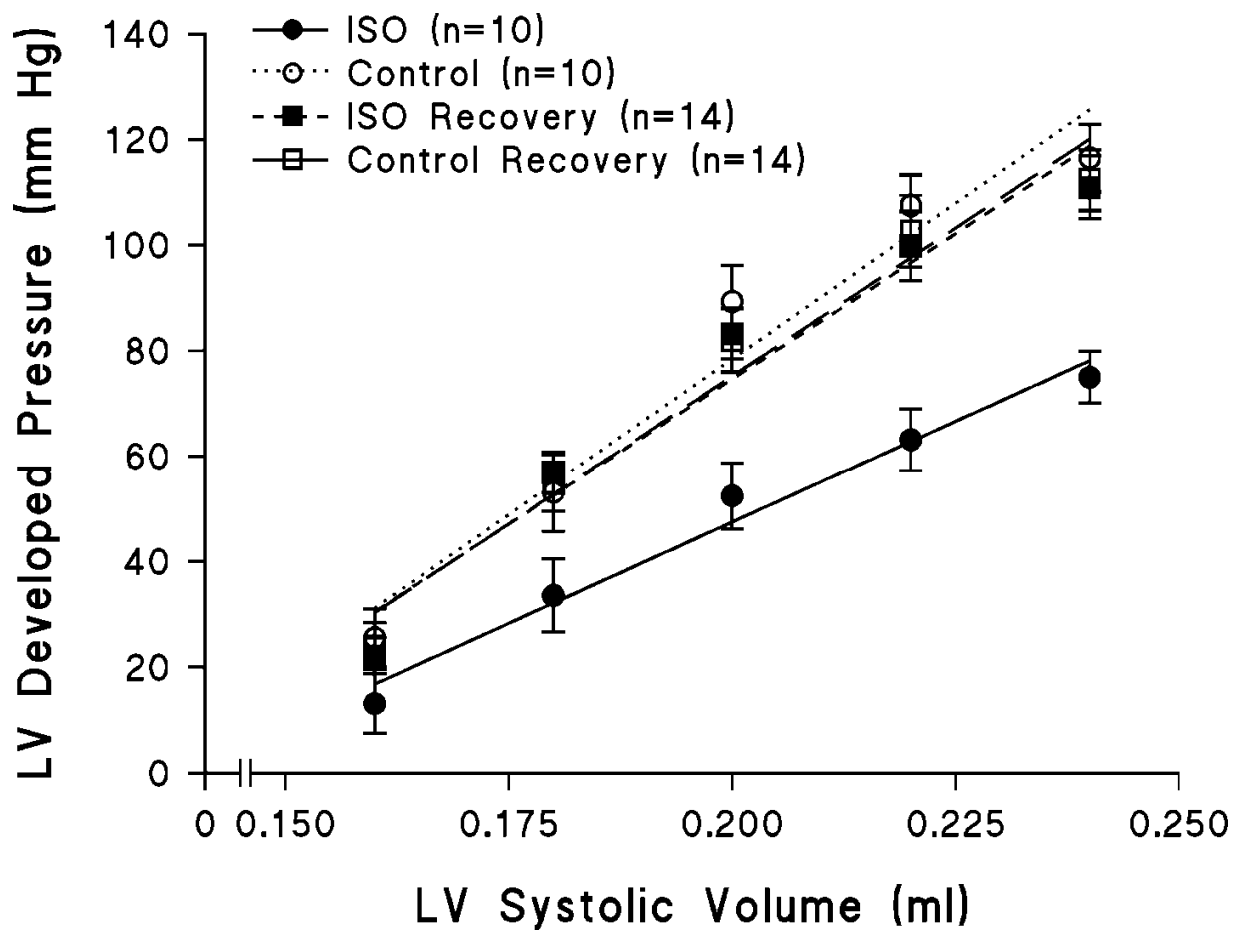


Figure 3.6 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on left ventricular (LV) systolic pressure-volume relations in rats. Statistical comparisons of the slopes of these relations are given in Figure 3.7.

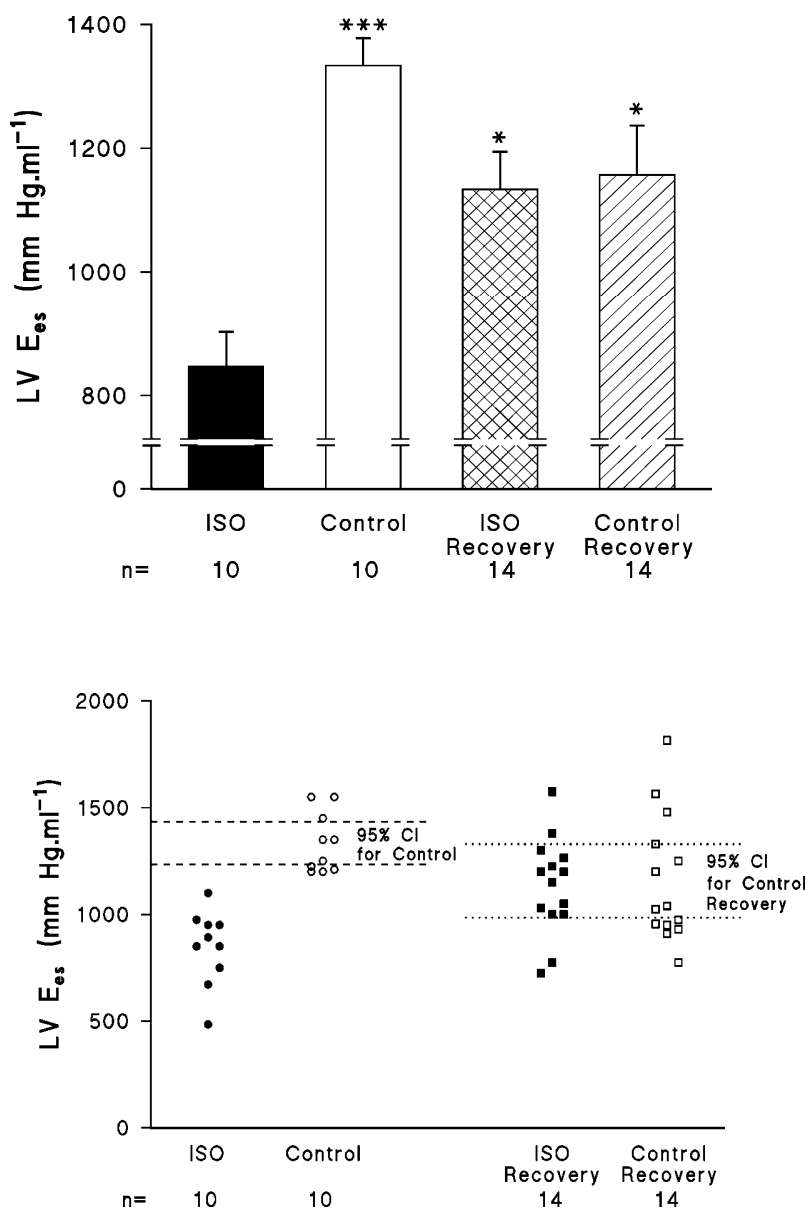


Figure 3.7 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on the slope (LV end systolic elastance-LV E_{es}) of the left ventricular systolic pressure-volume relations shown in Figure 3.6 in rats. The upper panel shows means and standard deviations and the lower panel shows individual data and 95% confidence intervals for control rat data. The lower panel therefore illustrates the proportion of experimental animals whose data lies at or outside of the 95% intervals for controls. * $p < 0.05$, *** $p < 0.001$ vs ISO. Statistical comparisons of proportions ISO or ISO Recovery rats that are at or below the lower 95% confidence intervals for controls are given in the text.

reduced left ventricular systolic chamber function as indexed by a right shift in the left ventricular systolic pressure-volume relation (Figure 3.6) and a decreased LV Ees (Figure 3.7) effects that were completely reversed 4 months after cessation of ISO (Figures 3.6 and 3.7).

Although 100% of rats had LV Ees values that lay below the lower 95% confidence interval for control rats at 6 months ($p < 0.02$ for comparison of proportion of rats within the control group that had LV Ees values that were noted to lie below the lower 95% confidence interval for control rats) (Figure 3.7), only 14.3% of rats 4 months after cessation of ISO administration had LV Ees values that were noted to lie below the lower 95% confidence interval for age-matched control rats ($p = 0.21$ for comparison of proportion of rats within the control group that had LV Ees values that were noted to lie below the lower 95% confidence interval for age-matched control rats) (Figure 3.7). Thus, withdrawal of ISO administration resulted in complete reversal of the attenuation of cardiac systolic chamber contractility as determined by *ex vivo* measurements assessed at controlled heart rates, under controlled loading conditions and at matched coronary flows.

3.5 Impact of cessation of chronic isoproterenol administration on left ventricular systolic myocardial contractility assessed *ex vivo*.

Figure 3.8 shows the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on left ventricular systolic stress-strain relations as determined in isolated perfused heart preparations. Figure 3.9 shows the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on the

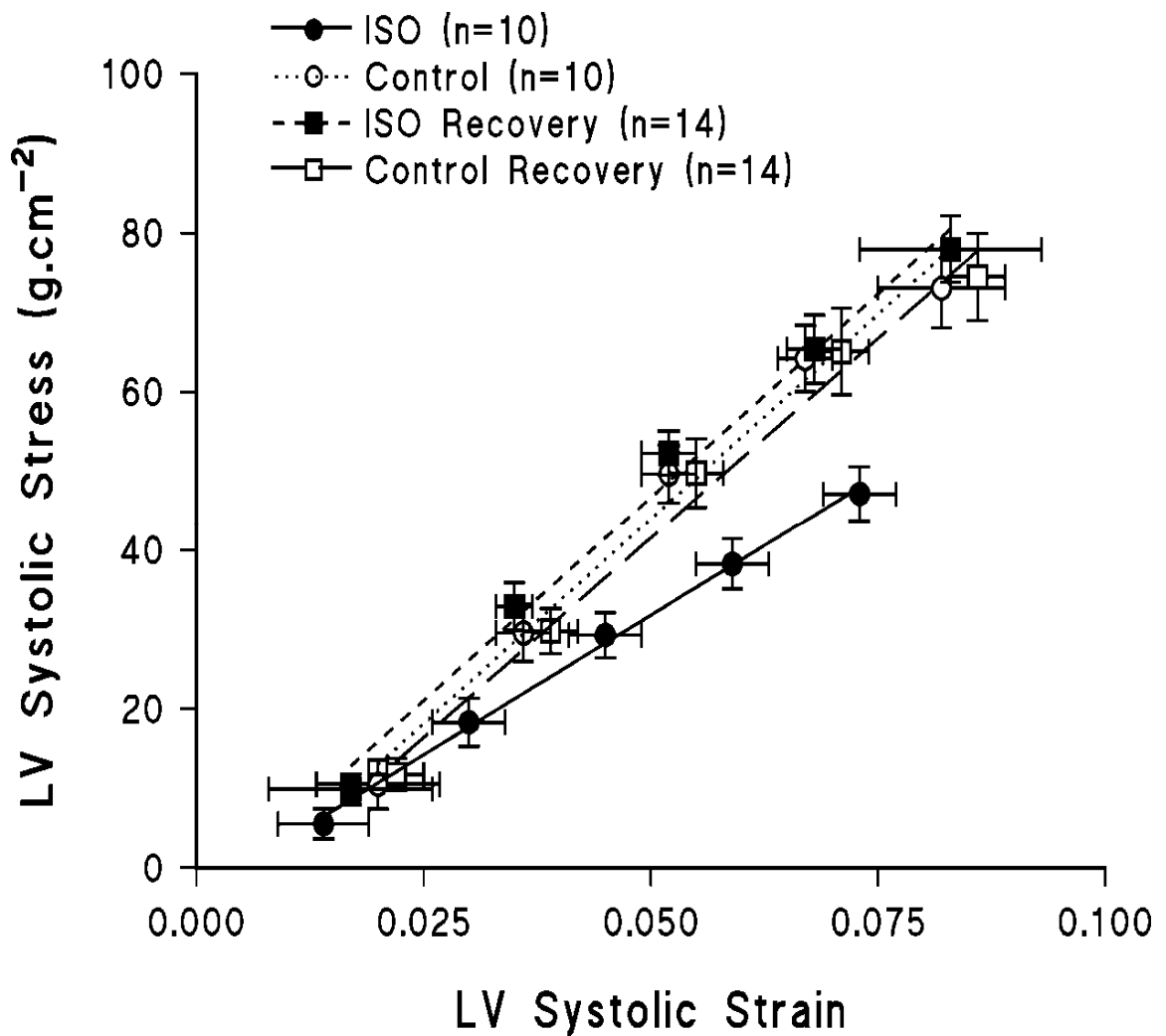


Figure 3.8 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on left ventricular (LV) systolic stress-strain relations in rats. Statistical comparisons of the slopes of these relations are given in Figure 3.9.

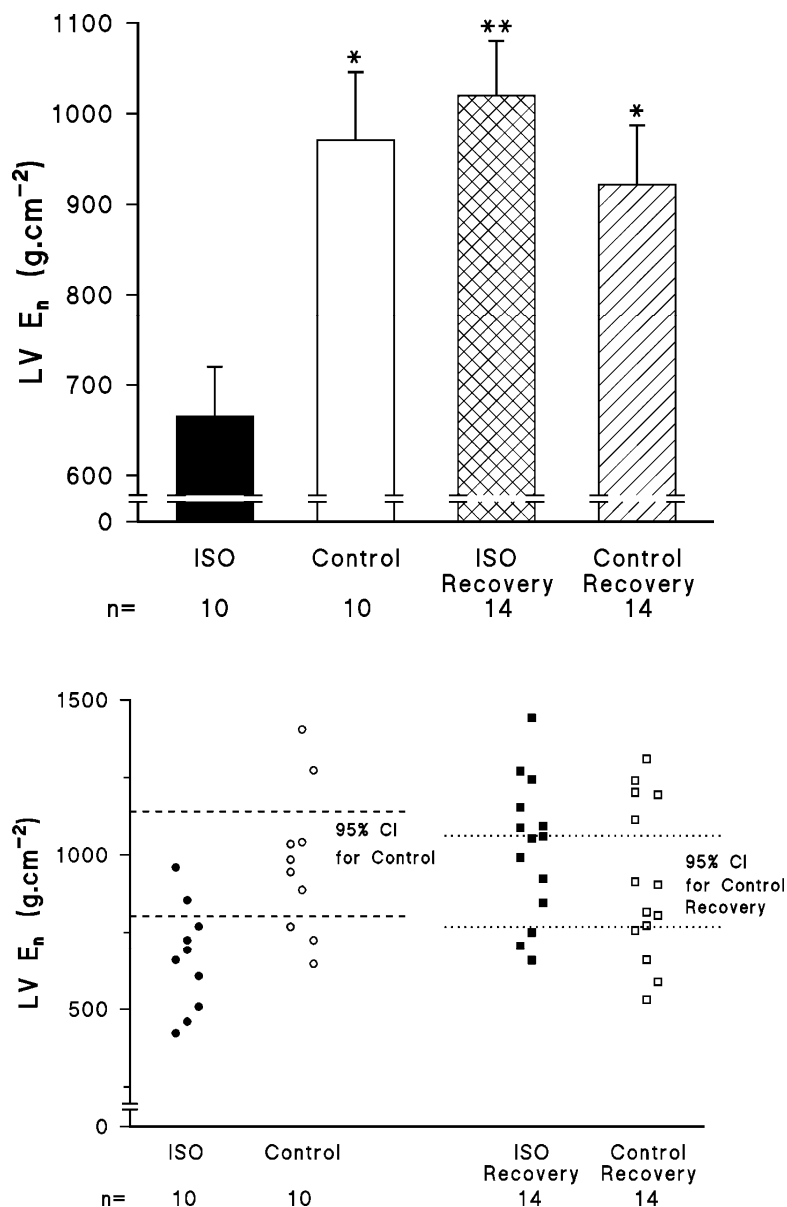


Figure 3.9 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on the slope (LV end systolic myocardial elastance-LV E_n) of the left ventricular systolic stress-strain relations shown in Figure 3.8 in rats. The upper panel shows means and standard deviations and the lower panel shows individual data and 95% confidence intervals for control rat data. The lower panel therefore illustrates the proportion of experimental animals whose data lies at or outside of the 95% intervals for controls. * $p < 0.05$, ** $p < 0.001$ vs ISO. Statistical comparisons of proportions ISO or ISO Recovery rats that are at or below the lower 95% confidence intervals for controls are given in the text.

slope (LV En) of these relations. Six months of daily ISO administration resulted in a reduced left ventricular systolic myocardial function as indexed by a right shift in the left ventricular systolic stress-strain relation (Figure 3.8) and a decreased LV En (Figure 3.9) effects that were completely reversed 4 months after cessation of ISO (Figures 3.8 and 3.9).

Eighty percent of rats had LV En values that lay below the lower 95% confidence interval for control rats at 6 months. However, this proportion was not statistically greater than the proportion of control rats with LV En values below the lower 95% confidence intervals ($p=0.07$ for comparison of proportion of rats within the control group that had LV En values that were below the lower 95% confidence interval for control rats) (Figure 3.9). Only 21.4% of rats 4 months after cessation of ISO administration had LV En values that were below the lower 95% confidence interval for age-matched control rats ($p=0.68$ for comparison of proportion of rats within the control group that had LV En values that were noted to lie below the lower 95% confidence interval for age-matched control rats) (Figure 3.7).

3.6 Impact of chronic isoproterenol administration and the cessation of isoproterenol administration on myocardial damage.

Table 3.3 shows the impact of 6 months of daily isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration on left ventricular necrosis (pathological score), cardiomyocyte apoptosis (TUNEL) and collagen concentrations (hydroxyproline). Chronic ISO administration did not modify either the pathological score or myocardial collagen concentrations. Moreover, as assessed 24 hours after the last dose of ISO, no evidence of cardiomyocyte apoptosis

was noted in rats receiving chronic ISO. After 4 months of recovery following cessation of ISO administration, cardiomyocyte apoptosis tended to be lower than those rats 4 months younger.

Table 3.3 Impact of chronic isoproterenol (ISO) administration and 4 months of recovery after cessation of ISO administration (recovery) on cardiac apoptosis, necrosis and total myocardial collagen concentrations.

	ISO (6 months) (n= 10)	Control (6 months) (n= 10)	ISO (6 months) + Recovery (n=14)	Control (6 months) + Recovery (n = 14)
Path Score (Necrosis)	1.4 ± 0.27	1.5 ± 0.22	1.6 ± 0.17	1.9 ± 0.25
% Apoptosis	0.205 ± 0.04	0.207 ± 0.03	0.097 ± 0.01*	0.137 ± 0.02
Total collagen [HPRO] µg.mg⁻¹	0.86 ± 0.03	0.84 ± 0.05	0.85 ± 0.07	0.92 ± 0.05

Path, pathological; [HPRO], myocardial concentration of hydroxyproline. *p<0.05 vs ISO (6 months) and Control (6 months) groups.

Chapter 4

Discussion

4.1 Summary of main findings

The main findings of the present study are as follows: After the induction of marked cardiac dilatation (as indexed by increases in LVEDD, right shifts in LV diastolic pressure-volume relations and increases in LV V_0 with the increase in LV V_0 being 2.5 times normal), pump dysfunction (as indexed by decreases in LV FS_{end}), and decreases in left ventricular chamber (as indexed by decreases in LV E_{es}) and myocardial (as indexed by decreases in LV E_n) contractility by daily administration of the β -adrenergic receptor agonist ISO for 6 months, withdrawal of ISO resulted in complete reversal of the adverse remodelling, pump dysfunction and contractile disturbances over a subsequent 4 month follow-up period. The adrenergic-induced left ventricular dilatation, pump dysfunction and contractile disturbances (chamber and myocardial) and the subsequent complete reversal of this process was documented not only with load and heart rate-dependent echocardiographic measurements obtained *in vivo*, but also with load and heart rate-independent measures of cardiac dilatation and pump dysfunction *ex vivo* under controlled conditions. Importantly however, isoproterenol-induced left ventricular dilatation, pump dysfunction and contractile disturbances in the present study were not associated with myocardial necrosis, or fibrosis. Although I could not document myocardial apoptosis and reversal of this process, these data will be considered in the context of the timing of the measurement. How do the results of the present study build on our previous understanding of the ability of adrenergic blockers to decrease cardiac cavity volumes through the process of reverse remodelling?

4.2 How does the present study build on our current understanding of reversal of adrenergic-induced cardiac dilatation and pump dysfunction?

The present study is the first to provide clear evidence to show that marked adrenergic-induced cardiac dilatation, pump dysfunction and contractile disturbances can be completely reversed when excessive adrenergic stimulation is removed. Unlike the present study where reversal of the deleterious effects of adrenergic activation was assessed after removal of the adrenergic stimulus, to address the question of the extent to which adrenergic-induced cardiac dilatation can be reversed, previous studies have focussed principally on the beneficial effects of adrenergic receptor blockade to reduce excessive adrenergic stimulation, on cardiac cavity dimensions in cardiac disease. In this regard, although pre-clinical studies have demonstrated the capacity of adrenergic receptor blockers to prevent the development of cardiac dilatation (Chan *et al.*, 2004, Hu *et al.*, 1998), the impact of adrenergic receptor blockade once the structural remodelling and contractile disturbances are fully established is minimal at best and by no means returns cardiac cavity dimensions back to normal values (Hu *et al.*, 1998, Gan *et al.*, 2007b). In this regard, the administration of a β -adrenoreceptor blocker late in the development of cardiac dilatation results in a diminished capacity to attenuate cardiac dilatation as compared to when initiating therapy early (Hu *et al.*, 1998). These studies (Hu *et al.*, 1998, Gan *et al.*, 2007b) therefore challenge the possibility that adrenergic receptor blockade can reverse cardiac dilatation once fully established.

In contrast to only preventing or attenuating progressive cardiac dilatation, which generally characterises the beneficial effects of renin-angiotensin system blockers (Konstam *et al.*, 1992, Konstam *et al.*, 1993, Greenberg *et al.*, 1995, St. John Sutton *et al.*, 1997, Wong *et al.* 2002), as summarised in Table 1.2, in the clinical setting β -

adrenergic receptor blockers reduce cardiac cavity dimensions and volumes in patients with heart failure and established cardiac dilatation (Waagstein *et al.*, 1989, Eichhorn *et al.*, 1990, Gilbert *et al.*, 1990, Woodley *et al.*, 1991, Hall *et al.*, 1995, Heesch *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2000, Lotze *et al.*, 2001, Bello *et al.*, 2003, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007) As compared to the angiotensin-converting enzyme inhibitors, the non-selective α and β -adrenoreceptor blocker, carvedilol produces a greater beneficial effect on cardiac cavity dimensions (Khattar *et al.*, 2001). These beneficial effects of adrenergic blockade on cardiac cavity dimensions and volumes have been noted for the non-selective α and β -adrenoreceptor blocker, carvedilol as well as for the selective β -adrenoreceptor blocker, metoprolol (Table 1.2), thus indicating that the benefits that accrue from adrenergic receptor blockade on cardiac cavity dimensions in heart failure mainly occur through β -adrenoreceptor blockade. However, these effects by no means achieve normalisation of the cavity volumes or dimensions (see Table 1.2 for a summary of cardiac chamber volumes and internal diameters before and after β -adrenergic receptor blockers therapy).

In contrast to an inability of β -adrenergic receptor blockade to show complete reverse remodelling in both preclinical and clinical studies, I have been able to show that complete reversal of β -adrenoreceptor-mediated cardiac dilatation, pump dysfunction and chamber and myocardial contractile disturbances can occur. One possible explanation for the apparent discrepancies between the present study and studies conducted with β -adrenergic receptor blockers in heart failure is that the model of adrenergic-induced cardiac dilatation and pump dysfunction evaluated in the present study simply does not reflect what happens in the clinical heart failure condition where β -

adrenergic receptor blockers produce benefits. Indeed, at a clinical level in heart failure, increases in circulating adrenaline and noradrenaline concentrations are noted and sympathetic activation to the heart and other organs increases (see chapter 1 for a summary), effects that are in-part targeted by α and β -adrenoreceptor blockers. In contrast, in the present study an increase in circulating concentrations of a β -adrenergic receptor agonist was employed to induce cardiac dilatation and pump dysfunction and I failed to assess the impact of α and β -adrenoreceptor blockers on the model. However, it is difficult to conceive of how the model studied in the current dissertation cannot be seen as in some way as able to mimic the human condition as the β -adrenergic receptor blockers bisoprolol and metoprolol (which do not target any other adrenergic receptors) produce beneficial effects on outcomes and cardiac dimensions in heart failure (see chapter 1 for summary).

Assuming that the model of adrenergic-induced cardiac dilatation and pump dysfunction explored in the present dissertation does mimic the human heart failure condition, another obvious question that arises is whether in the present study the extent of cardiac dilatation can be considered to be compatible with that noted in many of the clinical studies described in Table 1.2. This question is important, as normalisation of cavity volumes may occur if the extent of the dilatation is not as advanced. Indeed, as indicated in Table 1.2 when baseline left ventricular volumes are lower, final volumes after β -adrenoreceptor blocker therapy may lie in the normal range (Malfatto *et al.*, 2007). Is the extent of cardiac dilatation and pump dysfunction noted in the present study compatible with more marked cardiac dilatation noted in many clinical studies?

Although it is difficult to extrapolate cardiac dimension data obtained in rodents to the clinical setting, there is no question that proportionately, the left ventricular diastolic volume changes noted in the present study are of clinical importance. In this regard, as

with the present study where LV V_0 increased from on average ~ 0.16 mls in the control group to on average ~ 0.40 mls in the group receiving β -adrenergic stimulation for 6 months, in clinical studies assessing the impact of adrenergic blockade on cardiac cavity volumes, the baseline volumes in patients with heart failure were approximately 1.3-1.9 times that considered to be normal (Hall *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Capomolla *et al.*, 2000, Groenning *et al.*, 2000, Waagstein *et al.*, 2003, Rahko *et al.*, 2005, Malfato *et al.*, 2007). In the present study the volume increase in response to β -adrenergic stimulation was 2.5 times greater than the control group, volume increases that may therefore even exceed that noted in clinical studies. Despite the potentially greater extent of the left ventricular chamber volume changes noted in the present study as compared to clinical studies (Waagstein *et al.*, 1989, Eichhorn *et al.*, 1990, Gilbert *et al.*, 1990, Woodley *et al.*, 1991, Hall *et al.*, 1995, Heesch *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2000, Lotze *et al.*, 2001, Bello *et al.*, 2003, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007), LV V_0 returned to control values in the present study after cessation of the β -adrenergic agonist. In contrast, in clinical studies, long-term blockade of adrenergic receptors failed to normalise left ventricular chamber volumes. Assuming that the model studied in the present dissertation does in-part mimic the human heart failure condition, the question therefore arises as to why chronic β -adrenergic receptor blockade cannot normalize cardiac cavity size in clinical studies of heart failure when the current study suggests that complete reversal should be achievable?

4.3 Why is adrenergic receptor blockade unable to completely reverse cardiac dilatation and pump dysfunction?

A number of potential explanations could account for the inability of adrenergic receptor blockers to completely reverse cardiac dilatation and pump dysfunction. First, in clinical (Hall *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007, Lotze *et al.*, 2001)(Table 1.2) and pre-clinical (Hu *et al.*, 1998, Gan *et al.*, 2007a, 2007b, Li *et al.*, 2007) studies where the impact of β -adrenergic receptor blockade on cardiac cavity dimensions and pump function has been evaluated, it is impossible to document whether complete myocardial β -adrenergic receptor blockade has been achieved. Indeed, it is unlikely that in many patients receiving β -adrenergic receptor blockers, because of the negative inotropic and hypotensive effects of these agents, or through alternative side effects such as bronchoconstriction, that maximal doses of β -adrenergic receptor blockers can be achieved and tolerated by many patients with heart failure (Fowler *et al.*, 2007, Egred *et al.*, 2005). In this regard, it is possible that the higher the dose of β -adrenergic receptor blocker employed the better the outcomes. No clear dose-response relationship between β -adrenergic receptor blocker use and changes in cardiac structure and function in pre-clinical (Yaoita *et al.*, 2002) or some clinical (Colucci *et al.*, 2007) studies, and similarly no clear dose-response relationship between β -adrenergic receptor blocker use and outcomes in some clinical studies (CIBIS II, 1999, Packer *et al.*, 2001, MERIT-HF, 1999) has been documented. However, there is indeed a survival advantage and decreased hospitalisations when the dose of β -adrenergic receptor blockers is relatively close to target doses (CIBIS II, 1999, Packer *et al.*, 2001, MERIT-

HF, 1999). Furthermore, dose-related improvements in left ventricular function and survival have been reported on in one study for carvedilol (Bristow *et al.*, 1996) and patients treated with high doses of β -adrenergic receptor blockers gain a greater benefit than patients treated with low doses (Lenzen *et al.*, 2005). In contrast to the use of β -adrenergic receptor blockers, where complete adrenergic receptor blockade may not occur, using the approach employed in the present study, I could ensure that excessive β -adrenergic receptor activation was largely attenuated, simply by removing the adrenergic stimulus. Hence, a lack of ability to completely reverse cardiac dilatation through the use of β -adrenergic receptor blockers may simply reflect an inability to completely block adrenergic receptors.

The second reason that may account for the inability of adrenergic receptor blockers to completely reverse cardiac dilatation and pump dysfunction in clinical (Waagstein *et al.*, 1989, Eichhorn *et al.*, 1990, Gilbert *et al.*, 1990, Woodley *et al.*, 1991, Hall *et al.*, 1995, Heesch *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2000, Lotze *et al.*, 2001, Bello *et al.*, 2003, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007) and one pre-clinical (Hu *et al.*, 1998) study, is that in progressive heart failure a number of neurohumoral and inflammatory changes may occur which could contribute toward progressive cardiac dilatation and pump dysfunction over and above that produced by adrenergic activation (Mann, 1999). An inability to completely reverse cardiac dilatation through the use of β -adrenergic receptor blockers may therefore also reflect the inability of these agents to target all neurohumoral and inflammatory mechanisms responsible for chronic heart failure. In contrast, in the present study, the only stimulus for cardiac dilatation was excessive β -adrenergic receptor activation, and hence reversal of the

deleterious chamber remodelling process was dependent only on removing this stimulus. However, in one pre-clinical study (Gan *et al.*, 2007b) cardiac dilatation produced by adrenergic activation (isoproterenol) was not reversed by β -adrenergic receptor blockade. Nevertheless, in contrast to our study where doses of isoproterenol were employed that failed to produce myocardial necrosis and fibrosis, in this previous study (Gan *et al.*, 2007b) isoproterenol administration produced marked myocardial fibrosis.

The third reason that may account for the inability of adrenergic receptor blockers to completely reverse cardiac dilatation and pump dysfunction is that in progressive heart failure in the aforementioned clinical (Waagstein *et al.*, 1989, Eichhorn *et al.*, 1990, Gilbert *et al.*, 1990, Woodley *et al.*, 1991, Hall *et al.*, 1995, Heesch *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2000, Lotze *et al.*, 2001, Bello *et al.*, 2003, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007) and pre-clinical (Hu *et al.*, 1998) studies an initiating event such as hypertension, myocardial infarction or myocarditis may have caused marked and irreparable myocardial damage well beyond that which is produced by excessive adrenergic activation. An inability to completely reverse cardiac dilatation through the use of β -adrenergic receptor blockers in some patients may therefore simply reflect the presence of extensive pre-existing cardiac damage. In contrast, in the present study, the stimulus for cardiac dilatation was excessive β -adrenergic receptor activation produced by exogenous agonist administration and not the presence of pre-existing cardiac disease. Hence reversal of the deleterious chamber remodelling process was dependent only on removing this exogenous stimulus.

In summary, in none of the aforementioned clinical (Waagstein *et al.*, 1989, Eichhorn *et al.*, 1990, Gilbert *et al.*, 1990, Woodley *et al.*, 1991, Hall *et al.*, 1995, Heesch

et al., 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2000, Lotze *et al.*, 2001, Bello *et al.*, 2003, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007) and pre-clinical (Hu *et al.*, 1998, Gan *et al.*, 2007a, 2007b, Li *et al.*, 2007) studies can the presence of residual cardiac dilatation be taken to indicate that changes to the myocardium produced by excessive adrenergic activation cannot be completely reversed by adrenergic receptor blockade. Indeed, assuming that the model studied in the present dissertation does indeed in some way mimic the human heart failure condition, in this regard, the present study provides clear evidence to show that even after inducing marked cardiac dilatation and pump dysfunction through chronic β -adrenergic receptor activation, removal of the adrenergic stimulus can completely reverse the chamber dilatation and pump dysfunction.

4.4 Reversal of adrenergic-induced cardiac dilatation: Can this be attributed to normalisation of diastolic pressure-volume relations?

In clinical studies that have assessed the capacity and extent of adrenergic blockade to decrease cardiac dilatation (Waagstein *et al.*, 1989, Eichhorn *et al.*, 1990, Gilbert *et al.*, 1990, Woodley *et al.*, 1991, Hall *et al.*, 1995, Heesch *et al.*, 1995, Quaife *et al.*, 1996, Doughty *et al.*, 1997, Groenning *et al.*, 2000, Capomolla *et al.*, 2000, Metra *et al.*, 2000, Lotze *et al.*, 2001, Bello *et al.*, 2003, Metra *et al.*, 2003, Waagstein *et al.*, 2003, Toyama *et al.*, 2003, Pieske, 2004, Rahko *et al.*, 2005, Malfatto *et al.*, 2007, Gundogdu *et al.*, 2007), cardiac dilatation has been defined by the magnitude of the increase in cardiac cavity volumes or dimensions measured. As highlighted in chapter 1, this approach does not account for the fact that cardiac chamber dimensions or volumes can

change not only with true cardiac remodelling (a shift in the diastolic pressure-volume relationship), but also with alterations in heart rate (a decreased heart rate increases cavity volumes because the time for filling is extended), preload (an increased blood volume increases cavity volumes), afterload (an increased afterload decreases pump function and hence increases blood remaining in the ventricle at the end of each beat), or myocardial contractility (a decreased pump function increases blood remaining in the ventricle at the end of each beat). In contrast to prior studies that have described the impact of adrenergic receptor blockade on reverse remodelling in established cardiac dilatation, by reporting only on cardiac cavity dimensions or volumes (see Table 1.2) (Gan et al., 2007b) in the present study I have confirmed the beneficial effects of withdrawal of the adrenergic stimulus on adverse cardiac chamber remodelling, with measures of diastolic pressure-volume relationships assessed under controlled conditions (in paced hearts with comparable coronary flow rates).

In the present study true reverse remodelling of the chamber after cessation of chronic β -adrenoreceptor activation accounted for decreases in left ventricular cavity dimensions. In this regard, chronic β -adrenoreceptor activation resulted in a right shift in the diastolic pressure-volume relationship and this change was completely reversed four months after cessation of isoproterenol administration. This finding suggests that the beneficial effects of adrenergic receptor blockade on cardiac cavity dimensions or volumes in previous clinical (see Table 1.2) or preclinical studies (Gan *et al.*, 2007b) could be accounted for by a right shift in cardiac diastolic pressure-volume relationships. Obviously this would only apply if the current model of adrenergic-induced cardiac dilatation does indeed in-part mimic the human heart failure condition. Although in the present study the ability to show that chronic β -adrenergic receptor stimulation produces increases in cavity volumes through right shifts in diastolic pressure-volume relationships

is entirely consistent with a number of previous studies published by our group (Woodiwiss *et al.*, 2001, Badenhorst *et al.*, 2003b, Osadchii *et al.*, 2007), the present study is the first to show that this change can indeed be completely reversed by removal of the adrenergic stimulus.

4.5 Reverse remodelling or changes in myocardial contractile function as a determinant of an improved pump function?

As discussed in the introduction to the present dissertation, an important debate that has accompanied the topic of adverse cardiac chamber remodelling is whether cardiac dilatation is simply a consequence and hence an index of decreases in myocardial systolic function, or whether cardiac dilatation is indeed a cause of cardiac pump dysfunction. In this regard, one argument is that a decreased myocardial systolic function attenuates pump function and increases blood remaining in the ventricle at the end of each beat. With time the ventricle adapts to accommodate the increases in blood volume and maintain normal filling pressures by producing a right shift in the diastolic pressure-volume relationship. This view holds that cardiac dilatation is just an index of the extent of myocardial systolic dysfunction rather than an essential pathophysiological mechanism responsible for pump dysfunction. Although there is currently a general consensus that cardiac dilatation is an important cause of pump dysfunction and hence of heart failure (Cohn *et al.*, 2000), there is no clinical evidence that segregates the impact of cardiac dilatation from that of myocardial systolic dysfunction on cardiac pump function or the presence of heart failure. Probably the strongest clinical evidence in this regard is that in the absence of myocardial infarction, or pre-existing heart failure, over an 11 year follow-up period, the development of congestive heart failure is predicted by

baseline left ventricular internal dimensions (Vasan *et al.*, 1997). However, in the clinical setting an intervention study specifically targeting chamber volumes without influencing myocardial systolic function is still required to establish this hypothesis. Nevertheless, preclinical studies conducted by members of our group have provided significant evidence to show that cardiac dilatation is a cause of pump dysfunction and heart failure. What is this evidence and how does the current study compare to the outcomes of these prior studies?

In a preclinical study conducted by members of our group, the presence of heart failure (identified from the presence of pulmonary congestion) and pump dysfunction (a reduced endocardial fractional shortening) in marked pressure overload hypertrophy produced by abdominal aortic banding was noted to occur in association with a combination of cardiac dilatation and myocardial contractile disturbances, whilst myocardial contractile disturbances alone were insufficient to account for the presence of pump dysfunction and heart failure (Norton *et al.*, 2002). Thus, without cardiac dilatation, pump dysfunction and heart failure may not occur in pressure overload states (Norton *et al.*, 2002). Further studies from members of our group have provided additional support for a critical role of cardiac dilatation in mediating pump dysfunction independent of myocardial contractile disturbances. Indeed, our group has demonstrated that chronic adrenergic stimulation can promote the transition from compensated cardiac hypertrophy to pump dysfunction in association with cardiac dilatation, but not with decreases in intrinsic myocardial contractile disturbances (Badenhorst *et al.*, 2003b, Gibbs *et al.*, 2004, Veliotes *et al.*, 2005, Veliotes *et al.*, 2010). Thus, preclinical studies have provided significant evidence to suggest that cardiac dilatation is a necessary prerequisite for the development of pump dysfunction and subsequent systolic heart failure at least in

pressure overload states and following excessive adrenergic activation. How does the present study differ from previous studies?

In the present study adrenergic-induced cardiac dilatation and pump dysfunction was associated with decreases in myocardial contractility (as indexed by a decrease in the load-independent measure, LV En). Moreover, the reversal of cardiac dilatation and the return of pump function to normal values was associated with a parallel return of myocardial contractile function back to normal levels. Thus, in the present study at least, adrenergic-induced pump dysfunction and its' reversal may be attributed to myocardial contractile disturbances rather than to cardiac dilatation. However, the more likely interpretation of the present data is that pump dysfunction and the reversal of the process following withdrawal of the adrenergic stimulus can be accounted for by both changes in myocardial contractility and in cardiac chamber dimensions. Importantly, in the present study I have taken care to determine myocardial systolic function using a load-independent measure of intrinsic myocardial contractile properties (that is left ventricular En) determined in hearts paced at the same rate and with comparable coronary flow rates.

4.6 Potential cellular mechanisms of reversal of adrenergic-induced cardiac dilatation.

As discussed in chapter 1 of the present dissertation a number of cellular mechanisms could explain adverse cardiac chamber remodelling. In the present study I evaluated some of these mechanisms including cardiomyocyte necrosis, apoptosis, and alterations in total myocardial collagen concentrations. Consistent with previous findings by our group in the model studied in the present dissertation, excessive cardiomyocyte

necrosis was not observed after chronic β -adrenergic receptor stimulation (Woodiwiss *et al.*, 2001, Badenhorst *et al.*, 2003b, Osadchii *et al.*, 2007, Veliotes *et al.*, 2005). Moreover, consistent with previous findings by our group in the model studied in the present dissertation, excessive cardiomyocyte apoptosis was not observed 24 hours after the last dose of isoproterenol (Veliotes *et al.*, 2005). What are the implications of these findings for the present study?

4.6.1 Cardiomyocyte necrosis

As previously demonstrated (Woodiwiss *et al.*, 2001, Badenhorst *et al.*, 2003b, Osadchii *et al.*, 2007, Veliotes *et al.*, 2005), cardiomyocyte necrosis is unlikely to be a major characteristic of the model of adrenergic-induced cardiac dilatation and pump dysfunction studied by our group. This is an important point as the conclusion that β -adrenoreceptor-mediated cardiac dilatation and pump dysfunction can be completely reversed, must carry the caveat that this may possibly only occur in situations where adrenergic stimulation is insufficiently robust to have produced cardiomyocyte necrosis. As previously shown (Benjamin *et al.*, 1989, Mann *et al.*, 1992, Teerlink *et al.*, 1994), cardiomyocyte necrosis is indeed a possible response to excessive β -adrenoreceptor activation and β -adrenergic blocker therapy attenuates this effect (Chan *et al.*, 2004, Pacca *et al.*, 2002). Nevertheless, how frequently sympathetic activation is sufficient to promote excessive cardiomyocyte necrosis in heart failure has not been identified. In our group's hands, doses of isoproterenol that cause significant cardiomyocyte necrosis (Teerlink *et al.*, 1994) result in extremely high mortality rates from sudden death in rats, and hence this model is not feasible for study in our laboratory.

4.6.2 Cardiomyocyte apoptosis

Cardiomyocyte cell death mediated by apoptosis, and the degree to which subsequent regeneration of cardiomyocytes via stem cells occurs, may determine the extent of deleterious remodelling of the myocardium (Yussman *et al.*, 2002). However, it may be argued that if cardiomyocyte apoptosis plays an important role in the adverse remodelling process following adrenergic activation, and that the myocytes have a limited capacity for mitosis, that reverse remodelling is unlikely to ever be complete if apoptosis is the fundamental mechanism responsible for cardiac dilatation. Nevertheless, it is becoming increasingly recognised that the heart has a considerable capacity to regenerate cells after an injury (Kajstura *et al.*, 1998, Beltrami *et al.*, 2001), and hence that even considerable cell death should not be seen as a limiting factor in the process of reverse remodelling. Does the current study support a role for cardiomyocyte apoptosis in mediating adrenergic-induced cardiac dilatation?

In the present study no relationship between adrenergic activation and cardiomyocyte apoptosis was noted. This finding however does not preclude the possibility that cardiomyocyte apoptosis plays a significant role in promoting β -adrenergic-mediated cardiac dilatation and pump dysfunction or that the reversal of this process depends on regeneration of cells. Indeed, in the present study the administration of the last dose of the β -adrenergic receptor agonist was given at least 24 hours prior to harvesting tissue for the assessment of cardiomyocyte apoptosis. This approach was employed to ensure that cardiac function measurements were not confounded by residual inotropic, chronotropic and lusitropic effects of isoproterenol. It is therefore possible that I may have missed the β -adrenergic receptor agonist-mediated cardiomyocyte apoptosis. Indeed, our group has previously demonstrated that when

assessing hearts within a short time period after injecting the last dose of isoproterenol, excessive cardiomyocyte apoptosis does indeed occur (Osadchii *et al.*, 2007, Veliotes *et al.*, 2010).

In the present study, support for a cardiomyocyte apoptotic process occurring in response to chronic adrenergic activation is the evidence to indicate that after cessation of the adrenergic stimulus, cardiac weight decreased to values lower than the control groups. This could only have occurred for one of two reasons. One possibility is that after cessation of the adrenergic stimulus, atrophy of cells to values lower than control cells occurs. However, the second possibility, which is the more likely of the two possibilities, is that after cessation of the adrenergic stimulus, although cell size returns to normal values, because of prior cell death mediated through adrenergic-induced apoptotic processes, cardiac weight decreases to values lower than control values. Clearly, this effect cannot be attributed to cardiomyocyte necrosis as pathological score and myocardial collagen concentrations were not increased. The only likely possibility is that β -adrenergic receptor-mediated cardiomyocyte apoptosis (Communal *et al.*, 1998, Singh *et al.*, 2001) (or autophagy) attenuated the number of viable cardiomyocytes and hence reduced cardiac weight to values lower than control values. Further work is therefore still required to evaluate changes in cell size at six months of isoproterenol administration and then at four months after cessation of the adrenergic stimulus.

4.6.3 Myocardial collagen changes

Although I was unable to show a relationship between myocardial collagen concentrations and cardiac dilatation or pump dysfunction in the present study, this does not preclude the possibility that interstitial changes could still explain adrenergic-induced

adverse chamber remodelling and the reversal thereof. In the present study because cardiac function was assessed in-part through the use of isolated, perfused heart techniques, and in our hands perfusion of hearts alters myocardial matrix metalloproteinase (MMP) expression and activity, I did not evaluate the role of myocardial expression and activity of matrix metalloproteinases (MMPs) in the reverse remodelling process. In this regard, as described in chapter 1 of the present dissertation, increases in myocardial MMP expression and activation may cause breaks in myocardial collagen and thus promote side-to-side cardiomyocyte slippage and cardiac dilatation. Indeed, increases in myocardial MMP expression and activity have been shown by members of our laboratory to accompany adrenergic-induced cardiac dilatation *in vivo* (Veliotis *et al.*, 2010). Obviously further work is required to evaluate whether cessation of adrenergic stimulation is accompanied by normalisation of myocardial MMP expression and activity.

4.6.4 Cardiomyocyte morphology

Adrenergic-induced cardiac hypertrophy may be an important mechanism responsible for cardiac dilatation. Indeed, as highlighted in chapter 1 of the present dissertation, through hypertrophic processes increases in cardiomyocyte length-to-width ratios could contribute toward adverse chamber remodelling (Zimmer *et al.*, 1990, Spinale *et al.*, 1991, Gerdes *et al.*, 1992, Gerdes & Capasso, 1995, Tamura *et al.*, 1998). However, in the present study I did not evaluate the role of cardiomyocyte length-to-width ratios in contributing toward reverse remodelling following cessation of adrenergic stimulation. Nevertheless, recent work from our laboratory, using both image analysis and flow cytometry performed on isolated cardiomyocytes, suggests that adrenergic-

induced cardiac dilatation is not accounted for by changes in cardiomyocyte length-to-width ratios (Veliotes *et al.*, 2010).

A lack of contribution of cardiomyocyte length-to-width ratios to the development of adrenergic-induced cardiac dilatation does not exclude the possibility that reversal of the adverse chamber remodelling process may depend in-part on alterations in cardiomyocyte morphology. In this regard, consistent with studies showing that adrenergic blockers decrease cardiac mass in heart failure (Hall *et al.*, 1995, Lowes *et al.*, 1999, Groenning *et al.*, 2000, Khattar *et al.*, 2001) in the present study reversal of adrenergic-induced chamber dilatation was closely associated with reversal of adrenergic-induced cardiac hypertrophy. In this circumstance, although changes in cardiomyocyte length-to-width ratios may not have occurred, by reducing cell length, cardiac cavity volumes could have been attenuated. Nevertheless, if cardiac hypertrophy was an important process in mediating reverse remodelling, it is likely that wall thickness would have changed. However, on echocardiography at least, although relative wall thickness values decreased with chronic adrenergic stimulation, absolute wall thickness remained unchanged throughout the study. Importantly, also against a role for adrenergic-induced cardiac hypertrophy in promoting cardiac dilatation is the evidence that in patients with heart failure receiving the β -adrenergic receptor blocker metoprolol, decreases in left ventricular volumes were noted after three months of metoprolol therapy, whilst left ventricular mass only decreased in this study by 18 months of metoprolol therapy (Hall *et al.*, 1995). These data suggest that the beneficial effects of adrenergic blockade on cardiac cavity volumes in heart failure can precede alterations in left ventricular mass. Further studies are therefore required to assess the role of cardiomyocyte morphology in the reverse remodelling process.

4.7 Potential cellular mechanisms of the reversal of adrenergic-induced myocardial contractile disturbances.

A number of cellular mechanisms could explain myocardial contractile disturbances produced by chronic adrenergic activation and the subsequent reversal of this process following withdrawal of the adrenergic stimulus. As discussed in sections 4.6.1 and 4.6.2 of the present dissertation, cardiomyocyte necrosis and apoptosis may occur following excessive adrenergic activation, changes which could account for a decrease in myocardial contractility. However, as previously pointed out in these sections, no evidence of cardiomyocyte necrosis was noted in the present study. Furthermore, although I was also unable to show an adrenergic-induced increase in cardiomyocyte apoptosis, I may have missed this change through the collection of tissue 24-hours after the last dose of isoproterenol. Hence, excessive cardiomyocyte apoptosis may explain the adrenergic-induced decrease in myocardial contractility. If this is indeed the explanation, to conceive of how withdrawal of the adrenergic stimulus could reverse the myocardial dysfunction one would have to consider the possibility that substantial reparative properties occur in the myocardium. In this regard, as pointed out in section 4.6.2, it is becoming increasingly recognised that the heart has a considerable capacity to regenerate cells after an injury (Kajstura *et al.*, 1998, Beltrami *et al.*, 2001).

An alternative potential mechanism by which chronic sympathetic activation may promote progressive decreases in myocardial contraction and subsequently a return of contractility to normal after cessation of the adrenergic stimulus, is through alterations in the sensitivity of the system to agonist stimulation. A number of authors have demonstrated that as a consequence of sympathetic over-activation, in failing human hearts a decrease in β -adrenoreceptor density leads to subsensitivity of the β -adrenergic

pathway and decreased β -adrenoreceptor-agonist-stimulated muscle contraction (Bristow *et al.*, 1982, Bristow *et al.*, 1986, Brodde *et al.*, 1986, Böhm *et al.*, 1988, Brodde *et al.*, 1989, Brodde, 1991, Steinfath *et al.*, 1991, Schotten *et al.*, 2000, Tevæearai & Koch, 2004). In support of a role of β -adrenergic receptor desensitization in-part explaining the reduced myocardial contractility associated with chronic adrenergic activation in the present study, is data from a previous study by our group demonstrating β_1 - and β_2 -adrenoreceptor inotropic down-regulation (attenuated contractile responses to dobutamine and salbutamol) after three months of isoproterenol administration (Osadchii *et al.*, 2007). However, in that study myocardial contractility was not decreased by three months of isoproterenol administration (Osadchii *et al.*, 2007), a finding that was explained by increases in myocardial norepinephrine release, to up-regulation of α -adrenoreceptor-mediated contractile effects as determined by phenylephrine responsiveness and to compensatory cardiac hypertrophy (Osadchii *et al.*, 2007). Whether a longer period of isoproterenol administration, such as in the present study, subsequently results in an attenuation of these compensatory changes and hence a reduction in myocardial contractility was not explored.

4.8 Clinical implications

The present study provides clear evidence to show that even with advanced adrenergic-induced cardiac dilatation, decreases in myocardial contractility and pump dysfunction, complete reversal of these changes may be achieved as long as the adrenergic stimulus is eliminated. The caveat is that this is possible in the absence of cardiomyocyte necrosis, but whether the same outcome can be achieved in patients in whom adrenergic activation is sufficiently robust to cause necrosis of cardiomyocytes

remains to be established. Assuming that the model of cardiac dilatation and pump dysfunction reported on in the present dissertation can indeed be considered to in-part mimic the human heart failure condition, these data therefore support the need to block β -adrenergic receptors or the downstream pathways that mediate cardiac dilatation in patients with heart failure associated with cardiac dilatation. In this regard, the use of β -adrenergic receptor blockers in patients with heart failure is not always optimal (Cleland *et al.*, 2002, Pont *et al.*, 2003, Rutten *et al.*, 2003, Komajda *et al.*, 2003, Murphy *et al.*, 2004, Lenzen *et al.*, 2005, Fernandes *et al.*, 2005, Bongers *et al.*, 2006, Fowler *et al.*, 2007, Sturm *et al.*, 2007, Kavookjian & Mamidi, 2008), possibly because of hypotensive effects produced by negative inotropic actions of β -adrenergic receptor blockers, particularly in patients with severe pump dysfunction, or because of the presence of co-morbid conditions where β -adrenergic receptor blockers are contraindicated (e.g. the presence of reactive airways disease) (Egred *et al.*, 2005). Future studies may therefore be required to identify alternative therapeutic interventions, specifically targeting downstream pathways responsible for adrenergic-induced cardiac dilatation, myocardial contractile disturbances and pump dysfunction, but pathways which do not influence cardiac or vascular smooth muscle function.

4.9 Strengths and limitations of the present study.

As with any study the outcomes of the present study should be evaluated in the context of the strengths and weakness. As previously indicated, a weakness of the study is that I assessed the effect of a β -adrenergic receptor agonist and its withdrawal, rather than trying to mimic the human heart failure condition where increases in circulating concentrations of both adrenaline and noradrenaline occur and where sympathetic

activation to some but not other organ systems also occurs. Hence, data obtained from the present model of cardiac dilatation and pump dysfunction may not apply to the human heart failure condition.

As previously emphasised, in the present study I used two approaches to measure cardiac chamber remodelling and pump function, each with its own strengths and weaknesses. The first approach was echocardiography performed in anaesthetised rats. The second approach was to construct diastolic and systolic pressure-volume and stress-strain relations in an isolated, perfused heart preparation. The strength of the echocardiographic measurements is that heart dimensions and pump function were measured in a filling and emptying ventricle, in the presence of an intact and functional neurohumoral system, and in blood perfused hearts. The limitation of using echocardiography is the presence of an anaesthetic with potential effects on cardiac function, and that the measurement is affected by the confounding effects of preload, afterload, coronary flow, and heart rate. In addition, on echocardiography left ventricular end diastole was taken as that point at which maximal diastolic diameter was achieved rather than identifying end diastole from the onset of the Q wave of the electrocardiograph recording. Although in human studies correlating echocardiographic images with electrocardiographic recordings allows for accurate identification of the exact end diastolic period of the cardiac cycle, in rats the much faster heart rate (4-5 times faster) results in a decreased ability to accurately time end diastole using electrocardiograph recordings. Nevertheless, at end diastole the heart is at peak filling volumes, and hence maximal diameter is likely to reflect diameters at end diastole.

The strength of the measurements obtained in the isolated, perfused heart preparation is that they are preload and afterload independent, and that these measurements were obtained at controlled heart rates and coronary flow rates.

Moreover, the confounding effects of anaesthesia are eliminated. The limitations of the isolated, perfused heart preparation include measurements performed under non-physiological conditions, and obtained in isovolumic preparations. Importantly, however, despite the strengths and weaknesses of each approach, the same outcomes were reproduced with both. In addition to considering the strengths and weaknesses of the whole organ assessments, it is similarly important to consider the strengths and weaknesses of the measurements designed to assess the cellular mechanisms of the reverse remodelling. In this regard, most of the deficiencies of these measurements have been discussed in aforementioned sections.

4.10 Conclusions

In conclusion, data provided from this study provide clear evidence to indicate that in the absence of myocardial necrosis, even marked adrenergic-induced cardiac dilatation, contractile disturbances and pump dysfunction are completely reversible.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2008/19/04

APPLICANT: Mr. HL Roux Booysen

SCHOOL: Physiology

DEPARTMENT:

LOCATION: Medical School

PROJECT TITLE: **Reverse remodelling and the mechanism thereof following adrenergic induced cardiac dilatation and pump dysfunction in rats**

Number and Species

102 M 200-250g Sprague Dawley rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20080506. This approval remains valid until 20100506

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Liaison with the CAS over the supply of animals. The technique needs to be sterile

Signed: _____



(Chairperson, AESC)

Date: 12/05/2008

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____



(Registered Veterinarian)

Date: 12/05/2008

cc: Supervisor: Gavin Norton
Director: CAS

Works 2000/lain0015/AESCCert.wps
