

# **The Transition from Hypertensive Hypertrophy to Left Ventricular Systolic Chamber Decompensation**

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## **Abstract**

Hypertensive left ventricular hypertrophy (LVH) increases the risk for the development of heart failure with systolic chamber dysfunction. However, the exact mechanisms and hence best therapeutic approach to prevent this transition process is uncertain. One potential mechanism is through excessive  $\beta$ -adrenergic receptor ( $\beta$ -AR) activation, but the risks of  $\beta$ -AR blocker therapy may outweigh the benefits. Since activation of  $\beta$ -AR augments function of the renin-angiotensin-aldosterone system, I therefore explored whether mineralocorticoid receptor (MR) blockade prevents the transition from hypertensive LVH to systolic chamber decompensation produced by excessive  $\beta$ -AR activation, and the mechanisms thereof. The role of hypertensive LVH as a predisposing factor to systolic chamber decompensation post-myocardial infarction (MI) is controversial. In the present thesis I therefore also evaluated this question.

The effect of spironolactone (SPIRO, 80 mg.kg<sup>-1</sup>.day<sup>-1</sup>), an MR blocker, on LV chamber remodelling and function was evaluated in spontaneously hypertensive rats (SHR) in whom decompensation was induced by administering a low dose of the  $\beta$ -AR agonist, isoproterenol (ISO) for 4.5 months. ISO administration resulted in an increased urinary aldosterone excretion and LV cavity dimensions, a right shift in LV diastolic pressure-volume relations, and a decreased LV relative wall thickness without further enhancing an increased myocardial norepinephrine (NE) release in SHR. ISO reduced LV systolic chamber function (decreased LV endocardial fractional shortening and the slope of the LV systolic pressure-

volume relationship) without modifying intrinsic myocardial systolic function (as assessed from LV midwall fractional shortening and the slope of systolic stress-strain relationship). SPIRO abolished ISO-induced chamber dilatation, wall thinning and systolic dysfunction, but failed to modify blood pressure, volume preloads, intrinsic myocardial systolic function, or myocardial NE release. These results suggest that MR activation, through load-independent effects, may be critical in mediating the transition from compensated hypertensive LVH to dilatation and LV systolic chamber dysfunction.

In SHR, ISO increased myocardial matrix metalloproteinase (MMP)-2 activity (zymography) after only 4-5 days of administration, a change that was associated with MMP-2, but not TIMP expression. The increased MMP-2 activity persisted until 4.5 months of the study and these changes were prevented by SPIRO. At 4.5 months, ISO resulted in increased non-cross-linked, but not cross-linked myocardial collagen concentrations in SHR, an effect that was abolished by SPIRO. Although at 4.5 months ISO administration was not associated with an increased cardiomyocyte apoptosis (TUNEL), an early (4-5 days) ISO-induced apoptotic effect was noted, which was prevented by SPIRO. Neither ISO nor SPIRO influenced cardiomyocyte length (image analysis and flow cytometry) in SHR. Thus MR blockade may prevent the adverse effects of  $\beta$ -AR activation in hypertensive LVH through alterations in the cardiac interstitium and cardiomyocyte apoptosis.

Six-to-seven months after ligation of the left anterior descending coronary artery, LV myocardial systolic function as assessed from % shortening of the non-infarcted lateral wall segmental length determined over a range of filling pressures (ultrasonic transducers placed in the lateral wall in anaesthetized, open-chest, ventilated rats) and % thickening of the posterior wall (echocardiography) was reduced in infarcted SHR (SHR-MI) ( $p < 0.05$ ), but not in normotensive Wistar Kyoto (WKY-MI) animals as compared to corresponding controls (SHR-Sham, WKY-Sham). This change in regional myocardial function in SHR-MI, but not in WKY-MI, occurred despite a similar degree of LV dilatation in SHR-MI and WKY-MI rats and a lack of difference in LV relative wall thinning, LV wall stress, apoptosis (TUNEL) or necrosis (pathological score) between SHR-MI and WKY-MI rats. Although the change in regional myocardial function in the SHR-MI group was not associated with a greater reduction in resting global LV chamber systolic function (endocardial fractional shortening- $FS_{end}$  and end-systolic elastance [LV  $E_{es}$ ] determined in the absence of an adrenergic stimulus), in the presence of an ISO challenge a reduction in LV  $E_{es}$  in SHR-MI compared to WKY-MI and SHR and WKY-Sham rats was noted ( $p < 0.04$ ). These data suggest that with chronic MI, the hypertensive heart is susceptible to development of viable tissue myocardial dysfunction, a change which cannot be attributed to excessive chamber dilatation, apoptosis or necrosis, but which in-turn, contributes toward a reduced cardiac adrenergic-inotropic reserve.

The present thesis therefore suggests that MR blockade may prevent the transition from hypertensive LVH to systolic chamber decompensation, and that pre-existing hypertensive LVH increases the susceptibility to a depressed LV regional myocardial systolic function in the non-infarcted LV myocardium subsequent to MI, an effect that translates into a reduced inotropic reserve.

## **Declaration**

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy, in the School of Physiology, Faculty of Health Sciences, University of Witwatersrand, Johannesburg. The work contained in this thesis has not been submitted for any degree or examination in this university or any other university.

I certify that the study contained in this thesis has the approval of the Animal Ethics Committee of the University of Witwatersrand, Johannesburg. The ethics approval numbers are: 2006/37/4, 2004/43/4, 2004/59/4, 2002/37/5, 2002/39/5 and 99/01/2b.

.....

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.....

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.....

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Signed this .....<sup>th</sup> day of October, 2013

In memory of my father  
Dr. George Demetrios Veliotis  
(1949-2009)

## **Publications and presentations arising from this thesis**

### **Publications:**

Veliotis DGA, Norton GR, Correia RJ, Strijdom H, Badenhorst D, Brooksbank R and Woodiwiss AJ. Impact of aldosterone receptor blockade on the deleterious cardiac effects of adrenergic activation in hypertensive rats. *J Cardiovasc Pharmacol*. 2010;56:203-211.

Veliotis DGA, Woodiwiss AJ, Deftereos D, Gray D, Osadchii O and Norton GR. Aldosterone receptor blockade prevents the transition to cardiac pump dysfunction induced by  $\beta$ -adrenoreceptor activation. *Hypertension*. 2005;45:914-920.

Norton GR, Veliotis DGA, Osadchii O, Woodiwiss AJ and Thomas DP. Susceptibility to systolic dysfunction in the myocardium from chronically infarcted spontaneously hypertensive rats. *Am J Physiol Heart and Circ Physiol*. 2008;294:H372-H378.

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Veliotis D, Badenhorst D, Osadchii O, Dube N, Woodiwiss AJ and Norton GR. (2003). Spironolactone prevents beta-adrenergic-induced cardiac dilatation in hypertensive rats. *Cardiovascular Journal of South Africa* (March Supplement):19.

Badenhorst D, Veliootes D, Osadchii O, Dube N, Maseko M and Norton GR. (2003). Beta-adrenergic-induced cardiac dilatation precedes myocardial dysfunction in hypertensive rats. *Cardiovascular Journal of South Africa* (Supplement): 25.

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### **List of abbreviations**

|                   |                                                |
|-------------------|------------------------------------------------|
| AESC              | Animal ethics screening committee              |
| AR                | Adrenergic receptor                            |
| ATP               | Adenosine triphosphate                         |
| $\beta$ -AR       | Beta-adrenergic receptor                       |
| BP                | Blood pressure                                 |
| BW                | Body weight                                    |
| cDNA              | Complementary deoxyribonucleic acid            |
| CNBr              | Cyanogen bromide                               |
| DBP               | Diastolic blood pressure                       |
| DHBA              | Dihydroxybutyric acid                          |
| DNA               | Deoxyribonucleic acid                          |
| EF                | Ejection fraction                              |
| Ees               | End systolic elastance                         |
| ELISA             | Enzyme-linked immunosorbent assay              |
| EMMPRIN           | Extracellular matrix metalloproteinase inducer |
| En                | Systolic myocardial elastance                  |
| FS <sub>end</sub> | Endocardial fractional shortening              |
| FS <sub>mid</sub> | Midwall fractional shortening                  |
| HOPE              | Heart Outcomes Prevention Evaluation (trial)   |
| HPRO              | Hydroxyproline                                 |
| HR                | Heart rate                                     |
| iNOS              | Inducible nitric oxide synthase                |
| ISO               | Isoproterenol                                  |

|                |                                                         |
|----------------|---------------------------------------------------------|
| LIFE           | Losartan Intervention For Endpoint reduction (study)    |
| LV             | Left ventricular                                        |
| LVDP           | Left ventricular developed pressure                     |
| LVED           | Left ventricular end diastolic                          |
| LVEDD          | Left ventricular end diastolic diameter                 |
| LVED h         | Left ventricular end diastolic wall thickness           |
| LVED h/r       | Left ventricular end diastolic relative wall thickness  |
| LVEDL          | Left ventricular end diastolic segment length           |
| LVEDP          | Left ventricular end diastolic pressure                 |
| LVED PWT       | Left ventricular end diastolic posterior wall thickness |
| LVED r         | Left ventricular end diastolic radius                   |
| LV $E_{es}$    | Left ventricular end systolic elastance                 |
| LVES           | Left ventricular end systolic                           |
| LVESD          | Left ventricular end systolic diameter                  |
| LVES h         | Left ventricular end systolic wall thickness            |
| LVES h/r       | Left ventricular end systolic relative wall thickness   |
| LVESL          | Left ventricular end systolic segment length            |
| LVESP          | Left ventricular end systolic pressure                  |
| LVES PWT       | Left ventricular end systolic posterior wall thickness  |
| LVES r         | Left ventricular end systolic radius                    |
| LVH            | Left ventricular hypertrophy                            |
| $LVM_{inappr}$ | “Inappropriate” left ventricular hypertrophy            |
| LV $V_0$       | LV volume at LV diastolic pressure of 0 mm Hg           |
| MI             | Myocardial infarction                                   |

|       |                                                   |
|-------|---------------------------------------------------|
| MMP   | Matrix metalloproteinase                          |
| MR    | Mineralocorticoid receptor                        |
| NE    | Norepinephrine                                    |
| PWT   | Posterior wall thickness                          |
| RWT   | Relative wall thickness                           |
| SBP   | Systolic blood pressure                           |
| SERCA | Sarcoplasmic/endoplasmic reticulum calcium ATPase |
| Sham  | Sham operation                                    |
| SHR   | Spontaneously hypertensive rat                    |
| SPIRO | Spironolactone                                    |
| TdT   | Terminal deoxynucleotidyl transferase             |
| TIMP  | Tissue inhibitor of metalloproteinase             |
| TUNEL | TdT dUTP nick end labeling                        |
| WKY   | Wistar Kyoto rat                                  |

## **Preface**

Hypertension is a prevalent condition in the general population, and left ventricular hypertrophy (LVH) is present in a significant portion of these patients. Contrary to the previously held belief that LVH is protective in reducing cardiac wall stress, it has become apparent that this is not always true. Indeed, LVH increases the risk for cardiovascular events, including heart failure, in patients with hypertension. In this regard hypertensive LVH may predispose to heart failure associated with either systolic or diastolic dysfunction. However, the mechanisms responsible for the transition from compensated LVH to LV dysfunction are not necessarily just related to blood pressure (BP). Hence, it is possible that antihypertensive therapy designed not only to decrease BP, but also to modify the non-BP-related mechanisms responsible for this transition process, should be employed to treat patients with hypertensive LVH.

Recent evidence suggests that a critical mechanism responsible for the transition from hypertensive LVH to systolic chamber decompensation is excess  $\beta$ -adrenergic receptor (AR) activation. However, the beneficial effects of  $\beta$ -AR blockers in patients with hypertensive LVH may not outweigh alternative potential deleterious effects. Hence, it may be more beneficial to target downstream or parallel pathways to prevent the transition from hypertensive LV to LV systolic chamber dysfunction. In this regard, in the present thesis I examined the possibility that blocking mineralocorticoid receptors using the antihypertensive agent, spironolactone, may have beneficial effects in preventing the transition

from hypertensive LVH to systolic chamber decompensation mediated by excessive adrenergic stimulation in an animal model of hypertension.

An alternative possible mechanism that may account for the transition from compensated hypertensive LVH to systolic chamber decompensation is that LVH may not only increase the risk for myocardial infarction (MI), but that pre-existing LVH may increase the chances that MI results in systolic LV chamber decompensation. As this notion is highly controversial, in the present thesis I also further explored this possibility in an animal model of hypertensive LVH.

In the present thesis, in chapter 1, I have first reviewed the scientific literature that describes our current understanding of the transition from hypertensive LVH to systolic chamber decompensation. This review is designed to lead the reader through a series of arguments that support the hypotheses tested in the present thesis. Chapters 2-4 consist of a series of semi-independent chapters, each divided into “abstract”, “introduction”, “methods”, “results” and “discussion” sections, describing the studies performed in the present thesis, the data obtained, and the implications of these data. Finally, in chapter 5, I provide a summary and concluding statement of the work described in the present thesis.

In support of this thesis I am either first or second author on three papers emanating from the present thesis with second authorship indicating an equal contribution to this paper as the first author. These papers have been published in the journal *Hypertension* (Veliotes et al

2005), the *Journal of Cardiovascular Pharmacology* (Veliotis et al 2010), and the *American Journal of Physiology* (Norton, Veliotis, et al 2008).

## **Chapter 1**

**Hypertensive Left Ventricular Hypertrophy as a  
Cause of the Progression to Systolic Chamber  
Decompensation.**

### **1.1 Introduction.**

There is substantial evidence to support a role for hypertension as a major cause of heart failure. Indeed, hypertension is one of the most common comorbidities in heart failure (Levy et al 1996) and hypertension may occur in approximately 40% of patients with systolic dysfunction and heart failure (Davies et al 2001). In primary care settings, approximately 48% of patients diagnosed with heart failure have hypertension (Cleland et al 2002). Moreover, in clinical trials of heart failure, hypertension is one of the four most frequently cited comorbidities (Krum and Gilbert 2003). The role of blood pressure (BP) in heart failure is highlighted by the ability of antihypertensive therapy to prevent the development of heart failure (Dahlof et al 1991, Kostis et al 1997, MRC Working Party Medical Research Council 1992). Despite the evidence implicating hypertension as a primary driving force for heart failure, many issues regarding the exact mechanisms of this effect remain unresolved. In particular there is little consensus as to the precise role of hypertension-induced left ventricular hypertrophy (LVH) in the pathophysiology of hypertensive heart failure.

Hypertrophy of the left ventricle (LV) in hypertension is traditionally considered to be a compensatory response of the LV to chronic pressure overload. This is in accordance with the law of LaPlace where in a sphere, wall tension (stress) is proportional to the product of pressure and internal radius and inversely proportional to wall thickness. Therefore an increased wall thickness to radius ratio in LVH will maintain a normal LV wall stress

in the face of increments in pressure generated within the LV cavity (Grossman et al 1975). In principle the theory that LVH may be an appropriate compensatory response is inarguably correct. However significant clinical evidence has accumulated in the more recent past to indicate that LVH may not be considered an entirely compensatory change. Indeed, LVH predicts cardiovascular outcomes beyond BP (Casale et al 1986, Drazner et al 2004, Gardin et al 2001, Ghali et al 1998, Levy et al 1990, Levy et al 1994, Verdecchia et al 1996, Verdecchia et al 2001) and a reduction of LV mass during antihypertensive therapy predicts improved cardiovascular outcomes beyond BP changes (Devereux et al 2004a, Mathew et al 2001, Okin et al 2004). Therefore, in the light of this evidence (Casale et al 1986, Devereux et al 2004a, Drazner et al 2004, Gardin et al 2001, Ghali et al 1998, Levy et al 1990, Levy et al 1994, Mathew et al 2001, Okin et al 2004, Verdecchia et al 1996, Verdecchia et al 2001) most guidelines for the diagnosis and management of hypertension specify that when risk stratifying patients with hypertension, the presence of LVH should be sought (Chobanian et al 2003, Mancia et al 2007, Whitworth 2003, Williams et al 2004).

In spite of the considerable clinical evidence supporting the inclusion of measurements of LV mass in risk stratification of patients with hypertension, there is nevertheless still debate as to the pathophysiological significance of LVH *per se*. LVH may either point towards BP-independent adverse cardiac effects, or LVH may merely be a more sensitive measure of the temporal effects of BP over time which is

not accurately assessed by measures of BP. Thus the question still remains as to whether the presence of hypertensive LVH represents an intermediate cardiac phenotype that is causally linked to cardiovascular morbidity and mortality. In the following section I will review the evidence to suggest that LVH may be an intermediate phenotype that is causally related to cardiovascular morbidity and mortality and highlight some of the discrepancies that challenge this notion.

## **1.2 Is LVH causally linked to cardiac decompensation?**

If LVH signals BP-independent adverse cardiac effects, then the expected morbid events associated with LVH should be cardiac-related, such as through the development of cardiac decompensation and heart failure. Indeed, a number of clinical studies have demonstrated that LVH is an independent predictor of the development of heart failure (Aurigemma 2001, Casale et al 1986, Gardin et al 2001, Gottdiener et al 2000, Levy et al 1990, Levy et al 1996). Nevertheless, these relationships between LVH and heart failure (Aurigemma 2001, Casale et al 1986, Gardin et al 2001, Gottdiener et al 2000, Levy et al 1990, Levy et al 1996) are not necessarily cause-effect relationships as there are no studies that have specifically targeted LVH therapeutically and demonstrated an ability to prevent the development of heart failure. Therefore, the role of LVH as a cause of heart failure remains controversial. The following section will highlight the evidence both for and against LVH as a pathophysiological process

responsible for heart failure. First, however, I will discuss the potential theories that suggest that hypertensive LVH could promote the development of cardiac decompensation and heart failure.

### **1.2.1 Potential mechanisms through which LVH may promote cardiac decompensation.**

Hypertensive LVH may be associated with an increased myocardial oxygen demand (Bache et al 1999, Jung et al 1998, Kannel et al 1970, Strauer 1979). However an accompanying proportional increase in coronary blood flow may not occur due to limitations of coronary flow reserve produced by the adverse effects of hypertension (Cimini et al 1989, Gosse and Clementry 1995, Lorell et al 1987, Marcus et al 1979). As a consequence of alterations in myocardial oxygen supply-to-demand ratios and thus a reduced capacity of the sarcoplasmic reticulum to sequester calcium (an energy requiring process), hypertensive LVH may lead to impaired filling of the LV due to a decreased early-diastolic relaxation (Calderone et al 1995, Gibson et al 1980, Kass et al 2004, Shapiro and McKenna 1984). Furthermore, subsequent to either reparative myocardial fibrosis, induced by tissue necrosis possibly in-part as a consequence of alterations in myocardial oxygen supply-to-demand ratios (Tsotetsi et al 2001), or reactive fibrosis (Brilla et al 1996, Weber et al 1990), hypertensive LVH may be associated with increased myocardial diastolic stiffness (Conrad et al 1995, Norton et al 1997) and a diminished

late-diastolic compliance (Norton et al 1997), changes which could contribute towards LV diastolic chamber dysfunction in hypertensive LVH. In addition, in LVH re-expression of the cardiomyocyte fetal gene programme occurs which includes repression of genes important for myocardial relaxation (e.g. SERCA2) and contraction (e.g.  $\alpha$  myosin heavy chain) and overexpression of genes responsible for programmed cell death (apoptotic pathways) (Frohlich et al 2011). These changes may also contribute toward an impaired ability of the myocardium to contract and relax and hence produce either systolic or diastolic LV chamber dysfunction. Activation of specific protein kinases in LVH may induce apoptosis (Liu et al 2000, Wang et al 1998) with an ensuing further decline in intrinsic myocardial contractility and myocardial systolic function (Brooks et al 1997). Not only is there excessive apoptosis in LVH, but also an increase in cardiomyocyte autophagy, a change that may contribute toward further cell death and myocardial contracture (Frohlich et al 2011). Left ventricular hypertrophy may also be associated with an impaired contractility of viable cardiomyocytes (Neyses and Vetter 1989) an effect that may be explained by a number of cellular and molecular mechanisms. As a consequence of a shift in substrate utilisation from fatty acids to carbohydrates, the hypertrophied heart has been proposed to be an energy-compromised organ with diminished adenosine triphosphate (ATP) production; and hence is sensitive to lipotoxicity and contractile dysfunction through the accumulation of intracellular fatty acids (Bugger et al 2010). Moreover, hypertensive LVH may diminish LV systolic chamber

function as a consequence of chamber dilatation which develops subsequent to reparative interstitial changes (Bing et al 1995, Norton et al 2002, Tsotetsi et al 2001) or apoptosis (Liu et al 2000, Wang et al 1998). Thus, a host of potential pathophysiological changes may occur in LVH that may explain a transition from compensated to decompensated LVH. What is the evidence, therefore, to support a causal role of LVH in the pathophysiology of LV dysfunction?

### **1.2.2 What evidence supports or opposes the theory that LVH is causally related to systolic cardiac chamber decompensation?**

With respect to the question of whether LVH is causally related to cardiac decompensation, few studies have been able to segregate the adverse effects of BP on cardiac function from those potentially mediated by the presence of LVH *per se*. Although not extensive, there is nevertheless some pre-clinical and clinical evidence that provides insights into this question. These pre-clinical and clinical findings either supporting or refuting the view that LVH mediates cardiac decompensation will be reviewed in the following section. The focus of this discussion will be on evidence to either support or refute the notion that LVH promotes the progression to systolic rather than diastolic cardiac chamber decompensation. In this regard, the focus of the work presented in the present thesis was on the role of LVH in promoting systolic rather than diastolic cardiac function and hence it is more relevant to discuss this

issue. Furthermore, the question of the role of LVH is as cause of diastolic dysfunction is less controversial than the role of LVH as a cause of systolic chamber decompensation and hence the latter issue warrants more consideration.

#### **1.2.2.1 Pre-clinical evidence supporting or opposing the theory that LVH is causally related to systolic chamber decompensation.**

There are a number of pre-clinical studies, too many to cite in the present thesis, that have demonstrated that the development of LVH is associated with LV dysfunction and that regression of LVH is associated with improvements in pump or diastolic function (see review paper, Frohlich et al 2011). However, as previously indicated, with the exception of a few studies, none of these studies have been able to segregate the adverse effects of BP or LV loading conditions on the heart from those potentially produced by LVH *per se*. Nevertheless, revealing insights as to whether LVH *per se* could be causally related to the progression to heart failure have been provided by one pre-clinical study (Esposito et al 2002). In this pre-clinical study the striking outcome was the finding that genetic modifications targeting molecules that influence sympathetic nervous system activation, and that reduce the LV hypertrophic response to a pressure overload state in mice, attenuate the development of LV systolic chamber decompensation (Esposito et al 2002). A bold interpretation of

this study (Esposito et al 2002) could be that an attenuation of pressure overload-induced increases in LVM prevented the transition to systolic cardiac chamber decompensation. Alternatively, these data may also be interpreted as an indication that the genetic variations themselves, which reduced sympathetic nervous system activity, were protective. Indeed, as shall be highlighted in subsequent discussion, the sympathetic nervous system is well recognized as mediating adverse effects on the heart. Nevertheless, these data (Esposito et al 2002) dispel the notion that LVH is a necessary compensatory response to maintain cardiac systolic chamber function in pressure overload states. However, they do not necessarily imply a deleterious effect of LVH *per se*.

In contrast to evidence from pre-clinical studies that suggests that LVH may promote the transition to systolic cardiac chamber decompensation, there are pre-clinical studies which do not support this standpoint. Indeed, in rats, following chronic pressure-overload, the extent of LVH may be equivalent in animals with, as opposed to those without, evidence of left heart failure and systolic chamber decompensation (Norton et al 2002). Thus we must question whether LVH independently governs the progression to cardiac systolic chamber decompensation. Nevertheless the authors of that study (Norton et al 2002) could not rule out the possibility that those animals identified as having compensated LVH may have been in an earlier phase of LV systolic decompensation. Indeed, in that study myocardial systolic function in rats without heart

failure was diminished, but this change was nevertheless equivalent to that determined in rats with evidence of left heart failure (Norton et al 2002).

Further evidence opposing a role of LVH as a critical mediator in the development of cardiac decompensation comes from data which indicate that the progression from compensated LVH to either diastolic decompensation (Norton et al 1997), or systolic decompensation and cardiac dilatation (Tsotetsi et al 2001), can be attenuated by antihypertensive agents that do not regress LVH. If LVH was a critical mediator in the transition to cardiac decompensation, we would expect that in the presence of persistent LVH, animals would develop some degree of either diastolic (Norton et al 1997) or systolic chamber (Tsotetsi et al 2001) dysfunction in comparison to animals which did not receive antihypertensive therapy. However, the authors of these studies (Norton et al 1997, Tsotetsi et al 2001) could not exclude the possibility that LVH in the presence of an elevated BP promotes cardiac decompensation. Nevertheless, at the very least, these studies suggest that LVH is not a change which in its own right triggers the transition to cardiac decompensation (Norton et al 1997, Tsotetsi et al 2001).

#### **1.2.2.2 Clinical evidence supporting or opposing the theory that LVH is causally related to systolic chamber decompensation.**

Is there clinical evidence to either support or dispel the notion that LVH promotes LV systolic chamber decompensation? In keeping with the classical tenet that LVH is a compensatory response to increases in LV load and myocardial systolic dysfunction, an increased LV mass is associated with an unchanged LV systolic chamber function as indexed by ejection fraction (EF) (Aurigemma et al 1995, de Simone et al 1994, Shimizu et al 1991). Moreover, LVH may even be associated with an enhanced EF for that predicted by wall stress (Hartford et al 1985). Thus, earlier studies favour LVH being a compensatory change rather than a pathophysiological process responsible for cardiac decompensation.

There are few longitudinal studies which provide insights into the significance of the relationship between LV mass and cardiac decompensation. In this regard, in a cohort of the Cardiovascular Health Study (CHS) which was followed for approximately five years, (Drazner et al 2004), independent of traditional risk factors, including conventional BP measurements, LVH was associated with the development of a decreased LV EF. Since a reduced LV EF in asymptomatic patients is associated with the subsequent development of heart failure (Wang et al 2003), it is possible that an increase in LV mass may be a critical pathophysiological mechanism which drives the transition to heart failure. However,

intervention studies demonstrating that regression of LVH with therapy is associated with an improved LV systolic chamber function would be required to put this question to rest. What have intervention studies demonstrated?

There are few intervention studies that provide insights into the pathophysiological role of the relationship between LVH and cardiac systolic chamber decompensation. In keeping with the classical tenet that LVH is a compensatory response to increases in LV load and myocardial systolic dysfunction, on-treatment decreases in LV mass (Perlini et al 2001) have been shown to be associated with an unchanged LV systolic chamber function as indexed by EF. Whether a significant number of patients had a reduced LV systolic chamber dysfunction to begin with in this study (Perlini et al 2001) may however be questioned. Further insights may nevertheless be gained from the Losartan Intervention For Endpoint reduction (LIFE) study.

The multicentre LIFE study evaluated a large sample of hypertensive patients with electrocardiographic evidence of LVH. The LIFE study found a greater regression of LV mass in the patient group treated with an angiotensin II receptor blocker in combination with other antihypertensive agents, as compared to the patient group receiving a  $\beta$ -adrenoreceptor blocker together with other antihypertensive agents (Okin et al 2003). As compared to the  $\beta$ -adrenoreceptor blocker-treated group, patients receiving the angiotensin II receptor blocker experienced a greater reduction in overall cardiovascular mortality (Dahlof et al 2002).

Surprisingly though, the secondary end-point of heart failure was not reduced to a greater extent in the angiotensin II receptor blocker treated group (Dahlof et al 2002). Nevertheless, in a pre-specified subgroup of 1195 patients with diabetes mellitus, there was a reduction in heart failure in the angiotensin II receptor blocker-treated arm of approximately 40%, as compared to the  $\beta$ -adrenoreceptor blocker group (Lindholm et al 2002). However, in the echocardiographic subgroup of the LIFE study, on-treatment decreases in LV mass were related to reductions rather than increases in indices of LV systolic chamber function, despite improvements in indices of myocardial systolic function (Wachtell et al 2002). Thus, the LIFE Study provides contradictory evidence for a pathophysiological role for LVH as a cause of cardiac systolic chamber decompensation.

Are there additional clinical intervention studies that provide some insight into the role of LVH as a causal factor in the development of heart failure? Notably, in the Heart Outcomes Prevention Evaluation (HOPE) trial, angiotensin-converting enzyme inhibition both reduced LV mass and improved LV systolic chamber function beyond BP control (Lonn et al 2004), providing some additional evidence that LVH may be causally related to LV dysfunction.

### **1.2.2.3 Inappropriate LVH may be causally related to systolic chamber decompensation.**

One proposal for the transition from compensated LVH to LV systolic chamber decompensation is that LVH in-keeping with work load (i.e. stroke work = blood pressure x stroke volume) is compensatory (de Simone et al 1998), whilst that exceeding workload (i.e. “inappropriate” LVM [ $LVM_{inappr}$ ]) contributes toward decompensation. Indeed a number of studies have demonstrated an inverse relationship between  $LVM_{inappr}$  and LV systolic chamber function (Celentano et al 2001, Chinali et al 2006, Chinali et al 2007, Cioffi et al 2011, de Simone et al 2004, Palmieri et al 1999, Palmieri et al 2001). However, whether  $LVM_{inappr}$  is associated with a reduced systolic chamber function independent of and more strongly than absolute or indexed LVM was until recently, uncertain. Some prior studies conducted in select clinical samples had demonstrated inverse correlations between LV systolic chamber function and both absolute LVM as well as  $LVM_{inappr}$  (Palmieri et al 1999, Palmieri et al 2001) and in one of these studies LVM was equally as strongly inversely correlated with EF as  $LVM_{inappr}$  (Palmieri et al 2001). This finding is at odds with the notion that it is only LVM beyond stroke work that explains decompensation. Thus, whether inverse relationships between  $LVM_{inappr}$  and EF are independent of absolute LVM was, until recently, unclear. Moreover, prior relationships between  $LVM_{inappr}$  and LV systolic chamber function were demonstrated in cross-sectional studies (Celentano et al 2001, Chinali et al 2006, Chinali et

al 2007, Cioffi et al 2011, de Simone et al 2004, Palmieri et al 1999, Palmieri et al 2001). Conclusions regarding cause and effect cannot be drawn from cross-sectional studies. Indeed, relationships between  $LVM_{inappr}$  and LV systolic chamber function may reflect residual confounding effects or compensatory increases in LVM as a consequence of systolic dysfunction (reverse causality). Although one previous study had reported that on-treatment regression but not persistence of  $LVM_{inappr}$  is associated with an improved EF (Muiesan et al 2007), whether increases in EF in the participants showing regression of  $LVM_{inappr}$  in that study were independent of or stronger than changes in LVM or LVMI was, until recently, also uncertain. However, two recently published studies by our laboratory have provided clarity on these issues. In this regard, in a large randomly selected community-based study our group have demonstrated that the strong relationship between  $LVM_{inappr}$  and EF is indeed independent of LVM or LVMI and additional confounders (Libhaber et al 2013). Moreover, in a further study our group have shown a strong relationship between antihypertensive treatment-induced decreases in  $LVM_{inappr}$  and increases in EF independent of LVM or LVM index (Woodiwiss et al 2012). These data provide support for the notion that LVH is indeed a cause of LV systolic chamber decompensation, but that this relationship only occurs when LVH exceeds that predicted from workload (Libhaber et al 2013, Woodiwiss et al 2012). Thus, it may require more advanced forms of LVH for LVH to translate into LV systolic chamber decompensation.

**1.3 Are there BP-independent changes which coexist with LVH that may promote the progression to systolic chamber decompensation?**

Supposing that cardiomyocyte growth in LVH does not promote myocardial abnormalities and that LV mass *per se* is not merely a more sensitive proxy of temporal changes in BP control than clinical assessments of BP, is there an alternative potential explanation of the BP-independent relationship between LV mass and cardiac systolic chamber decompensation in hypertension? It is possible that increments of LV mass in hypertension may be associated with a number of additional changes that are not as a consequence of increments in BP, but which contribute toward cardiac systolic chamber decompensation. In this regard, in the present thesis I posed the hypothesis that increases in LV mass in hypertension possibly via sympathetic activation, are associated with excess mineralocorticoid receptor activation independent of BP effects. I further hypothesized that sympathetic-induced excess mineralocorticoid effects in LVH predispose to the transition to systolic cardiac chamber decompensation, independent of effects on BP. I additionally hypothesized that LVH in systemic hypertension, not only increases the risk of myocardial infarction with ensuing heart failure, but may indeed amplify the likelihood of LV systolic chamber dysfunction occurring post-myocardial infarction as a consequence of disproportionate

post-myocardial infarction-induced viable tissue myocardial systolic dysfunction.

Therefore based on the aforementioned hypotheses, in the subsequent sections of the present chapter I will review the scientific literature that supports the opinion that relationships may exist between sympathetic activation and either LV mass or systolic chamber or myocardial decompensation. Secondly, I will review the evidence that proposes that a relationship between mineralocorticoid receptor activation, possibly induced by sympathetic activation and either LV mass or systolic chamber or myocardial decompensation may occur. Thirdly, I will summarise the evidence which supports the theory that in addition to LVH increasing the risk of subsequent myocardial infarction, LVH may additionally predispose to exaggerated abnormalities of myocardial function in viable non-infarcted tissue post-myocardial infarction and that this translates into reductions in LV systolic chamber function.

### **1.3.1 Is there an independent relationship between either sympathetic activity or mineralocorticoid excess and left ventricular mass?**

There is important evidence to suggest that sympathetic activation or mineralocorticoid receptor stimulation is associated with LVH. In the subsequent discussion I will first highlight the evidence in support of the notion that sympathetic activation is associated with LVH. I will then

summarise the data which indicates that increased myocardial mineralocorticoid receptor activation occurs in pressure-overload hypertrophy.

#### **1.3.1.1 Excess sympathetic activation is noted in pressure overload hypertrophy.**

Several lines of evidence point towards the occurrence of excessive sympathetic activation in pressure overload hypertrophy. In addition to evidence demonstrating an increase in general sympathetic activity in patients with hypertensive LVH (Agabiti-Rosei et al 1987), there is also support for a notion that regional myocardial sympathetic activity is also augmented. Indeed, cardiac adrenergic spillover, estimated using coronary sinus norepinephrine concentrations, is elevated in patients with hypertensive hypertrophy prior to the development of heart failure (Kelm et al 1996, Schlaich et al 2003). The increased myocardial norepinephrine spillover in pressure overload hypertrophy may be a consequence of both reduced cardiac norepinephrine re-uptake, mediated through as yet unidentified mechanisms (Rumantir et al 2000), as well as locally augmented cardiac sympathetic nervous system activity (Rumantir et al 2000, Simpson et al 1991).

Support for the theory that increased sympathetic activation may give rise to the development of LVH in pressure overload states is obtained from the following studies. Firstly that an early response to aortic

banding in rats is an upregulation of the renin-angiotensin system during the development of LVH (Akers et al 2000), and secondly from the finding that transgenic animal models with decreased adrenergic activation have a reduced LV mass after aortic banding (Esposito et al 2002). Moreover,  $\beta$ -adrenoreceptors blockade may regress LVH in hypertension independent of effects on BP (Gosse et al 1990, Ostman-Smith 1995).

#### **1.3.1.2 Excess mineralocorticoid receptor activation is noted in pressure overload hypertrophy.**

Contrary to the earlier perception that renal tubular epithelial cells are the sole localization of mineralocorticoid receptors, there is now sufficient evidence to demonstrate the presence of mineralocorticoid receptors as a trait of cardiac myocytes (Funder 1997, Funder 2010, Lombes et al 1995, Messaoudi and Jaisser 2011, Pearce and Funder 1987, Young et al 1994). Furthermore, the myocardium has the ability to produce aldosterone, independent of the circulating renin-angiotensin system (Messaoudi et al 2012, Silvestre et al 1998, Takeda et al 2000a, Young et al 2001). Supporting this notion, biologically significant concentrations of aldosterone have been measured in the coronary perfusate of isolated heart preparations (Silvestre et al 1998, Takeda et al 2000a, Takeda et al 2000b). Therefore amplified myocardial mineralocorticoid receptor activation may possibly occur through either upregulation of the circulating peripheral renin-angiotensin-aldosterone

system, or via local myocardial generation of aldosterone in LVH.

Pre-clinical evidence lends support for the theory that excessive mineralocorticoid receptor activation arises in pressure-overload states and hypertensive LVH. First, in pressure-overload states induced by aortic constriction, circulating plasma aldosterone concentrations are elevated (Ganong and Mulrow 1962, Morris et al 1977). However, in aortic constriction the increase in aldosterone concentration is likely to occur as result of a reduced renal perfusion. Secondly, even after accounting for differences in heart size, genetically hypertensive rats with LVH have an augmented myocardial aldosterone production (Takeda et al 2000a, Takeda et al 2000b). Furthermore, in hypertensive LVH in rats, augmentation of myocardial aldosterone production is coupled to enhanced activity and expression of the rate limiting aldosterone synthase enzyme (CYP11B2), an effect reversed by treatment with an angiotensin-converting enzyme inhibitor (Takeda et al 2000b).

What is the evidence potentially implicating excessive myocardial mineralocorticoid receptor activation in the development of LVH? There is significant pre-clinical evidence to support this supposition. LVH is stimulated by mineralocorticoid receptor activation (Takeda et al 2000b, Takeda et al 2002) and in rats, chronic peripherally administered aldosterone induces LVH independent of any effect on BP (Young et al 1994, Young et al 1995). Additionally, LVH is produced independent of BP elevation in transgenic animal models that have augmented mineralocorticoid activation (Qin et al 2003). Furthermore, without

reducing BP, mineralocorticoid receptor antagonism reduces LVH (Qin et al 2003, Takeda et al 2000b) and with the ablation of macrophage mineralocorticoid receptors, blood pressure increases in response to vasoconstrictor stimuli, but the extent of LVH is diminished (Usher et al 2010). Nevertheless, there are some pre-clinical studies which do not substantiate the notion that mineralocorticoid activation, independent of BP effects, may promote LVH. Indeed, blockade of mineralocorticoid receptors (Kuster et al 2005) or ablation of cardiomyocyte mineralocorticoid receptors (Lothar et al 2011) has minimal effects on the extent to which LVH develops post aortic banding in mice, despite the ability of mineralocorticoid receptor antagonism to attenuate the degree of myocardial damage mediated by the chronic pressure-overload (Kuster et al 2005). Furthermore, ablation of macrophage mineralocorticoid receptors decreases the blood pressure response to exogenous mineralocorticoid administration without preventing the development of LVH (Rickard et al 2009).

Despite the large quantity of available pre-clinical evidence demonstrating a potential association between LV mass and mineralocorticoid receptor activation, even beyond BP effects, there are only limited clinical studies which support this relationship in human pathophysiology. In this regard, in some studies of hypertensive patients, relationships between LV mass and circulating plasma aldosterone concentrations have been noted (Delles et al 2003, Duprez et al 1993, Schunkert et al 1997). Furthermore, even after matching hypertensive

patients for BP level, the prevalence of LVH in patients with primary hyperaldosteronism is higher than that of patients with essential hypertension (Rossi et al 1996). Moreover, the addition of a mineralocorticoid receptor blocker to an angiotensin-converting enzyme inhibitor in hypertensive patients results in additional regression of LVH without further decreasing BP (Sato et al 1999).

Downstream signaling pathways related to aldosterone receptor activation in LVH have been described. Principally aldosterone has been demonstrated to exhibit both genomic and nongenomic receptor signaling characteristics. The principal mediators of the pathological actions of aldosterone in LVH are serum- and glucocorticoid-regulated kinase 1 (Das et al 2012, Martin-Fernandez et al 2011). Adverse cardiac remodeling induced by adrenoreceptor activation in the presence of LVH is also mediated via serum- and glucocorticoid-regulated kinase 1 (Martin-Fernandez et al 2012), thus supporting the hypothesis that adrenoreceptor activation upregulates mineralocorticoid activity. The relationship between the sympathetic nervous system and mineralocorticoids is however complex. Although, traditionally,  $\beta$ -adrenergic receptor activation upregulates components of the renin-angiotensin-aldosterone system; more recently the reverse has also been found to be true. In the myocardium, elevated aldosterone levels result in increased levels of myocardial norepinephrine potentially via reduced norepinephrine reuptake (Silvestre et al 1999). Indeed, during the development of pressure overload cardiac hypertrophy activation of the renin-angiotensin-

aldosterone system is an early response which is followed by an increase in cardiac norepinephrine content (Akers et al 2000). Further downstream targets of aldosterone receptor activation which may be involved in mediating adverse cardiac remodeling include enhanced epidermal growth factor receptor expression (Krug et al 2003) and increased calcineurin activity (Grossmann et al 2010a, Grossmann et al 2010b).

A potential role for mineralocorticoid receptor activation in the myocardium nevertheless raises the question as to whether mineralocorticoid receptor activation not only promotes the development of LVH, but also induces myocardial damage or dysfunction and hence contributes to promoting the transition from compensated LVH to LV failure. In the following discussion I shall summarise the evidence to suggest that mineralocorticoid receptor activation may also mediate deleterious effects on the hypertrophied heart which are not related to LV mass *per se*. This evidence led me in the present thesis to explore the possibility that the transition from hypertensive LVH to systolic chamber dysfunction may be mediated through mineralocorticoid receptor activation, and that these effects may be through mechanisms that do not involve LV mass changes.

**1.3.2 In LVH, both sympathetic activation and mineralocorticoid excess may promote the transition to systolic chamber decompensation.**

There is increasing evidence to suggest that sympathetic activation or mineralocorticoid receptor stimulation mediates the progression of compensated LVH to cardiac decompensation. In the subsequent discussion I will first highlight the major evidence which supports the theory that sympathetic activation may provoke the transition from compensated LVH to cardiac failure. This section will not take the form of a critical review of the literature, since it is not one of the major aims of the present thesis. I will, however, provide a critical review of the scientific literature which supports the view that myocardial mineralocorticoid receptor activation potentially promotes the transition from compensated LVH to cardiac failure.

**1.3.2.1 Sympathetic activation may promote the transition from compensated LVH to systolic chamber decompensation.**

Substantial evidence now indicates that excessive sympathetic nervous system activation promotes the progression of heart failure, once cardiac decompensation is already established. Indeed, plasma noradrenaline and adrenaline concentrations are considerably increased in patients with heart failure (Anand et al 2003, Cohn et al 1984, Esler et al

1997, Francis et al 1993, Hasking et al 1986, Kluger et al 1982, Sigurdsson et al 1994, Swedberg et al 1990) and these concentrations are related to the severity of pump dysfunction (Kluger et al 1982), the functional class (Sigurdsson et al 1994) and mortality (Anand et al 2003, Cohn et al 1984, Francis et al 1993, Swedberg et al 1990) in heart failure. Additionally, adrenergic receptor blockade with either carvedilol, a non-selective  $\alpha$ - and  $\beta$ -adrenergic receptor blocking agent, or metoprolol, a selective  $\beta$ -adrenergic receptor blocking agent improves LV systolic chamber function in patients with heart failure (Capomolla et al 2000, Doughty et al 1997, Doughty et al 2004, Gerson et al 2002, Groenning et al 2000, Gundogdu et al 2007, Hall et al 1995, Khattar et al 2001, Lowes et al 1999, Malfato et al 2007, Metra et al 2003, Olsen et al 1995, Paraskevaidis et al 2007, Quaife et al 1996, Rahko et al 2005, Ramahi et al 2001, Toyama et al 2003, Waagstein 1993, Waagstein et al 1993, Waagstein et al 2003). Furthermore, clinical studies have demonstrated improved survival benefits and reduced hospitalisations in patients with heart failure in response to a variety of  $\beta$ -adrenergic receptor blockers including carvedilol (Packer et al 1996, Packer et al 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 (CIBIS-II 1999, Packer et al 1996) 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.

There are no clinical studies as yet to support the concept that the transition to cardiac systolic chamber decompensation in LVH may be promoted by sympathetic activation. Importantly though, there are significant pre-clinical studies which demonstrate that the transition from compensated LVH to cardiac systolic chamber decompensation could nonetheless be promoted by sympathetic activation. Indeed, the development of cardiac dilatation and heart failure in response to pressure-overload states is significantly diminished in transgenic animals with a decreased adrenergic activity (Esposito et al 2002). Furthermore, in a hypertensive rat model, compensated LVH prematurely progresses to chamber dilatation and hence pump dysfunction in response to chronic  $\beta$ -adrenoreceptor activation (Badenhorst et al 2003b) and  $\beta$ -adrenoreceptor blockade, without altering BP, is able to prevent the transition from compensated hypertensive cardiac hypertrophy to heart failure with systolic chamber decompensation (Chan et al 2004). Notably, our group has previously shown that the principal mechanisms responsible for the premature transition from hypertensive LVH to pump dysfunction induced by chronic  $\beta$ -adrenoreceptor activation is through eccentric cardiac remodelling (cardiac dilatation), rather than through alterations of intrinsic myocardial dysfunction (Badenhorst et al 2003b, Booyesen et al 2012,

Osadchii et al 2007b).

In spite of the accumulating evidence to demonstrate that elevated sympathetic activation induces the transition from compensated hypertensive LVH to cardiac systolic chamber decompensation, there is unlikely to be a shift to significantly increase the use of  $\beta$ -adrenoreceptor blockers in hypertension. The reasons not to increase the use of  $\beta$ -adrenoreceptor blockers include their ability to increase the probability of users developing new onset diabetes mellitus (Bakris and Sowers 2004). Furthermore,  $\beta$ -adrenoreceptor blockers may not regress LVH (Devereux et al 2004b) or decrease central aortic BP (Williams et al 2006) as effectively as alternative antihypertensive agents. As a consequence, the use of agents that block the renin-angiotensin system or calcium channel blockers have a more pronounced benefit on cardiovascular events in comparison to the impact of  $\beta$ -adrenoreceptor blocker-based therapy (Dahlof et al 2002, Dahlof et al 2005). Therefore it is obvious that the potential benefits of  $\beta$ -adrenoreceptor blockers in preventing the transition to pump dysfunction in compensated LVH could be counteracted by potentially harmful adverse effects. Hence, in the absence of evidence of the availability of  $\beta$ -adrenoreceptor blockers that do not induce these potentially adverse effects, alternative pharmacological agents must be identified. In order to achieve this goal, targets parallel to or downstream from  $\beta$ -adrenoreceptors that are responsible for LV decompensation need to be identified. Therefore, I hypothesised in the present thesis that because sympathetic activation stimulates the renin-angiotensin-

aldosterone system (Saxena 1992, van Zwieten and de Jonge 1986), one potential downstream or parallel target of  $\beta$ -adrenoreceptors which may mediate the transition from compensated LVH to cardiac decompensation is through the effects of aldosterone acting on myocardial mineralocorticoid receptors. To test this hypothesis, in the present thesis I examined the effect of mineralocorticoid receptor blockade on an animal model of adrenergic-induced cardiac dilatation and systolic chamber decompensation associated with hypertensive LVH. These data and the implications thereof are described in chapter 2 and have been published in the journal *Hypertension* (Veliotis et al 2005). What is the evidence to suggest a role for mineralocorticoid receptor activation in mediating the transition from compensated LVH to cardiac systolic chamber decompensation? In the following discussion, I will review the available evidence and data which suggests that aldosterone triggers deleterious effects on the myocardium.

#### **1.3.2.2      Activation of mineralocorticoid receptors may promote the transition from LVH to systolic chamber decompensation.**

As indicated in the aforementioned discussion, mineralocorticoid receptors are present in cardiac myocytes (Funder 1997, Funder 2010, Lombes et al 1995, Messaoudi and Jaisser 2011, Young et al 1994) and the myocardium is able to locally synthesize aldosterone independent of

the circulating renin-angiotensin system (Messaoudi et al 2012, Silvestre et al 1998, Takeda et al 2000, Young et al 2001). Therefore, excessive activation of myocardial mineralocorticoid receptors may potentially provoke various deleterious cardiac effects which could result in cardiac decompensation. To-date a variety of adverse effects of aldosterone, or activation of the mineralocorticoid receptor at the myocardial tissue level, have been reported. These effects include myocardial fibrosis (Brilla et al 1993, Robert et al 1994) induced possibly through activation of mineralocorticoid receptors on cardiac fibroblasts (Brilla et al 1994), cardiomyocyte necrosis following mitochondrial alterations (Rocha et al 2000, Shahbaz et al 2011, Zia et al 2010), cardiomyocyte apoptosis (De Angelis et al 2002, Sam et al 2004, Sohn et al 2010) and an aldosterone-induced or mineralocorticoid receptor-mediated myocardial inflammatory reaction (Sun et al 2002). The role of mineralocorticoid receptors on macrophages has recently been highlighted (Rickard et al 2009, Usher et al 2010), and these effects may be attributed to mineralocorticoid receptor-induced increases in monocyte chemoattractant protein 1, transforming growth factor  $\beta$ 1, connective tissue growth factor, plasminogen activator inhibitor type 1, and a range of metalloproteinases (Marney and Brown 2007). These mineralocorticoid receptor-mediated effects may depend on the coexistence of alternative stimulants such as oxidative stress, angiotensin II, endothelin or salt administration (Gekle and Grossman 2009). In light of the evidence implicating aldosterone or mineralocorticoid receptors as a potential mediator of tissue-level damage in the

myocardium, what is the evidence to suggest that aldosterone and/or mineralocorticoid receptor activation can ultimately induce cardiac decompensation?

From a clinical standpoint, the role of aldosterone in already established heart failure is currently widely acknowledged. Indeed, in patients with heart failure, increased plasma aldosterone concentrations are associated with both mortality and the severity of heart failure (Dzau et al 1981, Swedberg et al 1990). In the failing human heart, an increased synthesis of aldosterone occurs in the myocardium (Hayashi et al 2001, Mizuno et al 2001). Blockade of mineralocorticoid receptors both improves cardiac function and reduces left ventricular dilatation in patients with chronic heart failure, and patients post-myocardial infarction (Cicoira et al 2002, Hayashi et al 2003, Kasama et al 2003, Modena et al 2001, Tsutamoto et al 2001). In heart failure patients, the addition of a mineralocorticoid receptor antagonist to standard heart failure therapy, including angiotensin-converting enzyme inhibitors and/or  $\beta$ -adrenoreceptor blockers, results in a significant reduction in mortality (Pitt et al 1999, Zannad et al 2011). Furthermore, mineralocorticoid receptor blockade in addition to standard therapy post myocardial infarction, including the use of both  $\beta$ -adrenoreceptor blockers and angiotensin-converting enzyme inhibitors, also results in a significant reduction in mortality (Pitt et al 2003). Notably, the ability of mineralocorticoid receptor blockade to reduce mortality in the study conducted in patients post-myocardial infarction, was greatest in the subset of patients (60% of

patients) with a history of hypertension (Pitt et al 2003). Hence, mineralocorticoid receptor antagonism may be of significant clinical benefit in patients with hypertensive LVH. Alternatively, the major therapeutic benefit of mineralocorticoid receptor antagonism may well be mediated via BP effects. Consequently, these clinical associations do not automatically imply a cause-effect relationship between myocardial mineralocorticoid receptor activation and heart failure. Does pre-clinical evidence support a haemodynamically-independent, cause-effect relationship between mineralocorticoid receptor activation and cardiac systolic chamber decompensation?

Emerging pre-clinical evidence indeed supports such a haemodynamically independent role for cardiac mineralocorticoid activation in the development of cardiac systolic chamber dysfunction. In this regard, excess cardiac mineralocorticoid receptor activation in a transgenic mice model, results in the development of LV systolic chamber dysfunction through BP-independent mechanisms (Qin et al 2003). Moreover, mineralocorticoid receptor blockade attenuates the development of cardiac dilatation and LV systolic chamber dysfunction in an animal model of chronic pressure-overload hypertrophy produced by aortic banding (Kuster et al 2005), a model where LV loads may not be influenced by vascular effects. Furthermore, mineralocorticoid receptor blockade or aldosterone synthesis inhibition attenuates the development of cardiac dilatation and LV systolic chamber dysfunction post myocardial infarction (Fraccarollo et al 2005, Fraccarollo et al 2008, Mulder et al 2008,

Wang et al 2004), an effect that may nevertheless still be mediated by decreases in LV loading conditions. In addition, conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene protects against the development of LV systolic chamber decompensation produced by chronic-pressure overload (Lothar et al 2011) or myocardial infarction (Fraccarollo et al 2011). Last, in an animal model of coronary microembolisation, mineralocorticoid receptor antagonism averts the development of cardiac dilatation and pump dysfunction, an effect not mediated through alterations in BP (Suzuki et al 2002). Thus, there is evidence to indicate that mineralocorticoid receptor activation may indeed promote the transition from cardiac hypertrophy to failure.

However, publications prior to and even in many instances subsequent to the publication of the work presented in the present thesis (Veliotes et al 2005, Veliotes et al 2010), may be characterized by a number of potential limitations with respect to the evidence favouring a role for mineralocorticoid receptor activation in promoting the transition to systolic cardiac chamber decompensation in pressure-overload states. These limitations were addressed as part of the present thesis. The following section therefore critically reviews the available evidence relevant to the work conducted in the present thesis. This critical review underscores the potential limitations of these prior studies from a perspective of the interpretation of the data.

#### **1.3.2.2.1 Limitations of the evidence favouring a role for mineralocorticoid receptor activation in promoting the transition from LVH to systolic chamber decompensation.**

Briefly, there are two main potential limitations of prior studies exploring the role of mineralocorticoid receptor activation in mediating the transition from LVH to LV systolic chamber decompensation. First, none of the studies evaluating the impact of mineralocorticoid receptor blockade or inactivation in pressure-overload states (Qin et al 2003, Kuster et al 2005, Lothar et al 2011) were able to completely identify the mechanisms responsible for the beneficial effects of mineralocorticoid receptor blockade on LV systolic chamber function. Second, as I will argue, none of the animal models employed to study the mechanisms of the beneficial effects of mineralocorticoid receptor blockade or inactivation on cardiac function in pressure-overload states (Qin et al 2003, Kuster et al 2005, Lothar et al 2011) addressed all of the issues of importance with respect to human hypertension. What evidence were these (Qin et al 2003, Kuster et al 2005, Lothar et al 2011) and other studies able to provide which lend insights into the potential mechanisms involved in the ability of mineralocorticoid receptor activation to promote the transition from LVH to systolic chamber decompensation?

#### **1.3.2.2.1.1 Does mineralocorticoid receptor blockade target contractile disturbances or adverse chamber remodelling?**

In none of the aforementioned studies conducted in either animals (Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Kuster et al 2005, Lothar et al 2011, Mulder et al 2008, Qin et al 2003, Suzuki et al 2002, Wang et al 2004) or humans (Kasama et al 2003, Pitt et al 1999, Pitt et al 2003, Tsutamoto et al 2001), were the authors able to identify whether the primary effect of mineralocorticoid receptor blockade or inactivation was on myocardial contractile disturbances with a subsequent reduction in cardiac chamber dimensions, or whether the primary effect was indeed on cardiac dilatation, which as a consequence, prevented pump dysfunction.

The question of whether mineralocorticoid receptor blockade or inactivation chiefly targets contractile dysfunction or cardiac chamber dilatation has important pathophysiological relevance. There is currently ample evidence to indicate that cardiac dilatation is a precursor of left ventricular dysfunction and clinical heart failure and not inevitably a result of an impaired myocardial contractility (Gaudron et al 1993, Pfeffer et al 1993, Vasan et al 1997). It is now well-recognized that cardiac chamber dilatation contributes towards the development of pump dysfunction and subsequent end-stage heart failure (Cohn 1995, Cohn et al 2000, de Kam et al 2002, Mann et al 1999). Indeed cardiac chamber dilatation is a

significant risk factor for morbidity and mortality in heart failure (Foley et al 1995, Gadsboll et al 1990, Lee et al 1993, Nestico et al 1985). Moreover, evidence produced by members of our laboratory indicates that LV systolic chamber dysfunction in heart failure is more closely associated with cardiac dilatation than with abnormalities in intrinsic myocardial function (Badenhorst et al 2003b, Booyesen et al 2012, Norton et al 2002, Osadchii et al 2007b, Veliotis et al 2005). What is the evidence to suggest that changes in the extent of chamber dilatation is an important determinant of outcomes?

Reductions of cardiac chamber volumes and dimensions have been noted in the treatment of heart failure (Doughty et al 1997). The reverse remodelling is in turn associated with a better long-term outcome, including survival (Levine et al 2000, Sharpe and Doughty 1998). Persistent cardiac chamber dilatation signals a poor outcome in heart failure to some extent because most pharmacological interventions, including angiotensin-converting enzyme inhibitors, have little effect on an extensively dilated LV (Levine et al 2000). Notably thus far, therapeutic agents have resulted in only limited reverse remodelling of the heart (Booyesen et al 2012). Thus, it is important to identify novel pharmacological approaches to prevent and reverse chamber dilatation. Indeed, mineralocorticoid receptor blockade or inactivation may produce beneficial effects on the myocardium principally through changes in cardiac dimensions. However, there is as yet no direct evidence to support this notion. Therefore, as part of the present thesis I assessed the impact

of mineralocorticoid receptor blockade on an animal model of LV systolic chamber dysfunction attributed to cardiac dilatation rather than alterations in intrinsic myocardial contractile disturbances (Badenhorst et al 2003b, Booyesen et al 2012, Osadchii et al 2007b). These data and the implications thereof are described in chapter 2 and have been published in the journal *Hypertension* (Veliotes et al 2005). In the following section of the present thesis I will review the available evidence which indicates that mineralocorticoid receptor blockade may in fact target cellular changes that principally affect adverse chamber remodelling.

#### **1.3.2.2.1.2 Could mineralocorticoid receptor blockade directly target the mechanisms responsible for adverse chamber remodelling?**

Several theoretical grounds support a notion that mineralocorticoid receptor blockade may target adverse chamber remodelling through direct mechanisms, rather than through effects secondary to an improvement in intrinsic myocardial contractile dysfunction. However, in order to suitably answer this question it is important to draw attention to the potential mechanisms responsible for adverse chamber remodelling (cardiac dilatation) and subsequently highlight evidence which indicates whether mineralocorticoid receptor activation may well target these mechanisms.

Cardiac chamber dilatation could be the consequence of an inappropriate hypertrophic process occurring in cardiomyocytes, where

increments in myocyte length exceed those of width, the result being chamber dilation (Gerdes 2002). This view is supported by the results of studies performed in various experimental models of cardiac remodelling and failure, including data obtained in ischaemic dilated cardiomyopathy (Anand et al 1997, Gerdes et al 1992, Gerdes and Capasso 1995, Zimmer et al 1990), hypertensive heart failure (Tamura et al 1998) and pacing-induced heart failure (Spinale et al 1991a). There are as yet no data supporting the notion that activation of mineralocorticoid receptors induces increments in cell length as opposed to cell width. Furthermore there is also no data relating to the impact of mineralocorticoid receptor inhibition on counteracting these potential effects on myocyte length. In the present thesis, I therefore assessed the possibility that mineralocorticoid receptor blockade modifies the ratio between cell length and width in a rat model of pump dysfunction induced by cardiac dilatation rather than contractile disturbances. These data and the implications thereof are described in chapter 3 and have been published in the *Journal of Cardiovascular Pharmacology* (Veliotes et al 2010).

Side-to-side slippage of cardiomyocytes has also been postulated as a mechanism resulting in cardiac chamber dilatation (Beltrami et al 1995, Linzbach 1960, Olivetti et al 1990). The favored hypothesis in this regard, is that degradation of collagen in the intercellular matrix would impair inter-cardiomyocyte attachment and thus favour slippage and left ventricular dilatation. Activation of matrix metalloproteinases (MMPs) or collagenases may induce degradation of myocardial collagen as

demonstrated in patients with congestive heart failure and in patients with reduced LV systolic function (Li et al 2001, Polyakova et al 2004, Reddy et al 2004, Spinale 2002, Spinale et al 2000) and in animal models of cardiac pump dysfunction (King et al 2003, Mujumdar et al 1999, Mukherjee et al 2003, Peterson et al 2001, Sakata et al 2004, Spinale et al 1998, Spinale et al 1999). The role of MMPs in mediating adverse chamber remodelling is strengthened by data demonstrating that MMP inhibition attenuates LV dilatation in heart failure in the spontaneously hypertensive rat (Peterson et al 2001), in animal models of pacing-induced heart failure (King et al 2003, Spinale et al 1999) and in animal models of myocardial infarction (Mukherjee et al 2003, Rohde et al 1999). Additionally, gene deletion of the tissue inhibitor of the matrix metalloproteinase-type 1 (TIMP-1), and hence failure of the inhibitory control on MMPs, leads to left ventricular dilatation in mice (Roten et al 2000).

Importantly, there is evidence to suggest an effect of mineralocorticoid receptor activation on MMPs. Indeed, aldosterone stimulates MMP release in isolated cardiomyocytes (Rude et al 2005). Moreover, blockade of mineralocorticoid receptors reduces the augmented MMP activity which accompanies chronic heart failure induced by coronary microembolization (Suzuki et al 2002) and the increased MMP/TIMP ratio that accompanies pressure-overload hypertrophy (Kuster et al 2005). In contrast, however, aldosterone synthase inhibition or mineralocorticoid receptor blockade is unable to attenuate the increased MMP activity noted in viable myocardium post-myocardial infarction

(Mulder et al 2008). Furthermore, mineralocorticoid receptor blockade, although reducing infarct expansion post-myocardial infarction, is unable to modify MMP expression in the infarct zone (Fracarollo et al 2008). These results are nevertheless in contrast to the reduced MMP-2 and 9 activity in the infarct zone post-myocardial infarction in mice with deletion of the cardiomyocyte mineralocorticoid receptor (Fraccarollo et al 2011). However, in previous studies demonstrating a beneficial effect of mineralocorticoid receptor blockade on MMPs (Kuster et al 2005, Suzuki et al 2002) it is difficult to identify whether mineralocorticoid receptor blockade mediates the beneficial effects directly through actions on MMPs, or whether these effects are indeed secondary to improvements in contractile and hence LV systolic chamber function. Therefore, as part of the present thesis I evaluated whether mineralocorticoid receptor blockade can directly target MMP activation using an acute stimulus for activation of these enzymes demonstrated to promote the development of cardiac dilatation and a decreased LV systolic chamber function. These data and the implications thereof are described in chapter 3 and have been published in the *Journal of Cardiovascular Pharmacology* (Veliotes et al 2010).

The role of elevated myocardial collagen concentrations as a potential mechanism resulting in cardiac dilatation has been unclear until our laboratory published additional insights into this topic. What is the evidence that supports or refutes a role for myocardial collagen concentrations in the pathogenesis of cardiac dilatation? Following the use

of LV assist devices, a reduction in cardiac cavity dimensions is commonly coupled to increased rather than decreased total myocardial collagen concentrations (Li et al 2001, Madigan et al 2001, McCarthy et al 1995, Scheinin et al 1992). Furthermore, although pressure-overload-induced heart failure is associated with elevated total myocardial collagen concentrations (Gunga-Smith et al 1996), decreases as opposed to increases in total myocardial collagen concentrations accompany rapid pacing-induced (Spinale et al 1991b, Spinale et al 1998) and  $\beta$ -adrenergic-induced (Woodiwiss et al 2001) cardiac dilatation. Importantly, increased myocardial collagen synthesis may result in cardiac chamber dilatation if the phenotype of collagen produced is more susceptible to collagenase degradation (Badenhorst et al 2003a). Indeed, systolic chamber dysfunction and cardiac dilatation are associated with collagen of the non-cross-linked phenotype (Badenhorst et al 2003a, Capasso et al 1989, Gunga-Smith et al 1996, Spinale et al 1996, Woodiwiss et al 2001). Furthermore, attenuation of pressure-overload-induced cardiac dilatation has been achieved by genetically reducing the susceptibility of collagen to MMP degradation (Lindsay et al 2003).

Importantly, in the development of pump dysfunction induced by pressure-overload states (Kuster et al 2005) and coronary microembolization-induced chronic heart failure (Suzuki et al 2002), mineralocorticoid receptor blockade has been demonstrated to attenuate accompanying increments in myocardial collagen concentrations. In addition, the ability of mineralocorticoid receptor blockade to diminish the

extent to which cardiac dilatation and LV systolic chamber dysfunction occur in animal models of myocardial infarction is associated with a reduced myocardial fibrosis (Fraccarollo et al 2005, Mulder et al 2008 Wang et al 2004). Furthermore, the ability of conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene to protect against the development of LV dilatation and systolic chamber decompensation produced by myocardial infarction is associated with a reduced myocardial fibrosis (Fraccarollo et al 2011). However, suprisingly, the ability of conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene to protect against the development of LV dilatation and systolic chamber decompensation produced by pressure-overload is not associated with a reduced myocardial fibrosis (Lothar et al 2011). Hence, it is likely that alternative mechanisms explain the benefits of mineralocorticoid receptor inactivation on LV chamber dimensions and systolic chamber function. In this regard, whether the beneficial effects of mineralocorticoid receptor inactivation on total myocardial collagen concentrations or content are associated with modifications of the phenotypic characteristics of collagen has not as yet been determined. Thus, in the present thesis I also explored the possibility that mineralocorticoid receptor blockade could mediate beneficial effects on cardiac dilatation by modifying the qualitative rather than the quantitative characteristics of myocardial collagen. These data and the implications thereof are described in chapter 3 and have been published in the *Journal of Cardiovascular Pharmacology* (Veliotes et al 2010).

Cardiomyocyte cell death induced either through apoptosis or necrosis may provide an alternative explanation for cardiac dilatation, although the mechanism of the effect is unclear. Cell death may conceivably reduce syncytial connections and thereby encourage cardiomyocyte slippage. What is the effect of aldosterone or mineralocorticoid receptor activation on cardiomyocyte apoptosis? Mineralocorticoid receptor blockade reduces cardiomyocyte apoptosis which occurs in pump dysfunction induced by pressure-overload states (Kuster et al 2005). In addition, the ability of cardiomyocyte mineralocorticoid receptor inactivation to attenuate the development of cardiac dilatation and LV systolic chamber dysfunction in animal models of myocardial infarction is associated with a reduced myocardial apoptosis (Fraccarollo et al 2011). However, surprisingly, the ability of conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene to protect against the development of LV systolic chamber decompensation produced by pressure-overload is not associated with a reduced myocardial apoptosis (Lothar et al 2011). Hence, whether a reduction of myocardial apoptosis is an important mechanism explaining the benefits of mineralocorticoid receptor inactivation on LV chamber dimensions and systolic chamber function is still uncertain. Unfortunately, in those animal models of LV systolic chamber decompensation where the protective effect of mineralocorticoid receptor inactivation was associated with beneficial effects on cardiomyocyte apoptosis, it remains unclear whether this effect is mediated via the direct actions of mineralocorticoid

receptor changes or whether these changes are merely secondary to beneficial effects on LV systolic chamber dysfunction. However, there is *in vitro* evidence to indicate that aldosterone mediates cardiomyocyte apoptosis (De Angelis et al 2002, Sohn et al 2010), thus suggesting that mineralocorticoid receptor blockade may be producing primary effects on cardiomyocyte apoptosis. *In vivo* confirmation of the role of mineralocorticoid receptor activation in mediating cardiomyocyte apoptosis is nevertheless still required. Therefore, as part of the present thesis I evaluated whether mineralocorticoid receptor blockade can directly inhibit cardiomyocyte apoptosis produced by an acute stimulus. These data and the implications for the role of mineralocorticoid receptor antagonism in preventing the transition to LV systolic decompensation are also described in chapter 3 and have been published in the *Journal of Cardiovascular Pharmacology* (Veliotis et al 2010).

#### **1.3.2.2.1.3 Animal models of the transition from hypertensive LVH to failure employed to assess the effect of mineralocorticoid receptor blockade.**

As previously indicated, various animal models have been utilized to assess the impact of mineralocorticoid receptor blockade on the transition from pressure-overload hypertrophy to pump dysfunction. However a number of potential limitations of the animal models studied exist. Several studies have reported on a beneficial impact of

mineralocorticoid receptor blockade on LV systolic function and chamber dimensions in pressure-overload hypertrophy (Kuster et al 2005, Lothar et al 2011). In these studies ascending aortic constriction was employed to elevate cardiac afterload (Kuster et al 2005, Lothar et al 2011). Indeed this model suitably excludes the potentially beneficial BP-mediated effects of aldosterone receptor blockade on the heart. However, it is also now well-recognized that aortic constriction results in acute and often striking increases in LV pressures with progression to cardiac LV systolic chamber decompensation and heart failure occurring within a rather brief time (Norton et al 2002). Importantly, this comparatively acute cardiac decompensation may not essentially characterize the decompensation that occurs in chronic pressure-overload states such as in primary hypertension. In primary hypertension, cardiac decompensation may take considerably longer to occur. Indeed, cardiac decompensation may take up to 26 months to evolve in a spontaneously hypertensive rat model (Tsotetsi et al 2001). Consequently, additional research is necessary to assess the role of mineralocorticoid receptor blockade on the transition from cardiac hypertrophy to LV systolic chamber dysfunction in more chronic models of pressure overload, whilst simultaneously segregating these effects from BP-mediated changes. Therefore, in this regard, as shall be described in chapter 2 of the present thesis, I evaluated the impact of mineralocorticoid receptor blockade on the transition from compensated cardiac hypertrophy in spontaneously hypertensive rats to LV systolic chamber dysfunction induced by chronic  $\beta$ -adrenoreceptor

activation. These data and the implications thereof are described in chapter 2 and have been published in the journal *Hypertension* (Veliotes et al 2005). This model of LV systolic chamber dysfunction and adverse chamber remodelling has recently been demonstrated by our group to develop systolic chamber dysfunction largely independent of BP effects and to closely mirror the LV systolic chamber dysfunction which occurs in aging spontaneously hypertensive rats (Badenhorst et al 2003b).

### **1.3.3 Is LVH a cardiac phenotype that exacerbates the progression to heart failure post-myocardial infarction?**

An additional consideration when evaluating the potential role of LVH as a cardiac phenotype that augments the progression to heart failure is the question of whether after myocardial infarction the presence of LVH exacerbates this transition process? In this regard it is now well-recognized that LVH is a risk factor for myocardial infarction. Indeed, almost 50 years ago the Framingham Heart Study used electrocardiographic markers of LVH to provide the first evidence to suggest a role for LVH as a predictor of myocardial infarction (Kannel et al 1961, Kannel et al 1970). These data were subsequently supported by evidence that echocardiographic LVH also predicts the development of myocardial infarction (Levy et al 1988, Levy et al 1990). Considerably more data has since emerged, which supports the concept that an increased LV mass is an independent predictor of myocardial infarction. In

the present thesis I have not addressed the pathophysiological role of LVH as a cause of myocardial infarction, and so a review of all of this literature goes beyond the scope of this thesis. Nevertheless, it is important to note that the association between baseline LVH and the development of myocardial infarction may merely be an epiphenomenon rather than a cause-effect relationship. Therefore, it is important to ascertain whether regression of LVH is associated with a reduced incidence of myocardial infarction. The evidence in this regard is controversial. Indeed, although regression of echocardiographic LV mass in the LIFE study was independently associated with a reduction in a number of cardiovascular end-points, it was not independently associated with a reduced incidence of myocardial infarction (Devereux et al 2004b). However, it is reassuring to note that regression of electrocardiographic LVH in the LIFE study was independently associated with reduced incidence of myocardial infarction (Okin et al 2004). However, this finding (Okin et al 2004) may be interpreted as indicating the electrocardiographic criteria for LVH signify something beyond LVH. Irrespective of whether LVH is causally related to myocardial infarction, there is nevertheless no doubt that an association exists. Hence one must consider whether the presence of LVH increases the probability of a worse outcome post myocardial infarction.

### **1.3.3.1 Left ventricular hypertrophy may increase the chance of systolic chamber dysfunction post-myocardial infarction.**

Although at the time of publication of the work on this topic explored in the present thesis (Norton, Veliotis et al 2008), there was no clinical evidence to support this notion, there was considerable pre-clinical evidence to show that reductions in LV systolic chamber function post-myocardial infarction may be exaggerated by hypertensive cardiac hypertrophy (Fletcher et al 1982, Fletcher et al 1986, Itter et al 2004, Jain et al 2002, Nass et al 2002, Nishikimi et al 1995). The excess systolic chamber dysfunction post-myocardial infarction could nevertheless be explained in-part through the adverse effects of afterload on infarct size. Indeed, in the presence of a higher BP, infarct size is increased (Nolan et al 1988, Pierard et al 1987). However, there is also evidence to suggest that the presence of LVH may augment the adverse remodelling process that occurs in non-infarcted cardiac tissue, the consequence being enhanced cardiac dilatation and subsequent worse systolic chamber function (Itter et al 2004, Jain et al 2002, Jilaihawi et al 2003). However, what had not been explored at the time of publication of the work on this topic in the present thesis (Norton, Veliotis et al 2008) is whether the chronically infarcted hypertensive heart is susceptible to a decrease in systolic function in the remaining non-infarcted and chronically remodelled viable myocardium. Is there evidence to suggest that hypertrophied tissue

may contribute to an exacerbated LV systolic chamber dysfunction post-myocardial infarction?

#### **1.3.3.2 Myocardial dysfunction may occur in non-infarcted tissue post myocardial infarction.**

Studies conducted in normotensive animals have established the role of a decreased regional myocardial systolic function in dilated, non-infarcted, but viable myocardial tissue post myocardial infarction (Cheung et al 1994, Davidoff et al 2004, Li et al 1995, Litwin et al 1992, Litwin et al 1994, Mill et al 1998, van der Velden et al 2004, Zhang et al 1999). These changes in regional myocardial systolic function may be attributed to a number of mechanisms involving changes in cardiomyocyte calcium handling, apoptosis, alterations in myosin isoforms, modifications in cardiomyocyte morphometry, cytoskeleton proteins and ion transport systems as well as changes in myocardial interstitial remodelling and non-myocyte factors (Gupta et al 2000, van der Velden et al 2004). However, whether these effects are exacerbated in LVH has not been evaluated. Is there evidence to suggest that LVH may exacerbate myocardial dysfunction in viable myocardial tissue post-myocardial infarction?

As compared to normotensive hearts, hypertensive hearts are more susceptible to alterations in viable tissue adrenergic signaling post-myocardial infarction (Kouchi et al 2000) and apoptosis in general (Liu et al 2000). These changes (Kouchi et al 2000, Liu et al 2000) may therefore

increase the chances of myocardial systolic dysfunction occurring in remote myocardial tissue post-myocardial infarction in the hypertrophied heart. Therefore as described in chapter 4 of the present thesis I also evaluated whether hypertensive cardiac hypertrophy renders the heart more susceptible to the development of intrinsic myocardial systolic dysfunction in viable non-infarcted cardiac tissue post-myocardial infarction. Secondly I explored the potential mechanisms thereof and whether this effect contributes toward global LV systolic chamber dysfunction.

#### **1.4 Summary of problem statements.**

- 1) Although there is significant evidence that sympathetic activation in LVH may contribute toward LV systolic chamber decompensation, the use of  $\beta$ -AR blockers in hypertension has become increasingly less attractive. Alternative downstream or parallel targets need identification which could be targeted therapeutically. One potential target is through blockade of mineralocorticoid receptors.
- 2) The mechanism through which LVH may progress to cardiac systolic chamber decompensation may be through either cardiac dilatation or decreases in intrinsic myocardial function. Whether mineralocorticoid receptor blockade targets cardiac dilatation and/or intrinsic myocardial function is uncertain. Moreover, the exact mechanisms that explain these effects are uncertain.

- 3) Although LVH is a risk factor for myocardial infarction and may increase the risk of progressing to cardiac systolic chamber decompensation, it is uncertain whether this effect could be mediated by a reduced intrinsic function of viable non-infarcted myocardial tissue.

### **1.5 Aims of the thesis.**

#### To determine:

- 1) Whether mineralocorticoid receptor blockade prevents the transition from compensated LVH to LV systolic chamber dysfunction and cardiac dilatation induced by sympathetic activation. The methodology, results and discussion related to this question are provided in chapter 2.
- 2) The mechanisms that may explain the ability of mineralocorticoid receptor blockade to prevent the progression from compensated LVH to LV systolic chamber dysfunction and cardiac dilatation induced by sympathetic activation. The methodology, results and discussion related to this question are provided in chapters 2 and 3.
- 3) Whether hypertensive LVH exacerbates reductions in intrinsic myocardial systolic function in viable myocardium post myocardial infarction. The methodology, results and discussion related to this question are provided in chapter 4.

## Chapter 2

### **Mineralocorticoid Receptor Blockade Prevents the Transition From Compensated Left Ventricular Hypertrophy to Systolic Chamber Decompensation Induced by $\beta$ -Adrenoreceptor-Activation.**

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**ABSTRACT**

Whether mineralocorticoid receptor activation contributes toward  $\beta$ -adrenoreceptor (AR)-mediated LV decompensation in hypertensive LVH and whether this is primarily through myocardial contractile effects or adverse chamber remodelling is uncertain. To address these questions, the effect of spironolactone (SPIRO, 80 mg.kg<sup>-1</sup>.day<sup>-1</sup>) on LV cavity dimensions, function and chamber remodelling was evaluated in spontaneously hypertensive rats (SHR) in whom decompensation was induced by administering a low dose of the  $\beta$ -AR agonist, isoproterenol (ISO), at 0.02-to-0.04 mg.kg<sup>-1</sup>.day<sup>-1</sup> for 4.5 months. ISO administered to SHR resulted in an increased 24-hour urinary aldosterone excretion and LV cavity dimensions, a right shift in LV diastolic pressure-volume relations, and a decreased LV relative wall thickness without further enhancing an increased myocardial norepinephrine (NE) release in SHR. ISO reduced LV systolic chamber function (decreased LV endocardial fractional shortening and the slope of the LV systolic pressure-volume relationship) without modifying intrinsic myocardial systolic function (as assessed from LV midwall fractional shortening and the slope of systolic stress-strain relationship). ISO only increased LV cavity volumes after prolonged periods of administration. SPIRO abolished ISO-induced chamber dilatation, wall thinning and pump dysfunction, but failed to modify blood pressure, volume preloads, intrinsic myocardial systolic function, or myocardial NE release. In conclusion, these results suggest that mineralocorticoid receptor activation, through load-independent

effects, may be critical in mediating the transition from compensated hypertensive LVH to dilatation and LV systolic chamber dysfunction induced by chronic  $\beta$ -AR activation. The mechanism of this effect may occur primarily through beneficial effects on adverse cardiac remodelling rather than through alterations in intrinsic myocardial contractile dysfunction.

## 2.1 Introduction.

Hypertension is a major risk factor for heart failure (Cleland et al 2002, Davies et al 2001, Krum and Gilbert 2003, Levy et al 1996). The mechanisms responsible for the transition from compensated left ventricular hypertrophy (LVH) in hypertension to heart failure are nevertheless unclear. Although there is evidence to indicate that adrenergic activation may mediate the transition to LV systolic chamber decompensation in pressure-overload hypertrophy (Badenhorst et al 2003b, Esposito et al 2002), the limited beneficial effects of  $\beta$ -adrenoreceptor (AR) blockers on LVH (Devereux et al 2004a) and central aortic BP (Williams et al 2006), the worse cardiovascular outcomes in  $\beta$ -AR blocker-based as compared to angiotensin-converting enzyme inhibitor/calcium channel blocker-based therapy (Dahlof et al 2002, Dahlof et al 2005), and the potential for  $\beta$ -AR blockers increasing the chance of new onset diabetes mellitus (Bakris et al 2004), is likely to curtail their use in hypertension.

An alternative mechanism that may mediate the progression from hypertensive LVH to cardiac systolic chamber decompensation is through mineralocorticoid receptor activation. Indeed, mineralocorticoid receptor blockade (Kuster et al 2005) or conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene (Lothar et al 2011) prevents the progression from pressure overload hypertrophy induced by aortic constriction to LV systolic chamber dysfunction.

However, whether mineralocorticoid receptor blockade is able to prevent cardiac decompensation in chronic hypertensive LVH, which may have a different natural history and pathophysiology to acute aortic constriction (Badenhorst et al 2003a, Badenhorst et al 2003b, Norton et al 1997, Norton et al 2002, Tsotetsi et al 2001) has not been determined. Furthermore, the mechanisms of the beneficial impact of mineralocorticoid receptor blockade on systolic chamber function in pressure-overload states are uncertain. In this regard, because sympathetic activation promotes increases in circulating aldosterone concentrations, mineralocorticoid receptor blockade may share the same cellular targets as adrenergic receptor blockade. Moreover, whether mineralocorticoid receptor blockade, in cardiac pathology that promotes LV systolic chamber dysfunction, mediates primarily beneficial effects on intrinsic myocardial systolic function, or adverse cardiac chamber remodelling has not been determined (Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Kuster et al 2005, Lothar et al 2011, Mulder et al 2008, Suzuki et al 2002, Wang et al 2004).

In the present study I therefore evaluated the effect of mineralocorticoid receptor blockade on  $\beta$ -AR-induced decompensation in hypertensive LVH and the mechanisms thereof. For this purpose the spontaneously hypertensive rat (SHR) was selected as a model of compensated LVH susceptible to  $\beta$ -adrenergic-induced LV decompensation (Badenhorst et al 2003b) and the impact of a mineralocorticoid receptor antagonist on  $\beta$ -AR-mediated LV dilatation and

LV systolic chamber dysfunction examined.

## **2.2 Methods.**

The present study was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institute of Health, publication no. 86-23, 1996), and was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (Approval numbers: 99:01:2b, 2002:37:5 and 2002:39:5).

### **2.2.1 Groups studied.**

Male spontaneously hypertensive rats bred in the Central Animal Services of the University of the Witwatersrand, were studied over a time period (14-to-18.5 months of age) prior to that when LV decompensation occurs in this rat strain (Tsotetsi et al 2001). Spontaneously hypertensive rats were assigned to groups that received either no treatment; twice daily intraperitoneal injections of the  $\beta$ -AR agonist, isoproterenol (ISO) (Imuprel, Adcock Ingram:  $0.02 \text{ mg.kg}^{-1}.\text{injection}^{-1} \approx 0.2 \text{ ml}$ ); daily spironolactone (SPIRO,  $80 \text{ mg.kg}^{-1}.\text{day}^{-1}$  suspended in a gelatine-meat extract cube), a mineralocorticoid receptor antagonist; or both ISO and SPIRO. Age-matched male Wistar Kyoto (WKY) control rats received no treatment as ISO has little effect on LV geometry and function in this rat strain

(Badenhorst et al 2003b). The model of LV dilatation studied has the advantage in that it is not associated with death related to heart failure when studied for up to 5 months from the time that ISO is initiated (Badenhorst et al 2003b) and hence allows for a clear interpretation of cardiac remodelling end-points.

The dose of SPIRO employed in the present study was selected from the outcome of a pilot study conducted in 14 month old SHR where SPIRO given orally at  $80 \text{ mg.kg}^{-1}.\text{day}^{-1}$  for 2 months decreased LV weight (in grams: SHR [n=7]  $=1.13\pm0.04$ , SHR+SPIRO [n=8]  $=0.98\pm0.02$ , WKY [n=8]  $=1.00\pm0.02$ ;  $p<0.01$ ) without reducing mean carotid arterial pressure (in mm Hg: SHR  $=118\pm6$ ; SHR+SPIRO  $=124\pm4$ ; WKY  $=91\pm8$ ;  $p<0.01$ ). Carotid artery pressures were measured with a Statham P25 pressure transducer coupled to a fluid-filled catheter system as previously described (Norton et al 1996, Norton et al 1997). The transducer dome and catheter had a uniform amplitude-frequency response up to 10 Hz as previously reported (Norton et al 1996).

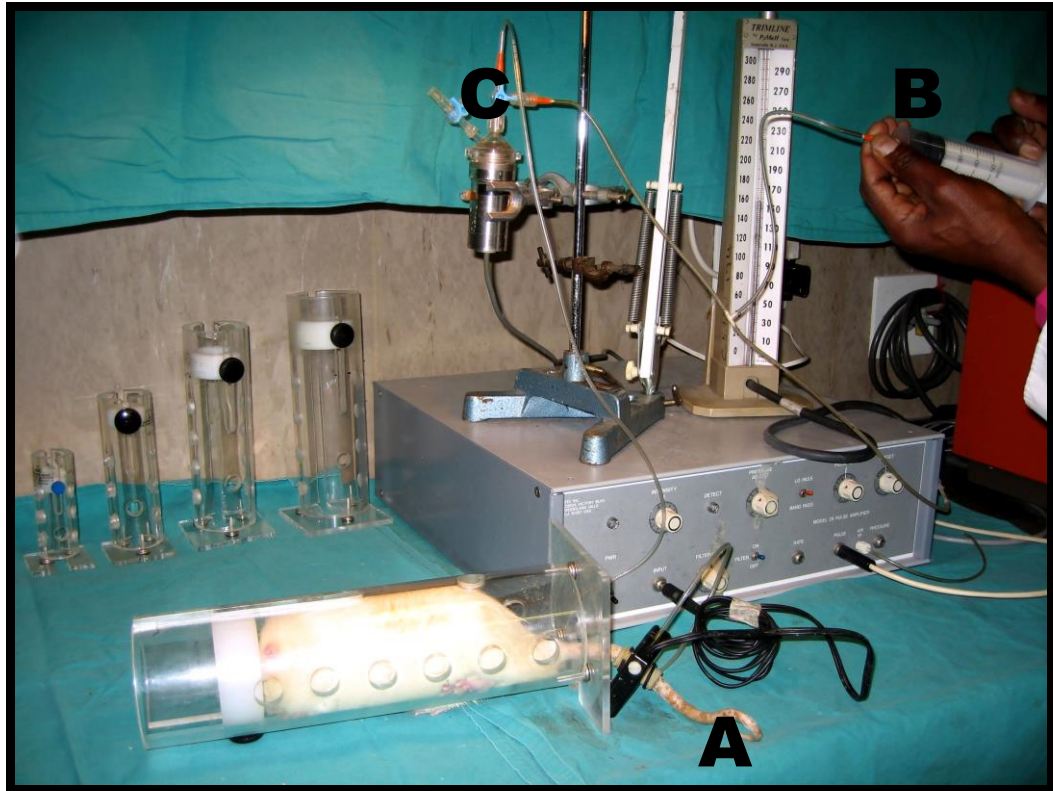
To ensure that SHR at the age studied represent the human condition with respect to myocardial norepinephrine (NE) release (Schlaich et al 2003), I evaluated myocardial NE release in 7 and 14 month old SHR and WKY rats as described in subsequent discussion.

As mineralocorticoid receptor blockade could modify LV cavity size through effects on myocardial NE release (Kasama et al 2003) or through alterations in blood volume mediated by a diuretic effect, I also studied SHR with  $\beta$ -AR-mediated LV dilatation to determine whether

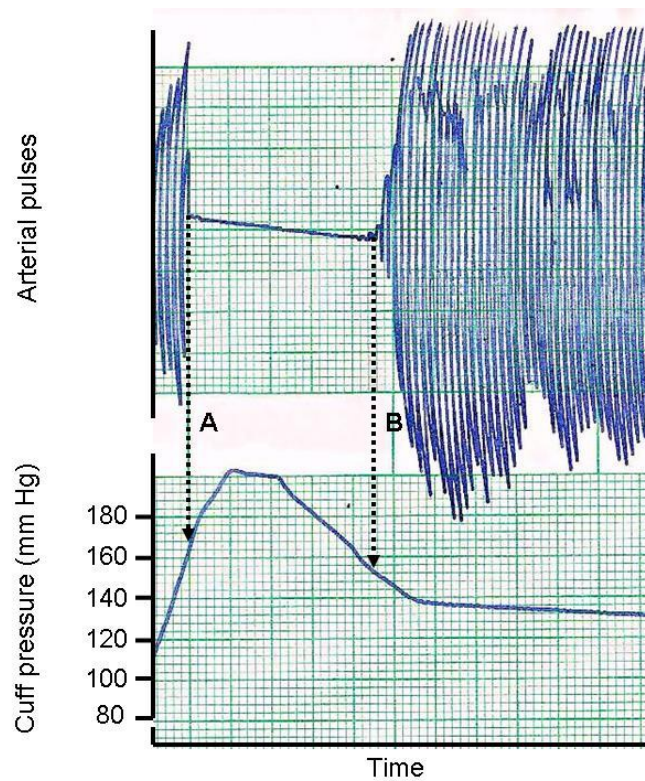
mineralocorticoid receptor blockade produced short-term actions on myocardial NE release or LV cavity dimensions. Thus, once clear evidence of LV dilatation was noted on echocardiography in SHR receiving ISO from 9 to 13.5 months (a 4.5 month period of ISO administration), rats were assigned to be given daily SPIRO or no treatment two weeks prior to termination of the study. At 14 months of age (5 months of ISO injections), LV cavity dimensions and myocardial NE release were measured.

### **2.2.2 Systolic blood pressures.**

Non-invasive systolic BP was assessed on three separate occasions in each group using a previously described tail-cuff technique (Norton et al 1993). Blood pressures were measured at least 24-hours after the last dose of ISO. Figures 2.1 and 2.2 illustrate the experimental protocol for the blood pressure measurements. In order to habituate the rats to the procedures they were placed in restrainers, the tail warmed and the tail cuff inflated every 15 minutes for an hour a day on five consecutive days prior to the first measurement. For BP measurements, the rat tails were pre-warmed until the tail artery pulse was detected by a photoelectric diode (Figure 2.2). A tail cuff coupled to a pressure transducer located proximal to the photoelectric diode was inflated until the tail artery pulse disappeared (Figure 2.2). Pressure in the cuff was slowly released until the



**Figure 2.1.** Approach used to measure tail artery systolic blood pressures in rats. Note the rat in restrainer on the left with the photoelectric diode and tail cuff around the tail (A). On the right air is being injected via a syringe (B) into an air filled system comprising a pressure transducer (C) coupled to both the tail cuff and a mercury manometer.

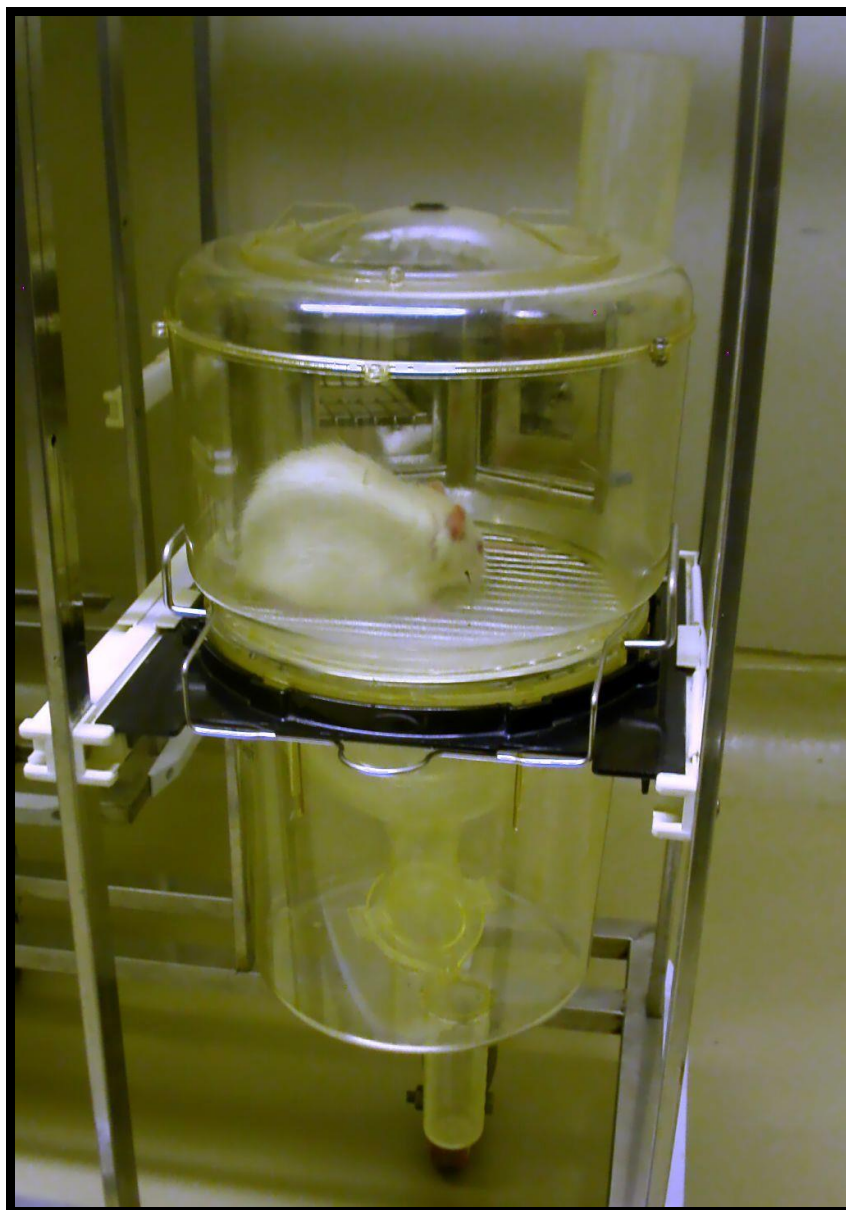


**Figure 2.2.** Illustrative traces of simultaneous tail pulse and tail cuff pressures recorded during systolic BP measurement in rats. The lower trace shows tail cuff pressure and the upper trace shows the pulse in the tail, just distal to the pressure cuff. At point A the pressure in the cuff is elevated sufficiently to obliterate the distal pulse. The pressure is then gradually reduced in the cuff until point B where the distal tail pulse reappears. The pressure at point B is thus the systolic BP.

pulse returned. The procedure was repeated a minimum of three times consecutively on the measurement day. The average pressure in the cuff at which the pulse returned was recorded as the systolic BP (Figure 2.2).

### **2.2.3 Urinary aldosterone excretion rates.**

In order to determine whether ISO increases mineralocorticoid production in SHR, urinary aldosterone excretion rates were determined after two months of daily ISO administration when normal LV dimensions were documented on echocardiography. Urine was collected using metabolic chambers (Figure 2.3) after a 72-hour adaptation period, over two consecutive 24-hour periods, in both untreated SHR (n=8) and SHR receiving ISO (n=8). Urine was acidified with glacial acetic acid ( $3 \mu\text{l} \cdot 30 \text{ ml}^{-1}$ ), stored at  $-20^\circ\text{C}$  for two weeks, and aldosterone concentration measured using a commercially available radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Aldosterone excretion rates were calculated as the mean of the product of urine volume excreted over 24-hours and urinary aldosterone concentrations. A pilot study established that measured values were consistent with values previously reported on (Takishita et al 1996). As urinary aldosterone excretion rates are not elevated in SHR (Herlitz et al 1983), comparisons between untreated SHR and WKY controls were not made.



**Figure 2.3.** Photograph of a rat in a metabolic chamber. Metabolic chambers designed to separate urine from faeces and food were used to collect urine samples from rats in order to measure 24-hour aldosterone excretion rates.

#### 2.2.4 Echocardiography.

Echocardiography on rats was performed at the end of the study at least 24-hours after the preceding dose of ISO using a 7.5 MHz transducer and a Hewlett Packard Sonos 2000 sector scanner (Figure 2.4) as previously described (Meyer et al 2001, Norton et al 2002) by a single assessor who was blinded to the study groups. Anaesthesia was induced by intraperitoneal injection of ketamine ( $75\text{mg.kg}^{-1}$ ) and xylazine ( $15\text{mg.kg}^{-1}$ ). Thereafter the rat's anterior thorax was shaved. The rat was placed prone in a container, positioning the shaved thorax over an inferiorly located window in the container. In order to obtain echocardiograph images the probe was positioned against the thorax through this window.

Using two-dimensional targeted M-mode echocardiography, a two dimensional short axis view of the left ventricle at the level of the papillary muscle was identified. The sector scanner was then used to identify the greatest width of the left ventricular short axis, and using this view, an M-mode image was obtained. An M-mode image was considered high quality if the both the anterior (septal) and the posterior wall endocardial surfaces were clearly visible throughout systole and diastole (Figure 2.5). Importantly, in the posterior wall measurements, care was taken to avoid using the endocardial surface of the papillary muscle as the endocardial border. In order to ensure quality control, measurements were made both on-line and off-line, using the computer assisted software and the images printed out of the M-mode traces respectively. Left ventricular posterior

wall thickness and internal dimensions were measured at the end of systole and diastole according to the leading edge method recommended by the American Society for Echocardiography (Sahn et al 1978) (Figure 2.5). Measurements of cardiac dimensions were taken and averaged over 3 consecutive beats.

Left ventricular anterior wall thickness was assumed to be equivalent to left ventricular posterior wall thickness for all calculations. This approach was adopted as left ventricular anterior wall thickness could not be measured due to an inability to obtain appropriate views of the right ventricular surface of the septal wall. The following equations were used for the determination of left ventricular endocardial ( $FS_{end}$ ) and midwall ( $FS_{mid}$ ) fractional shortening.

$$FS_{end} = \frac{100 \cdot (LVEDD - LVESD)}{LVEDD}$$

where:

LV EDD = left ventricular end diastolic internal diameter

LV ESD = left ventricular end systolic internal diameter

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

where:

LVED PWT = left ventricular end diastolic posterior wall thickness

LVES PWT = left ventricular end systolic posterior wall thickness

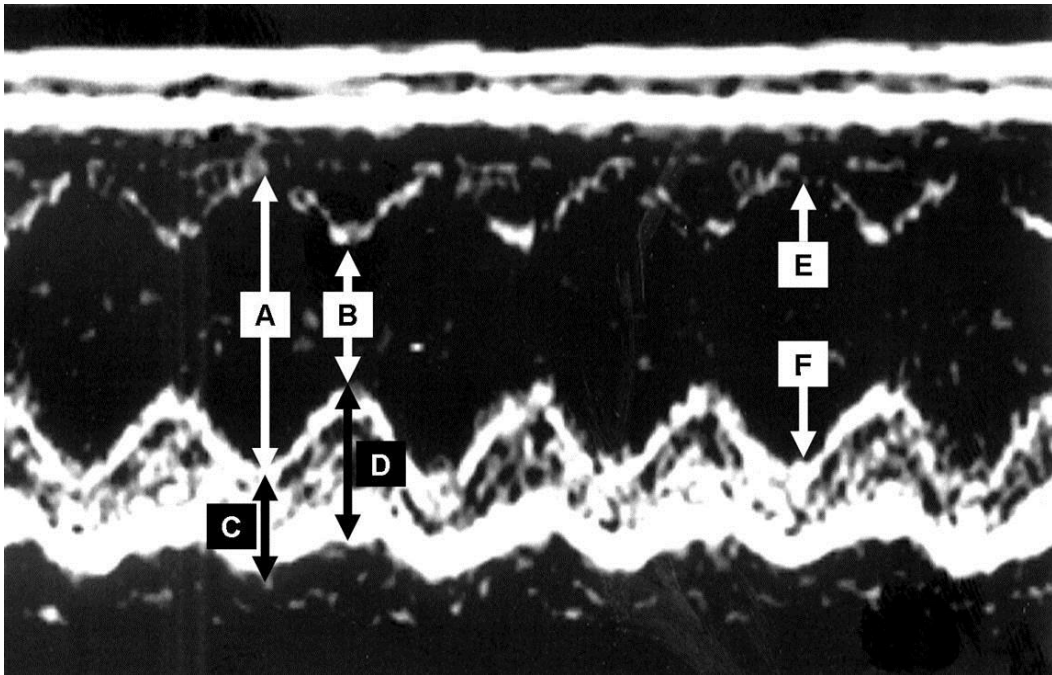
Left ventricular midwall and endocardial fractional shortening were utilized as indices of intrinsic myocardial and cardiac chamber systolic function respectively (Chung et al 1998, Norton et al 2002,). Since clear images of the endocardial surface for the entire circumference of the heart were not available throughout the cardiac cycle on two-dimensional imaging, left ventricular ejection fraction was not used as an index of left ventricular chamber systolic function. Diastolic function measured echocardiographically using transmitral early-to-late velocity ratios was not determined as the reproducibility of this measurement is poor in our hands. As with left ventricular ejection fraction, midwall and endocardial fractional shortening account for preload-dependent Frank-Starling effects. However neither is independent of heart rate or afterload.

#### **2.2.5 Left ventricular cavity size and geometry determined *in vivo* in open-chest rats.**

With left ventricular dilatation a reduced LV wall thickness-to-radius ratio ( $h/r$ ) may occur. Therefore  $h/r$  was determined *in vivo* over a range of filling pressures by measuring left ventricular short axis external diameters over a range of LV end diastolic pressure (LVEDP) values in anaesthetized, ventilated, open-chest rats using previously described and validated techniques (Norton et al 1997, Trifunovic et al 1995, Woodiwiss et al 1995). The rats were anaesthetized with ketamine ( $75\text{mg.kg}^{-1}$ ) and



**Figure 2.4.** In vivo measurements of left ventricular dimensions, structure and function were obtained on anaesthetised rats using a Hewlett Packard echocardiograph.



**Figure 2.5.** Typical two-dimensional targeted M-mode echocardiogram used to determine left ventricular dimensions. 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, E: Anterior (septal) endocardial surface, F: posterior endocardial surface.

xylazine ( $15\text{mg}\cdot\text{kg}^{-1}$ ). A saline-filled polyethylene PP25 catheter was inserted into the carotid artery for arterial BP measurements, to obtain blood samples and to infuse solutions. Positive pressure ventilation was initiated through a tracheostomy prior to performing a midline thoracotomy to expose the heart. Thereafter ventilation rates and volumes were adjusted to maintain a carotid arterial  $\text{PaO}_2$  of 90-110 mm Hg. Piezoelectric ultrasonic transducers placed across the short axis of the LV by means of a cradle designed and validated in our laboratory (Norton et al 1993, Norton et al 1997, Trifunovic et al 1995, Woodiwiss and Norton 1995) were used to measure the LV short axis external diameters throughout the cardiac cycle (Figure 2.6). Concurrent measurements of left ventricular end diastolic pressure (LVEDP) were recorded using a needle, inserted through the cardiac apex into the LV, coupled to a pressure transducer dome via a fluid-filled catheter (Figure 2.7). The amplitude-frequency response of the pressure transducer dome and catheter combination was uniform to 10 Hz (Norton et al 1997). Measurements of LVED external diameters were obtained over a wide range of measured LVEDP values. This was achieved by manipulating blood volume with Dextran, an iso-oncotic, isotonic solution, and via inferior vena cava occlusion. The iso-oncotic, isotonic solution was infused via the carotid artery catheter to increase LVEDP to values to between 10-15 mm Hg, during which time LVEDP and LVED diameters were measured and recorded (Figure 2.7). Thereafter inferior vena cava occlusion was used to reduce cardiac venous return to obtain LVED diameter measurements

over a range of LVEDP values of less than 5 mm Hg. Formulae previously described (Tsotetsi et al 2001) were employed to calculate LVED radius (r) and wall thickness (h) as follows:

$$\text{LVED } r = \sqrt[3]{\left(\frac{\text{LVED external diameter}}{2}\right)^3 - \frac{3 (\text{LVED wall volume})}{4 \pi}}$$

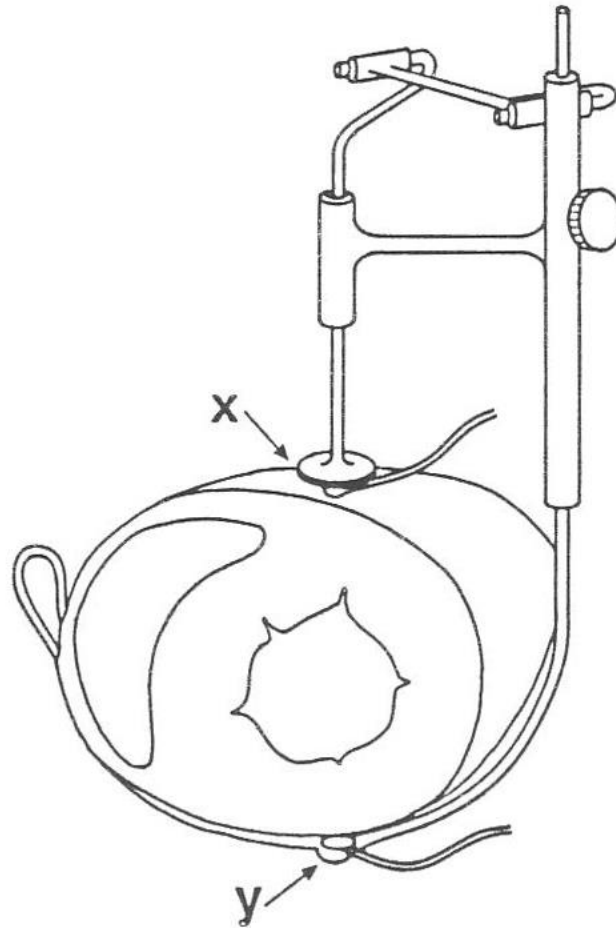
where:

$$\text{LV wall volume} = 0.943 \times \text{LV wet heart weight}$$

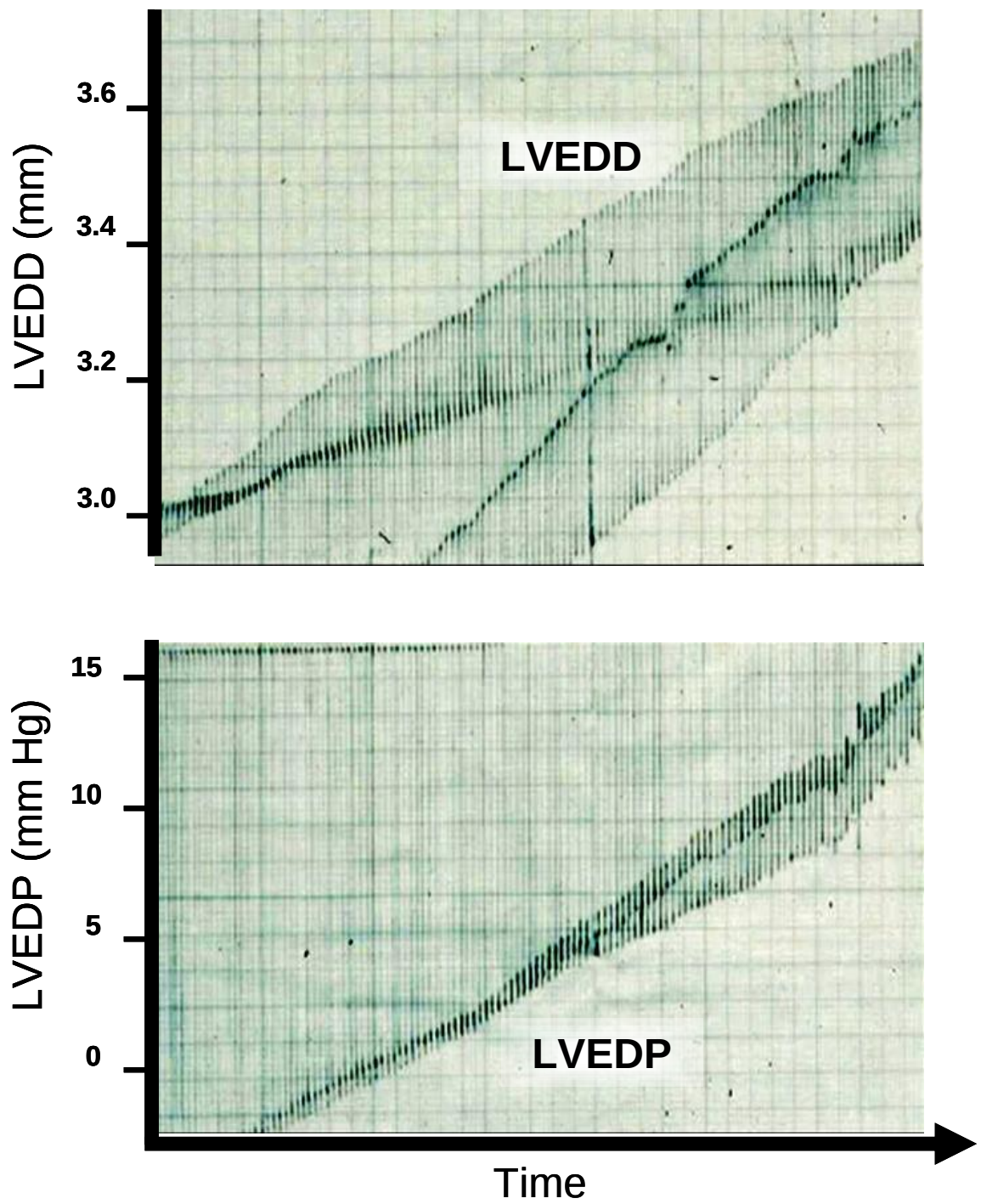
$$\text{LVED } h = \frac{(\text{LVED external diameter} - 2r)}{2}$$

$$\text{LVED } h/r = \frac{\text{LVED } h}{\text{LVED } r}$$

LVED h/r was used as an index of left ventricular relative wall thickness. In addition LV remodelling was further assessed from LVEDr<sub>0</sub>, the LVED r intercept of the LVEDP-LVED r relation. Importantly, an appropriate range of LVEDP and LVED external diameter measurements were obtained for all rats surviving the duration of the study.



**Figure 2.6.** Schematic illustration of the experimental approach used to determine left ventricular short axis dimensions. Measurements were made over a range of cardiac preloads. X: transmitting piezoelectric transducer and Y: receiving piezoelectric transducer.



**Figure 2.7.** Representative data recording obtained during infusion of an iso-oncotic, isotonic solution using the apparatus illustrated in Figure 2.6. LVED, left ventricular end diastolic; LVEDD, LVED diameter; LVEDP, LVED pressure.

### 2.2.6 Isolated perfused heart preparations.

Since with *in vivo* measurements one cannot fully account for variable cardiac loading conditions and heart rate and these may influence assessments of LV dimensions and systolic function, LV remodelling and function were also determined *ex vivo* under precisely controlled loading conditions and heart rate. Immediately following *in vivo* open-chest cardiac haemodynamic assessments, the rat hearts were excised and placed in an ice-cold physiological solution to maintain their viability prior to perfusion. Shortly thereafter the hearts were placed on a Langendorff apparatus and perfused, retrogradely via the aorta. A constant flow peristaltic pump was employed to drive fluid down the aorta toward the heart. As during perfusion the aortic pressure increases whilst the left ventricular pressure remains at 0 mm Hg, the aortic valve remains closed. Thus, during perfusion, fluid is only able to flow down the coronary arteries. Figures 2.8 and 2.9 illustrate the isolated, perfused organ system. The solution, which was used both to maintain the heart's viability prior to perfusion and for retrograde coronary perfusion consisted of (in mM) 118.0 NaCl; 4.7 KCl; 2.5 CaCl<sub>2</sub>; 25.0 NaHCO<sub>3</sub>; 1.2 KH<sub>2</sub>PO<sub>4</sub>; 1.2 MgSO<sub>4</sub> and 10.0 glucose. Before perfusion, the solution was carefully filtered through a size 0.45µm Millipore Durapore membrane filter, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> gas and adjusted to pH 7.4. For the duration of each study 95% O<sub>2</sub> and 5% CO<sub>2</sub> was constantly gassed through the perfusion solution.

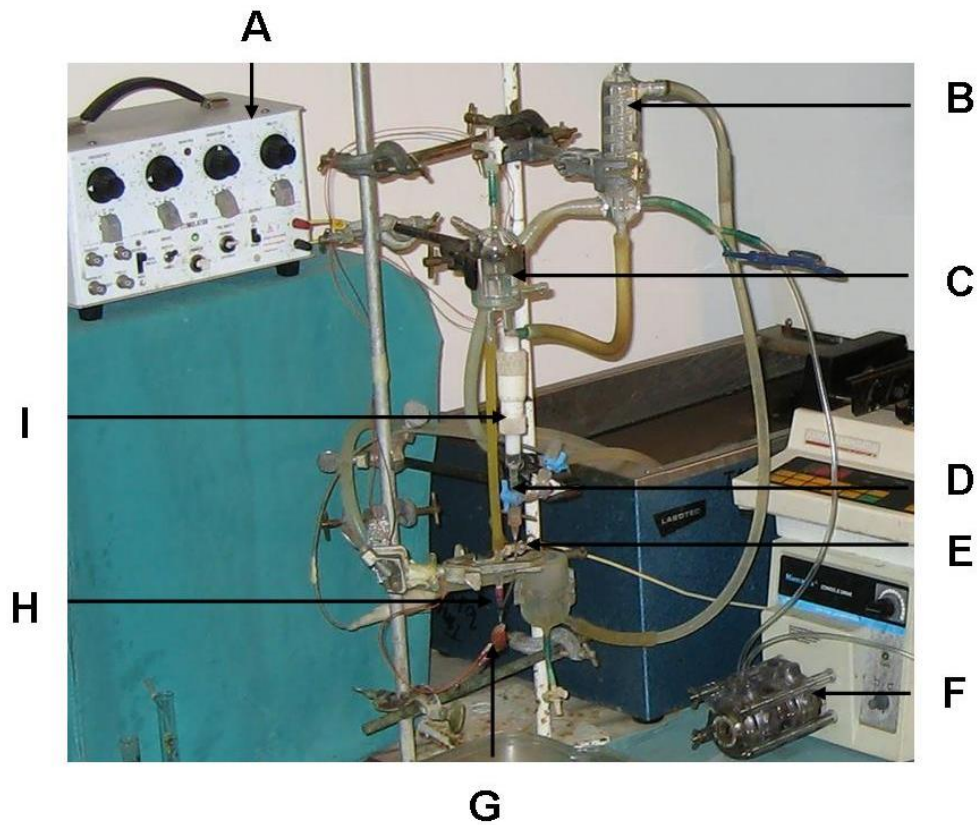
Retrograde perfusion of the hearts was maintained at a constant flow of  $12 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g wet heart weight}^{-1}$ . In order to accomplish this, the crude total heart weight (left ventricle, right ventricle, atria and large vessels) was measured prior to mounting the heart on the perfusion apparatus. Using timed coronary effluent samples as a measure of coronary flow rate, the speed of the peristaltic pump was then adjusted in order to achieve the appropriate coronary flow rate. A standard proportion of crude heart weight to left ventricular weight was assumed and then evaluated for each preparation at the end of the study. Using this approach myocardial viability is maintained via a constant coronary flow independent of left ventricular chamber function.

During perfusion, the perfusate supplying the heart passed through a tube surrounded by a water jacket through which warm water flows (Figure 2.8). The temperature of the perfusion solution was monitored via the coronary effluent, and was maintained at  $37^{\circ}\text{C}$  by controlling the temperature of the water flowing through the water jacket. A bubble trap was included in the apparatus proximal to the heart (Figure 2.8) in order to prevent air bubbles entering the coronary arteries.

After the heart was attached to the perfusion apparatus, platinum electrodes were applied to the right atrium and the apex of the heart. A Grass model SD9 (Astro Med Inc.) stimulator (Figure 2.8) was utilized to pace the hearts at  $360 \text{ beats} \cdot \text{min}^{-1}$ . Hearts were all paced at the same rate in order to prevent heart rate impacting on functional measurements. Conscious restrained rats *in vivo* typically have heart rates of

approximately 400-500 beats.min<sup>-1</sup>. A lower heart rate was employed in the present studies because the *ex vivo* studies are crystalloid rather than blood perfused preparations. Blood-perfused in contrast to crystalloid-perfused preparations contain a greater arterial oxygen content. Therefore physiological heart rates can only be employed in blood-perfused preparations. The heart rate selected was aimed to achieve maximal systolic function through mechanisms including the “Treppe” effect, without resulting in demand-induced myocardial ischemia. Diastolic left ventricular pressures characteristically increase in demand-induced ischemia, whereas contractile function decreases in low-flow ischemia (Umeda et al 2003). Studies have been performed in our laboratory to identify the pacing rate at which diastolic pressure begins to increase. In our hands, left ventricular diastolic pressures first begin to increase well above 360 beats.min<sup>-1</sup> in crystalloid perfused rat hearts. The pacing voltage used was approximately 10% higher than the threshold for spontaneous excitation (Norton et al 2002).

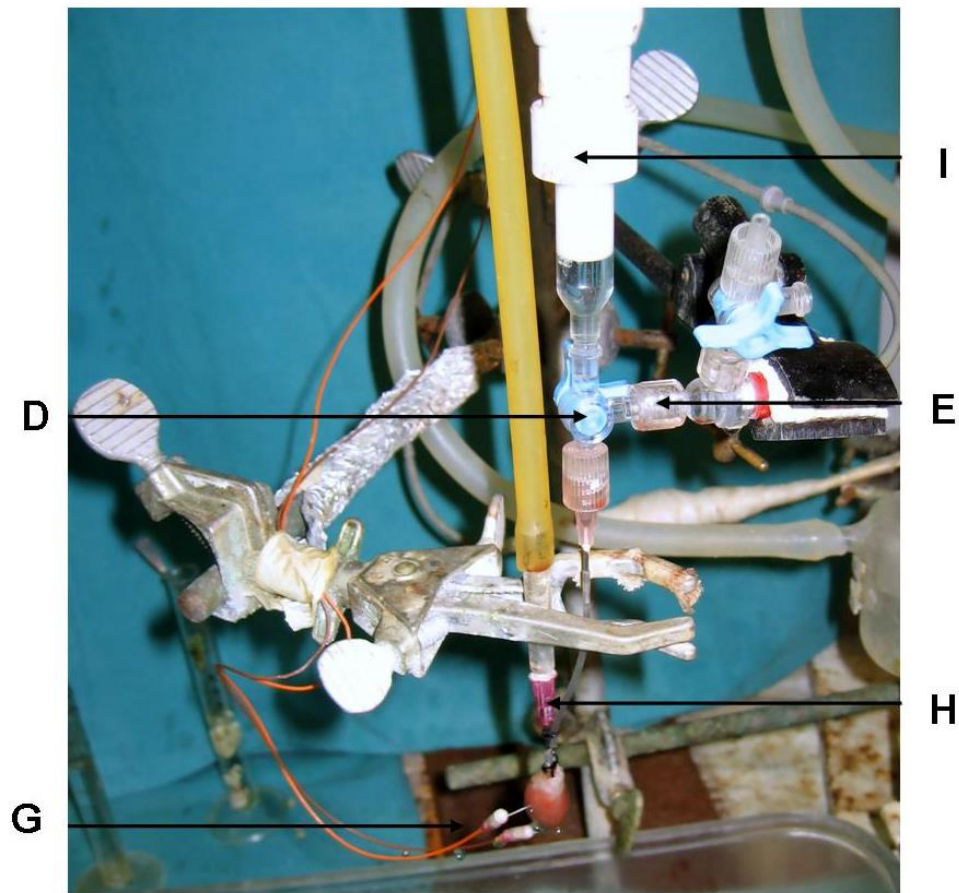
Left ventricular volumes and developed and diastolic pressures were measured using a latex balloon which was placed in the left ventricular chamber through the mitral valve (Figure 2.8 and 2.9). The balloon was coupled to both a pressure transducer and a micromanipulator via a three way tap and fluid-filled catheter system (Figure 2.9). The lumen of the latex balloon was large enough to accommodate volumes exceeding the maximal left ventricular volume expected of even a dilated rat ventricle.



**Figure 2.8.** Isolated, perfused heart apparatus used in *ex vivo* experiments to assess cardiac structure and function. A, pacing device; B, heated water jacket; C, bubble trap; D, three way tap open to E, H and I; E, pressure transducer; F, peristaltic pump; G, platinum electrodes attached to the isolated heart; H, fluid filled catheter attached to latex balloon which is inserted into the left ventricular lumen; I, micromanipulator.

The pressure-volume relationship of the balloon was such that the pressure in the balloon only started to increase at volumes considerably larger than the maximal left ventricular volume predicted to occur in a dilated rat ventricle. The balloon material and water in the balloon chamber were both included when assessing intraventricular volumes. The volume displaced by the balloon itself was determined as follows. First, the relationship between latex mass to displacement was determined by immersing a known large mass of latex in water and measuring the displacement thereof. Based on the weight of the balloon and the ratio of mass to displacement, the volume of the balloon was calculated. The balloon was completely emptied and then inserted into the left ventricular cavity. In order to empty the balloon the micromanipulator was removed, the three way tap opened to atmosphere, the balloon was compressed forcing water through the catheter and out of the open tap, and the three way tap was then closed ensuring that no air bubbles entered the catheter system and balloon (Figure 2.9).

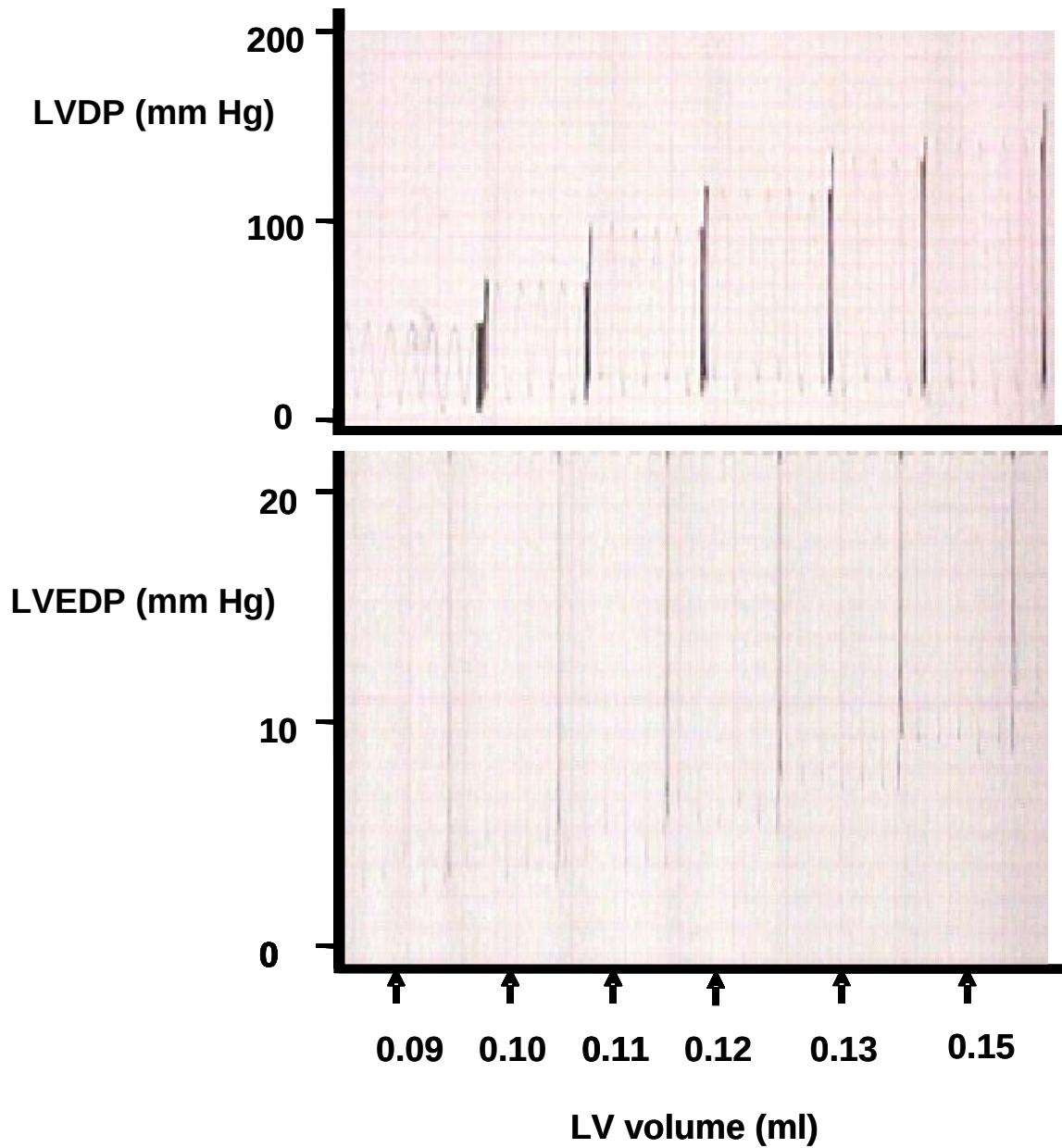
The Vernier scale on the micromanipulator enabled 0.005 to 0.01 ml increments in volume to be accurately injected into the balloon (Figure 2.9). Calibration of the micromanipulator was regularly performed by weighing 0.005 to 0.01 ml increments of fluid. Left ventricular pressures were determined at as many small increments in LV chamber volume as were possible. Developed and diastolic left ventricular pressures were recorded on a Hellige recorder (Figure 2.10). Developed left ventricular



**Figure 2.9.** Enlargement of a portion of the isolated perfused heart apparatus depicted in figure 2.8. Clearly visible here are D, the three way tap; E, the pressure transducer; G, platinum electrodes attached to the isolated heart; H, the fluid filled catheter connected to the balloon in the left ventricular lumen and I, the micromanipulator.

pressures were recorded on a different channel to diastolic left ventricular pressures which, due to the low pressure values, required an amplified calibration scale to ensure accurate recordings (Figure 2.10). A mercury manometer was used to calibrate the channel used to record left ventricular developed pressures. The left ventricular diastolic pressure measurement channel was calibrated using a specifically designed water-filled U-tube system (Norton et al 1996). The left ventricular developed and diastolic pressure recording channels were calibrated before and after each heart preparation. In order to assess adrenergic-inotropic reserve, left ventricular pressures were determined over a range of filling volumes initially in the absence of an inotropic stimulus, and then following exposure of the heart to perfusate containing  $10^{-7}$  M ISO. The minimum measured diastolic ventricular pressures in the isovolumic, isolated, perfused heart preparations used in this thesis were assumed to correspond with left ventricular end diastolic pressures in filling and emptying hearts.

Left ventricular diastolic pressure-volume relations were constructed to assess left ventricular dilatation (remodelling). Left ventricular dilatation was assessed statistically by comparing volume intercepts of the left ventricular diastolic pressure-volume relationships, i.e. LV  $V_0$  (left ventricular volume at a diastolic left ventricular pressure of 0 mm Hg) (Badenhorst et al 2003a, Badenhorst et al 2003b, Norton et al 2002, Woodiwiss et al 2001).



**Figure 2.10.** Typical recording of left ventricular developed (LVD) and diastolic (LVEDP) pressure obtained in isolated perfused heart preparations over a range of filling volumes.

The slope of the linear portion of the left ventricular developed pressure-volume relationship was used to quantify systolic chamber function. In an isovolumic preparation the linear portion of the developed systolic pressure-volume relationship is considered to be equivalent to the linear portion of the end systolic pressure-volume relationship in a heart that fills and ejects. This is because in an isovolumic preparation, peak systolic pressures are the same as end systole pressures. The slope of the left ventricular pressure-volume relationship (end systolic elastance;  $E_{es}$ ) is both preload and afterload-independent (Sagawa et al 1988). To identify LV developed pressure data points that could be included in the analysis of  $E_{es}$ , linear regression analysis was performed for individual rats. If the  $r^2$  value for the developed pressure-volume relationship was 0.95 or more with each point included, then these data were incorporated in the analysis. If the inclusion of subsequent data points reduced the  $r^2$  value, then these points were excluded. Utilizing this approach I was able to include the left ventricular developed pressures measured over the first 5 incremental LV volumes of 0.01 mls, to determine  $E_{es}$  in all rats.

Myocardial systolic elastance ( $E_n$ ), which is the slope of the systolic developed stress-strain relationship, was used to quantify intrinsic systolic myocardial function (Norton et al 2002, Badenhorst et al 2003b, Veliotes et al 2005). The impact of changes in left ventricular chamber geometry on systolic function is eliminated by converting pressure and volume values into stress and strain data (Weber et al 1988). Myocardial  $E_n$  is therefore the myocardial equivalent of left ventricular  $E_{es}$  and thus is also preload

and afterload-independent. Substantially more than five data points were able to be included in the analysis of  $E_n$  because the  $E_n$  relationship is linear. Additionally,  $E_n$  was also recalculated including only the first five left ventricular developed pressure and volume data to ensure that calculations of  $E_n$  were representative of data obtained for  $E_{es}$ . This sensitivity analysis provided essentially the same outcomes, which is not surprising as  $E_n$  is linear. Assuming a thick-walled, spherical left ventricle geometry, the developed stress and strain values were calculated from formulae previously described (Badenhorst et al 2003b, Norton et al 2002, Weber et al 1988).

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

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

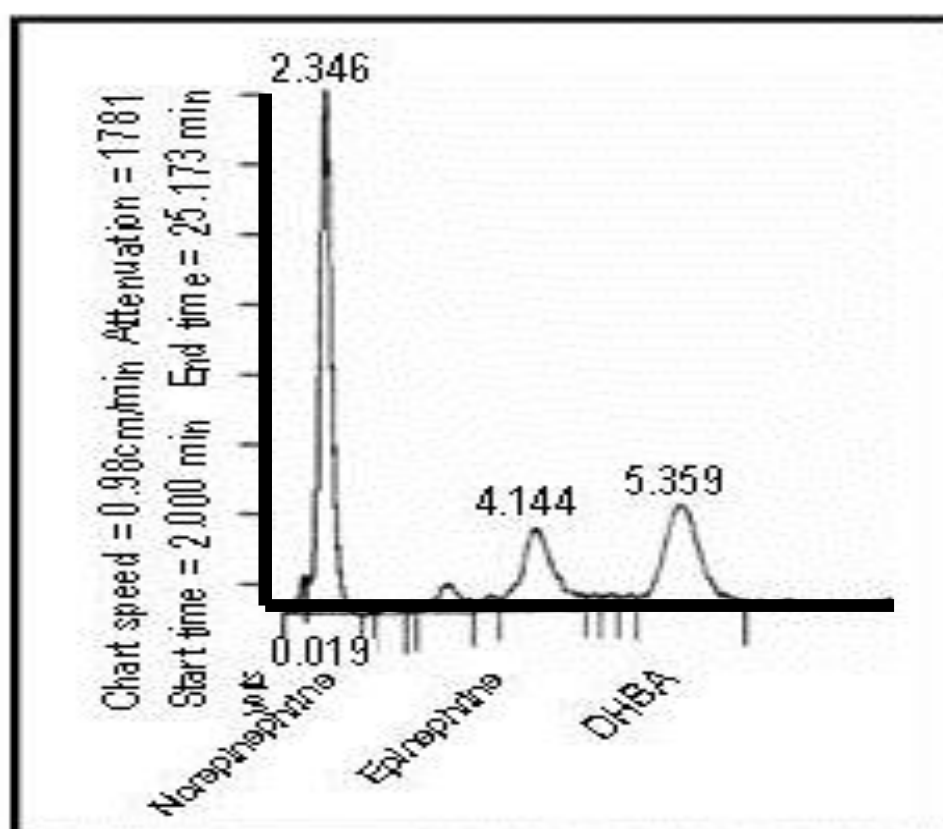
Where LVV is left ventricular volume and  $\text{LV } V_0$  is the volume intercept of the LV developed pressure-volume relationship (LVV when LV developed pressure = 0 mm Hg).

### 2.2.7 Myocardial norepinephrine.

In order to assess the impact of ISO and SPIRO on myocardial catecholamine release and to determine whether LVH is associated with excessive myocardial catecholamine release in SHR, coronary effluent NE

concentrations were determined from the isolated, perfused heart preparations prior to assessing cardiac function. While the hearts were perfused at a constant coronary flow rate of  $12 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}$  wet heart weight<sup>-1</sup>, coronary effluent samples were collected in pre-chilled containers over a minute. A pilot study conducted in SHR determined that NE release decreases when measured at increasing LV filling volumes from 0.20 ml ( $0.34 \pm 0.03 \text{ nmol} \cdot \text{ml}^{-1}$ ) to 0.23 ml ( $0.26 \pm 0.03 \text{ nmol} \cdot \text{ml}^{-1}$ ,  $p < 0.01$  versus 0.20 ml) to 0.27 ml ( $0.22 \pm 0.02 \text{ nmol} \cdot \text{ml}^{-1}$ ,  $p < 0.001$  versus 0.20 ml) (the range of filling volumes at which most hearts exhibited a wide range of developed pressures). Therefore measurements of NE release were all performed at filling volumes of 0.23 ml.

The coronary effluent was collected in a solution containing 0.01M  $\text{Na}_2\text{EDTA}$  and 0.025%  $\text{HClO}_4$  to prevent NE degradation. Norepinephrine was immediately extracted from 1 ml of coronary effluent using alumina (Sigma) adsorption with a Tris buffer at pH 8.6, eluted with 0.1 M  $\text{HClO}_4$  (Ganhao et al 1991), stored at  $-70^\circ\text{C}$  and NE concentrations determined using reversed phase, ion-exchange high performance liquid chromatography with electrochemical detection (Ganhao et al 1991). The equipment used and a typical data recording are shown in figure 2.11. The internal standard for catecholamine detection was dihydroxybutyric acid (DHBA). Standard curves for NE and DHBA were created on every day that measurements were made. The inter-assay variance for both NE (3.2%) and DHBA (1.9%) was low. Myocardial NE release was expressed



**Figure 2.11.** High performance liquid chromatography system used to assess the concentration of coronary effluent norepinephrine (upper panel). Typical example of data obtained using the above system while measuring various monoamine concentrations (lower panel).

as the concentration of NE in the effluent because all hearts were perfused at the same flow rate per gram of tissue.

#### **2.2.8 Data analysis.**

A one- or two-way (where necessary) ANOVA followed by a Tukey *post hoc* test was used to assess differences between groups. Lines of best fit for the cardiac function and other relations were determined by regression analysis. Unless otherwise specified all values in the text are represented as mean  $\pm$  SEM.

### **2.3 Results.**

#### **2.3.1 Blood pressure.**

Neither chronic ISO nor SPIRO administration significantly modified the increased systolic BP noted in SHR (e.g. at 18 months of age in mm Hg: SHR untreated =  $182 \pm 8$ , SHR+ISO =  $178 \pm 4$ , SHR+ISO+SPIRO =  $168 \pm 8$ , SHR+SPIRO =  $164 \pm 12$ , WKY =  $136 \pm 6$ ,  $p < 0.001$  for WKY versus other groups).

### **2.3.2 Heart weight.**

Spontaneously hypertensive rats had LVH as evidenced by increases in heart weight, LV weight and LV weight-to-body weight ratios. Chronic ISO administration further enhanced the increased LV weight noted in SHR (Table 2.1). In contrast, SPIRO decreased LV weight in SHR and prevented ISO-induced augmentation of LVH (Table 2.1).

### **2.3.3 Urinary aldosterone excretion rates.**

Isoproterenol administration, over two months, increased 24-hour urinary aldosterone excretion rates in SHR (in pg.gram of body weight<sup>-1</sup>.day<sup>-1</sup>: SHR = 49±2, SHR+ISO = 66±6, p<0.03).

### **2.3.4 Chamber dimensions in intact rats.**

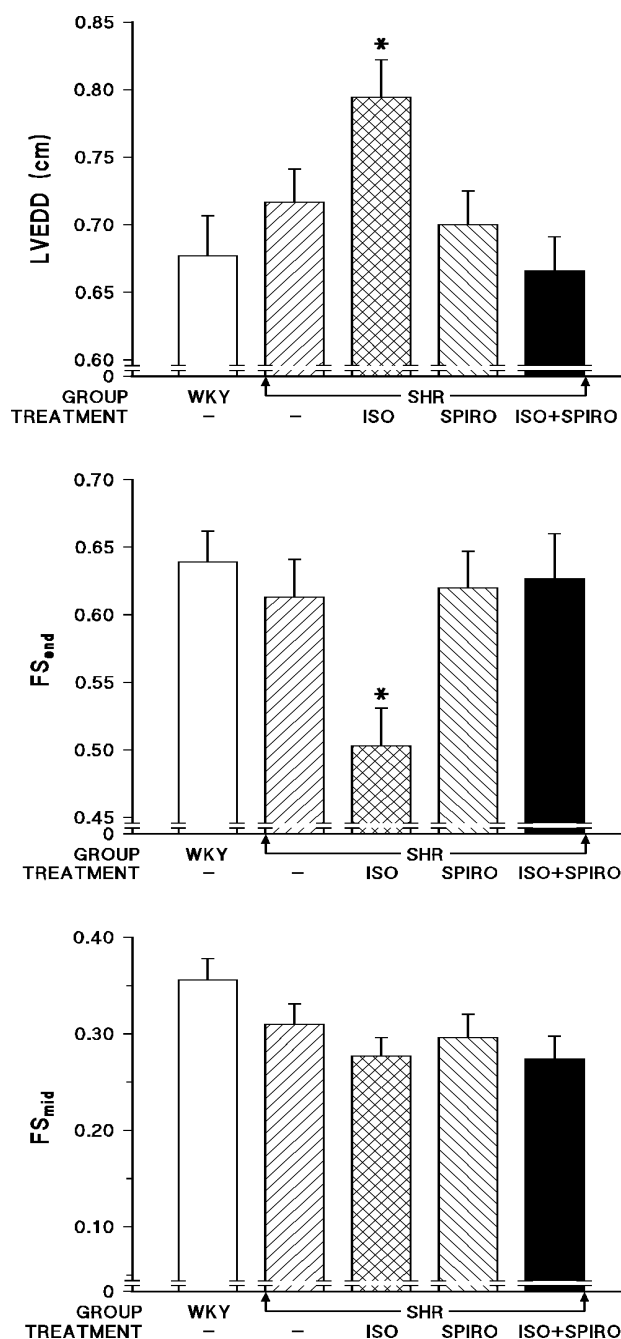
Chronic ISO administration increased LV end diastolic (LVEDD) and systolic diameters in SHR, an effect that was prevented by SPIRO (Figure 2.12 and Table 2.1). Although ISO augmented LV weight (Table 2.1) ISO failed to significantly modify LV wall thickness as a consequence of increases in LVEDD, a change that thins the LV wall (Table 2.1). In contrast to the ability of SPIRO to prevent  $\beta$ -AR-induced LV dilatation

**Table 2.1.** Effect of chronic administration of isoproterenol (ISO) and spironolactone (SPIRO) on left ventricular (LV) weight and dimensions in spontaneously hypertensive rats (SHR).

|                                               | WKY       | SHR         | SHR+ISO       | SHR+SPIRO   | SHR+ISO+SPIRO |
|-----------------------------------------------|-----------|-------------|---------------|-------------|---------------|
|                                               | n = 9     | n = 9       | n = 9         | n = 6       | n = 10        |
| Heart weight (g)                              | 1.45±0.06 | 1.64±0.05   | 2.11±0.14***† | 1.64±0.06   | 1.78±0.06     |
| LV weight (g)                                 | 1.08±0.04 | 1.32±0.06** | 1.56±0.09**†  | 1.18±0.05   | 1.32±0.04**   |
| Body weight (BW) (g)                          | 438±12    | 359±6**     | 353±10**      | 352±12**    | 355±7**       |
| LV/BW x 10 <sup>4</sup>                       | 2.45±0.14 | 3.66±0.14** | 4.41±0.34**†  | 3.35±0.10** | 3.65±0.11**   |
| LV end systolic diameter (cm)                 | 0.24±0.03 | 0.26±0.03   | 0.39±0.02**†  | 0.24±0.05   | 0.25±0.03     |
| LV posterior wall thickness <sup>#</sup> (cm) | 0.19±0.01 | 0.28±0.01** | 0.21±0.02†    | 0.24±0.03*  | 0.29±0.03**   |

<sup>#</sup> At end diastole; WKY, Wistar Kyoto control; LV V<sub>0</sub>, volume intercepts of LV diastolic pressure-volume relations; \*p<0.05,

\*\*p<0.01, \*\*\*p<0.001 versus WKY group, † p<0.01 versus other SHR groups.



**Figure 2.12.** Effect of chronic administration of spironolactone (SPIRO) on isoproterenol (ISO)-induced changes in left ventricular end diastolic diameter (LVEDD), LV pump function (endocardial fractional shortening,  $FS_{end}$ ), and LV myocardial function (midwall fractional shortening,  $FS_{mid}$ ) in spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto controls. \*  $p < 0.01$  versus other groups. See table 2.1 for sample sizes.

when initiated at the beginning of the study, two weeks of SPIRO administration, once LVEDD had increased, failed to attenuate these changes (in cm: SHR [n=18] =  $0.70 \pm 0.02$ , SHR+ISO [n=10] =  $0.82 \pm 0.02$ , SHR+ISO+SPIRO [n=9] =  $0.79 \pm 0.02$ ,  $p < 0.05$  versus untreated SHR).

### 2.3.5 Chamber remodelling.

As assessed in isolated, perfused heart preparations, ISO administered to SHR for 4.5 months resulted in a right shift in LV diastolic P-V relations and an increased LV  $V_0$  (Figure 2.13). These effects were prevented by SPIRO (Figure 2.13). Similarly, as assessed *in vivo*, ISO administered to SHR resulted in increases in LVED r noted over a range of LVEDPs ( $p < 0.01$ ), an effect abolished by the co-administration of SPIRO (Figure 2.14).

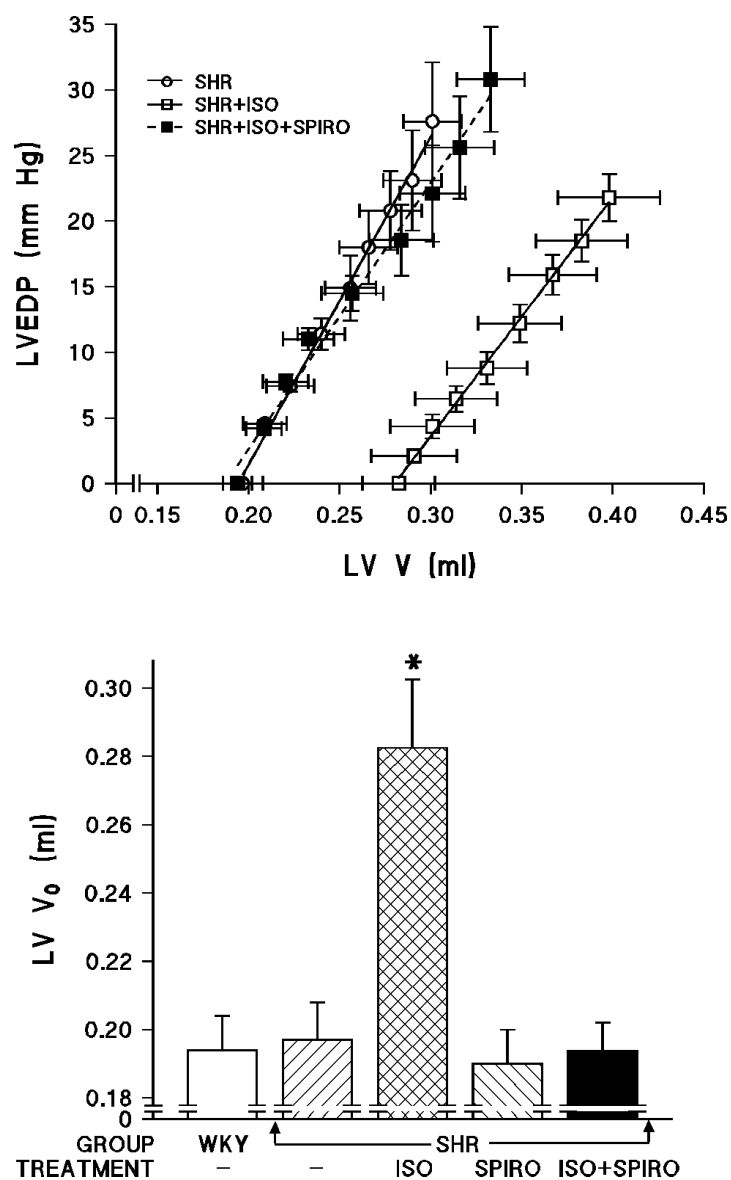
Consistent with concentric LVH, untreated SHR at 18.5 months of age had an increased LVED h/r (relative wall thicknesses) as compared to age-matched WKY controls (LVED h/r at 2 mm Hg shown in the lower panel of Figure 2.15). As a consequence of LV dilatation, ISO administered to SHR resulted in relative wall thinning, with marked decreases in LVED h/r noted over a range of LVEDP values (Figure 2.15) despite further increases in LV weight (Table 2.1). SPIRO administration abolished ISO-induced decreases in LVED h/r (Figure 2.15).

### 2.3.6 Systolic function.

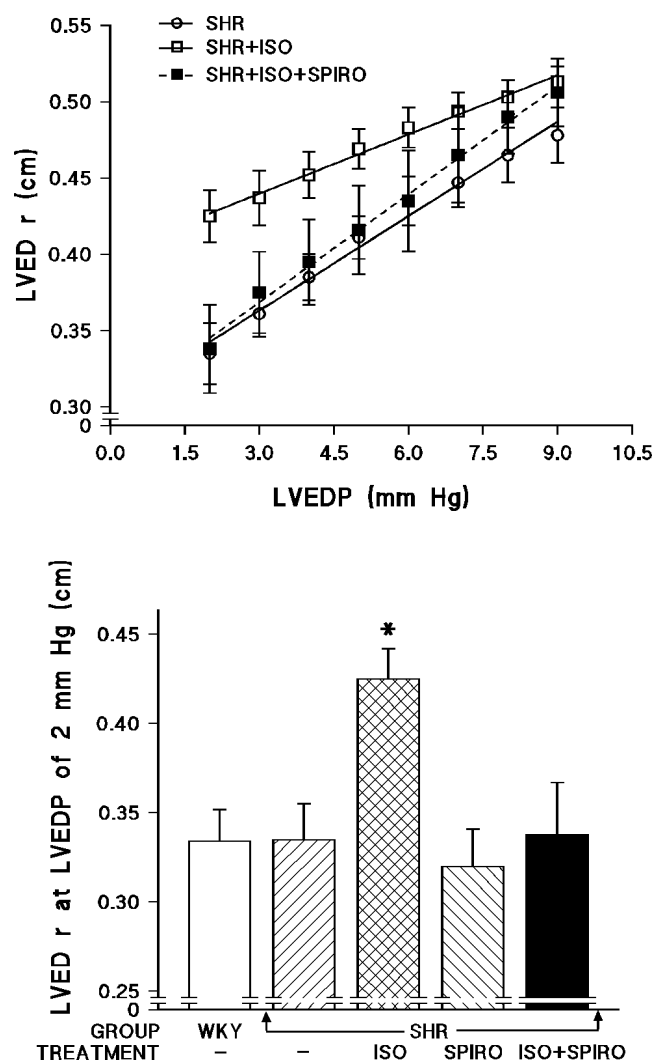
Chronic ISO administered to SHR decreased systolic chamber function as assessed both *in vivo* (Figure 2.12, LV FS<sub>end</sub>) and *ex vivo* (Figure 2.16, the slope [Ees] of the systolic P-V relations in the absence of an inotropic stimulus), an effect prevented by the co-administration of SPIRO (Figures 2.12 and 2.16). In contrast, neither ISO nor SPIRO modified intrinsic myocardial systolic function as assessed either *in vivo* (Figure 2.12; LV FS<sub>mid</sub>) or *ex vivo* (Figure 2.17 for systolic stress-strain relations and the slope of these relations [En] in the absence of an inotropic stimulus). Similar data were obtained when systolic chamber and myocardial function were assessed in the presence of  $10^{-7}$  M ISO (data not shown).

### 2.3.7 Myocardial norepinephrine.

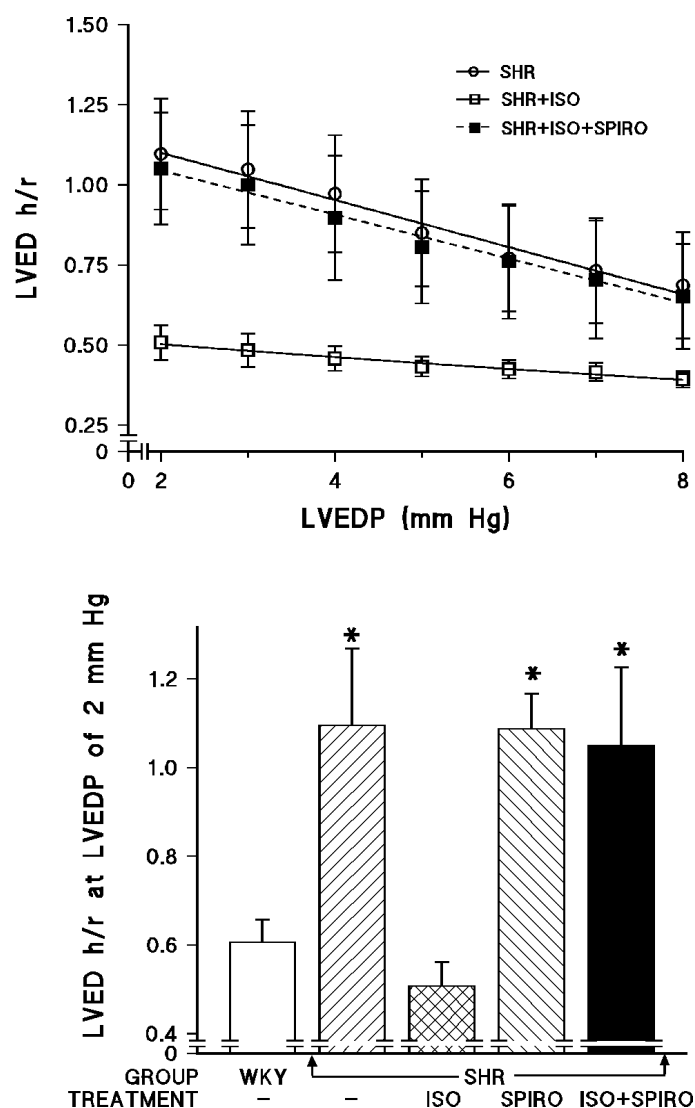
Myocardial NE release was significantly elevated in spontaneously hypertensive rats at 14 months of age (in nmol.ml<sup>-1</sup>: SHR at 14 months of age [n=12] =  $0.27 \pm 0.04$  nmols.ml<sup>-1</sup>, SHR at 7 months of age [n=16] =  $0.09 \pm 0.02$  nmols.ml<sup>-1</sup>, WKY [n=6] =  $0.08 \pm 0.02$  nmols.ml<sup>-1</sup>,  $p < 0.002$ ). Neither ISO (SHR+ISO at 14 months of age [n=10] =  $0.23 \pm 0.06$  nmol.ml<sup>-1</sup>) nor SPIRO (SHR+ISO+SPIRO [n=8] =  $0.32 \pm 0.07$  nmol.ml<sup>-1</sup>) given for two weeks prior to the termination of the study modified myocardial NE release.



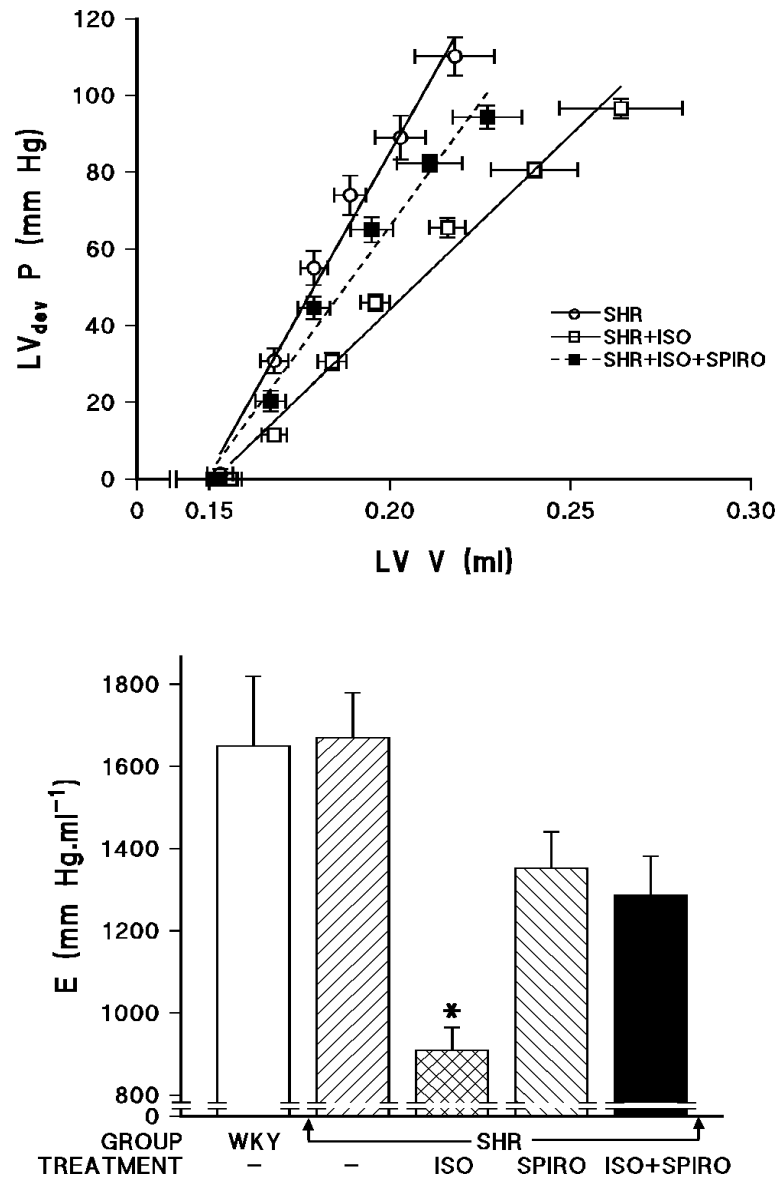
**Figure 2.13.** Effect of chronic administration of spironolactone (SPIRO) on isoproterenol (ISO)-induced changes in left ventricular end diastolic pressure-LV volume (LVEDP-LV V) relations (upper panel) and LV V at an LVEDP of 0 mm Hg (LV V<sub>0</sub>) (lower panel) in spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto controls. \*  $p < 0.01$  versus other groups. See table 2.1 for sample sizes.



**Figure 2.14.** Effect of chronic administration of spironolactone (SPIRO) on isoproterenol (ISO)-induced changes in left ventricular end diastolic pressure-internal radius (LVEDP-LVEDr) relations (upper panel) and LVEDr at an LVEDP of 2 mm Hg (lower panel) in spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto controls. \*  $p < 0.01$  versus other groups. LVEDr is greater in the SHR+ISO group compared to the other groups from 2-to-5 mm Hg ( $p < 0.05$ ). See table 2.1 for sample sizes.

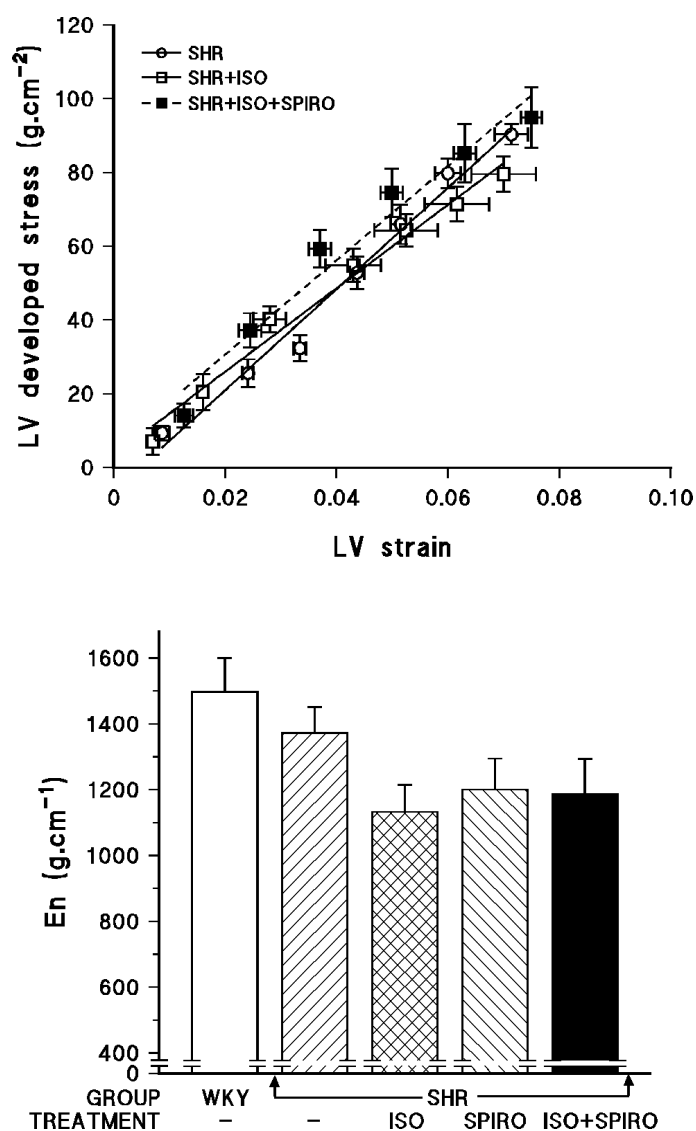


**Figure 2.15.** Effect of chronic administration of spironolactone (SPIRO) on isoproterenol (ISO)-induced changes in left ventricular end diastolic pressure-relative wall thickness (LVEDP-LVED h/r, LVED wall thickness-to-radius ratio) relations (upper panel) and LVED h/r at an LVEDP of 2 mm Hg (lower panel) in spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto controls. \*  $p < 0.01$  versus WKY and SHR ISO groups. LVED h/r is less in the SHR+ISO and the WKY groups compared to the other groups from 2-to-8 mm Hg ( $p < 0.05$ ). See table 2.1 for sample sizes.



**Figure 2.16.** Effect of chronic administration of spironolactone (SPIRO) on isoproterenol (ISO)-induced changes on left ventricular developed pressure-LV volume (LV<sub>dev</sub>P-LV V) relations (upper panel), and the mean slope of the relations (E<sub>es</sub>, systolic chamber function) (lower panel) in spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto controls.

\* p<0.01 versus other groups. See table 2.1 for sample sizes.



**Figure 2.17.** Effect of chronic administration of spironolactone (SPIRO) on isoproterenol (ISO)-induced changes in left ventricular developed stress-LV strain relations (upper panel), and the mean slope of the relations ( $E_n$ , systolic myocardial function) (lower panel) in spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto controls. There are no differences between groups. See table 2.1 for sample sizes.

## 2.4 Discussion.

The main finding of the present study was that the mineralocorticoid receptor antagonist, spironolactone, prevented the progression from compensated hypertensive LVH to LV dilatation (increases in LV end diastolic diameter as well as right shifts in LV diastolic pressure-dimension relations with wall thinning) and LV systolic chamber dysfunction induced in SHR by chronic  $\beta$ -adrenoreceptor activation. Neither an impact on BP, volume preload (as determined from dimension measurements made *in vivo* following short-term spironolactone administration), intrinsic myocardial systolic function (determined *in vivo* and *ex vivo*) nor NE release could explain the advantageous actions of spironolactone on LV dilatation and LV systolic chamber function in SHR.

The present study provides the first direct evidence to suggest that a fundamental mechanism responsible for the ability of chronic  $\beta$ -AR activation to promote the transition from compensated LVH to cardiac dilatation and systolic chamber dysfunction is through mineralocorticoid receptor stimulation. The results of the present study are consistent with the favourable impact of mineralocorticoid receptor antagonists or receptor inactivation on cardiac chamber dimensions in both human conditions and animal models of cardiac disease (Cicoira et al 2002, Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Hayashi et al 2003, Kasama et al 2003, Kuster et al 2005, Lothar et al 2011, Modena et al 2001, Mulder et al 2008, Suzuki et al 2002, Tsutamoto et al 2001, Wang et

al 2004). In this regard, however, these prior studies did not assess the impact of mineralocorticoid receptor blockade on LV diastolic P-V relations or on LV wall thickness-to-radius ratios assessed at controlled filling pressures (Cicoira et al 2002, Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Hayashi et al 2003, Kasama et al 2003, Kuster et al 2005, Lothar et al 2011, Modena et al 2001, Suzuki et al 2002, Tsutamoto et al 2001, Wang et al 2004). Consequently, it is difficult to identify from these previous studies whether mineralocorticoid receptor blockade mediated reductions in LV cavity dimensions through decreases in blood volume alone, or through beneficial effects on chamber remodelling. Importantly, in the present study, spironolactone not only reduced cavity dimensions as assessed *in vivo*, but prevented  $\beta$ -adrenergic-induced right shifts in the LV diastolic P-V relationship and an increase in the volume intercept of this relationship, as well as prevented the accompanying  $\beta$ -adrenergic-induced decrease in LV relative wall thickness assessed at controlled filling pressures. These changes in diastolic P-V relations and relative wall thickness characterize adverse chamber remodelling.

In the present study, chronic administration of a  $\beta$ -adrenoreceptor agonist to SHR induced LV systolic chamber dysfunction as determined from an afterload and heart rate-dependent, but preload independent assessment of systolic function *in vivo* (endocardial fractional shortening), as well as an afterload, preload and heart rate-independent assessment of systolic function *ex vivo* (systolic elastance-Ees) (Sagawa et al 1988). In

contrast, spironolactone prevented these adverse effects induced by chronic  $\beta$ -adrenoreceptor activation on LV systolic chamber dysfunction. Importantly, as previously demonstrated (Badenhorst et al 2003b), chronic administration of a  $\beta$ -adrenoreceptor agonist to SHR did not result in a decrease in myocardial systolic function as assessed from an afterload and heart rate-dependent, but preload independent assessment of myocardial systolic function determined *in vivo* (midwall fractional shortening), as well as an afterload, preload and heart rate-independent assessment of intrinsic myocardial systolic function assessed *ex vivo* (systolic myocardial elastance-En) (Sagawa et al 1988). Moreover, spironolactone had no effect on midwall fractional shortening or En. Consequently, in the present study the reduction in LV systolic chamber function induced by chronic  $\beta$ -adrenoreceptor activation in SHR could not be attributed to decreases in myocardial function. Furthermore, the beneficial actions of spironolactone on LV systolic chamber function in the present study could not be attributed to improvements in myocardial function. Rather, the actions of chronic  $\beta$ -adrenoreceptor activation and spironolactone in the present study on LV systolic chamber function could only be attributed to effects on structural remodelling.

The findings in the present study indicating that alterations in LV systolic chamber function are attributed to adverse chamber remodelling rather than to intrinsic myocardial systolic dysfunction, support the concept originally proposed by Cohn (1995) and subsequently substantiated by data obtained in human (Vasan et al 1997) and animal (Badenhorst et al

2003b, Booyesen et al 2012, Norton et al 2002) studies, that LV systolic chamber dysfunction can occur mainly as a consequence of cardiac dilatation rather myocardial contractile disturbances. The findings in the present study also provide the first direct evidence to indicate that mineralocorticoid receptor antagonists produce a primary effect on adverse cardiac chamber remodelling, which subsequently results in an improved LV systolic chamber function, rather than a beneficial effect on myocardial contractility which then reduces chamber dimensions. Unfortunately, previous studies assessing the impact of mineralocorticoid receptor antagonists or inactivation on cardiac function have not employed measurement approaches that allowed the authors to identify whether mineralocorticoid receptor blockade or inactivation primarily targets adverse cardiac chamber remodelling or whether the beneficial effects on LV systolic chamber function that were noted were as a consequence of improvements in myocardial contractile function (Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Kasama et al 2003, Kuster et al 2005, Lothar et al 2011, Mulder et al 2008, Suzuki et al 2002, Tsutamoto et al 2001, Wang et al 2004). In this regard, if mineralocorticoid receptor blockade improves myocardial contraction in cardiac pathology, this would explain associated decreases in cardiac cavity dimensions (Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Kasama et al 2003, Kuster et al 2005, Lothar et al 2011, Mulder et al 2008, Suzuki et al 2002, Tsutamoto et al 2001, Wang et al 2004).

Spironolactone is a non-selective mineralocorticoid receptor

antagonist in that it may also act on androgenic and glucocorticoid receptors (Rajagopalan and Pitt 2001, Rocha et al 2001). Thus, the beneficial effects of spironolactone noted in the present study could in-part be attributed to actions unrelated to mineralocorticoid receptors. However, as ISO and spironolactone modified the intercept, but not the slope of LV diastolic pressure-volume relations and androgenic steroids determine the slope, but not the intercept of these relations (Trifunovic et al 2005), the impact of spironolactone on adverse structural remodelling in the present study is unlikely to be attributed to androgen receptor effects. Furthermore, as glucocorticoids act as antagonists of aldosterone receptors in cardiac tissue and thus protect against aldosterone's ability to promote fibrosis and LVH (Qin et al 2003), the favorable effects of spironolactone may not be attributed to blockade of glucocorticoid actions on mineralocorticoid receptors. Nevertheless, under conditions of redox change/tissue damage/reactive oxygen species generation, cortisol may act as a mineralocorticoid receptor agonist, an effect that can be blocked by mineralocorticoid receptor blockers such as spironolactone (Funder 2010, Messaoudi et al 2012). It is therefore possible that the beneficial effects of spironolactone in the present study may be attributed to blockade of cortisol rather than aldosterone effects on the mineralocorticoid receptor.

Although spironolactone's beneficial effects on LV chamber dimensions and thus LV systolic chamber dysfunction noted in the present study are attributed to an ability to prevent adverse chamber remodelling, the mechanisms involved in the beneficial actions could still include

chronic effects on volume preloads through renal changes or on afterload effects via alterations in BP. Importantly, in the present study spironolactone given to rats with a dilated LV two weeks prior to assessing LV remodelling, failed to modify LV diameters. Thus, it is unlikely that spironolactone's beneficial effects on LV dimensions can be attributed to an impact on volume preloads through diuretic actions. Indeed, the beneficial effects of mineralocorticoid receptor antagonism on long-term survival and cardiovascular outcomes in patients with heart failure post-myocardial infarction were shown to be independent of its diuretic effects (Rossignol et al 2011). Moreover, spironolactone failed to significantly alter tail cuff systolic BP as determined on 3 separate occasions during the study. These data would suggest that spironolactone's beneficial effects are rather through a direct impact on the myocardium. Nevertheless, in the present study, neither 24-hour BP profiles, nor direct BP measurements obtained using telemetric measurements were assessed. Thus, alterations in BP may still have contributed in-part toward the beneficial actions of spironolactone on cardiac structure and function in the present study.

Mineralocorticoid receptor activation promotes cardiomyocyte apoptosis (De Angelis et al 2002, Sam et al 2004, Sohn et al 2010) and cardiomyocyte necrosis following mitochondrial alterations (Rocha et al 2000, Shahbaz et al 2011, Zia et al 2010), as well as mediates non-genomic effects on the myocardium to influence function (Funder 2010, Ngarmukos and Grekin 2001). The consequences of these changes could be either reductions in myocardial contractility with secondary increases in

cardiac cavity dimensions and hence right shifts in diastolic P-V relations or primary effects on cardiac cavity dimensions as a consequence of side-to-side slippage of cardiomyocytes induced by myocyte death. As indicated in the aforementioned discussion, in the present study although systolic chamber function was altered by ISO and spironolactone, intrinsic myocardial systolic function remained unchanged. Therefore whether ISO potentiated apoptosis, an effect that may have been attenuated by spironolactone, cannot be ruled out. If this were the case, apoptosis could still contribute toward side-to-side slippage of cardiomyocytes and hence promote chamber dilatation, an effect that may be attenuated by spironolactone. Indeed, our group has recently demonstrated that ISO administration at the doses employed in the present study may induce cardiomyocyte apoptosis (Osadchii et al 2007b). Whether mineralocorticoid receptor blockade can attenuate this effect is a question that was addressed in chapter 3 of the present dissertation.

Spironolactone may decrease excessive myocardial NE release in heart failure and as a consequence may prevent adverse remodelling (Kasama et al 2003). However, in the present study although SHR with LVH at 14 months of age had marked increases in myocardial NE release, a modification consistent with those reported on in human LVH (Schlaich et al 2003), neither ISO nor spironolactone influenced this change. Thus, it is unlikely that the beneficial effects of spironolactone in the present study can be attributed to modifications in myocardial NE release.

Importantly, although there is significant evidence that mineralocorticoid receptor blockade reduces cardiac cavity dimensions in clinical studies (Cicoira et al 2002, Hayashi et al 2003, Kasama et al 2003, Modena et al 2001, Tsutamoto et al 2001), thus suggesting that reverse remodelling is a potential mechanism explaining the benefits of this intervention, this effect has not been demonstrated in one recent clinical study conducted in patients with mild-to-moderate heart failure (Udelson et al 2010). In this regard, the present study may cast some insights into this finding. In that recent study (Udelson et al 2010) between 90-100% of patients were receiving background  $\beta$ -adrenergic receptor blocker therapy. As indicated by the present study, the adverse effects of  $\beta$ -adrenergic receptor activation are dependent on mineralocorticoid receptor activation. Thus, adding mineralocorticoid receptor blockers to patients already receiving background  $\beta$ -adrenergic receptor blocker therapy may produce no additional benefits to the LV chamber remodelling process.

In conclusion, the present results provide the first definitive evidence to suggest that the aldosterone receptor antagonist, spironolactone, may prevent the transition to LV systolic chamber dysfunction induced by excessive  $\beta$ -adrenergic activation in compensated hypertensive LVH. This action of spironolactone on LV systolic chamber dysfunction is attributed to a beneficial effect on adverse chamber remodelling rather than to alterations in intrinsic myocardial systolic function and occurs independent of both BP and volume preload changes. These data indicate that a basic mechanism responsible for the ability of

sympathetic activation to mediate the transition from compensated LVH to cardiac decompensation and LV systolic chamber dysfunction is through mineralocorticoid receptor activation. This finding also suggests that a useful therapeutic approach in hypertensive patients with LVH, in whom  $\beta$ -AR blockers have limited beneficial effects on LV mass (Devereux et al 2004a) and central aortic BP (Williams et al 2006) or may induce deleterious metabolic actions (Bakris and Sowers 2004) and hence potentially may result in worse cardiovascular outcomes as compared to other antihypertensive agents (Dahlof et al 2002, Dahlof et al 2005), may be through mineralocorticoid receptor blockade. Clinical studies are therefore required to evaluate whether mineralocorticoid receptor blockers may be especially advantageous in those with hypertensive LVH and asymptomatic left ventricular systolic dysfunction.

## Chapter 3

### **Mechanisms of the Beneficial Effect of Mineralocorticoid Receptor Blockade on the Transition to Chamber Dilatation in Compensated Cardiac Hypertrophy.**

Published in part:

Veliotis DGA, Woodiwiss AJ, Devereux D, Gray D, Osadchii O and Norton GR. Aldosterone receptor blockade prevents the transition to cardiac pump dysfunction induced by  $\beta$ -adrenoreceptor activation. *Hypertension*. 2005;45:914-920.

Veliotis DGA, Norton GR, Correia RJ, Strijdom H, Badenhorst D, Brooksbank R and Woodiwiss AJ. Impact of aldosterone receptor blockade on the deleterious cardiac effects of adrenergic activation in hypertensive rats.

*J Cardiovasc Pharmacol*. 2010;56:203-211

**ABSTRACT**

Although in hypertension  $\beta$ -adrenoreceptor activation promotes the transition from cardiac hypertrophy to LV systolic chamber dysfunction, the use of  $\beta$ -blockers in hypertension is controversial. However, the mineralocorticoid receptor blocker, spironolactone prevents  $\beta$ -adrenoreceptor-mediated increases in cardiac cavity size and LV systolic chamber dysfunction in hypertensive cardiac hypertrophy. The mechanisms of this effect nevertheless remain unresolved. I therefore evaluated the potential mechanisms responsible for the beneficial effects of spironolactone ( $80 \text{ mg.kg}^{-1}.\text{day}^{-1}$ ), on isoproterenol (ISO,  $0.02 \text{ mg.kg}^{-1}$  twice daily)-induced changes in left ventricular (LV) cavity size and LV systolic chamber function in spontaneously hypertensive rats (SHR). In SHR, ISO increased myocardial matrix metalloproteinase (MMP)-2 activity (zymography) after only 4-5 days of administration; a change that was associated with MMP-2, but not TIMP expression. The increased MMP-2 activity persisted until 4.5 months of the study and these changes were prevented by spironolactone therapy. Moreover, at 4.5 months, ISO resulted in increased non-cross-linked, but not cross-linked myocardial collagen concentrations in SHR, an effect that was abolished by spironolactone. Although at 4.5 months ISO administration was not associated with an increased cardiomyocyte apoptosis (TUNEL), an early (4-5 days) ISO-induced apoptotic effect was noted, which was prevented by spironolactone. Neither ISO nor spironolactone influenced cardiomyocyte length (image analysis and flow cytometry) in SHR. These

results suggest that mineralocorticoid receptor blockade may prevent the adverse effects of  $\beta$ -adrenoreceptor activation on cardiac dilatation and LV systolic chamber dysfunction in hypertensive cardiac hypertrophy largely through alterations in the cardiac interstitium and cardiomyocyte apoptosis.

### 3.1 Introduction.

Sympathetic over-activation in hypertensive left ventricular hypertrophy (LVH) (Agabiti-Rosei et al 1987, Schlaich et al 2003) may be a critical determinant of the progression to heart failure. Indeed, without influencing loading conditions, genetic modifications that reduce sympathetic activation attenuate (Esposito et al 2002) and chronic  $\beta$ -adrenoreceptor stimulation promotes (Badenhorst et al 2003b) the transition from hypertensive LVH to cardiac dilatation and LV systolic chamber dysfunction. Moreover, at a clinical level, present guidelines (Hunt et al 2009) acknowledge that in the treatment of hypertension,  $\beta$ -blockers are more effective than, for example, alpha-blockers and calcium channel blockers in preventing hypertensive heart failure. Despite this evidence,  $\beta$ -blockers may increase the chances of new-onset diabetes mellitus (Bakris and Sowers 2004) and reduce LVM (Devereux et al 2004a) and central blood pressures (Williams et al 2006) less effectively than other agents (Williams et al 2006), changes that may offset their beneficial effects. Indeed,  $\beta$ -adrenoreceptor blocker-based therapy is less effective at reducing cardiovascular outcomes than other antihypertensive agents (Dahlof et al 2002, Dahlof et al 2005). The potential solution to this conundrum may lie in the use of mineralocorticoid receptor antagonists, as I have shown that independent of changes in blood pressure or volume-preloads on the heart, mineralocorticoid receptor blockade may prevent the ability of  $\beta$ -adrenergic receptor activation to promote the transition from

LVH to systolic chamber dysfunction in hypertension (chapter 2, Veliotis et al 2005). Importantly, in that study I provide clear evidence that this beneficial effect is principally on the LV remodelling process (LV dilatation) rather than on intrinsic myocardial systolic function.

Although I have demonstrated the aforementioned potential advantages of mineralocorticoid receptor blockade (chapter 2, Veliotis et al 2005), the mechanisms that explain the ability of mineralocorticoid receptor activation to modify the adverse effects of  $\beta$ -adrenergic activation require elucidation. In this regard, the activity and expression of matrix metalloproteinases (MMP), enzymes that are activated in cardiomyocytes by  $\beta$ -adrenergic stimulation (Menon et al 2005), and which contribute toward cardiac dilatation and LV systolic chamber dysfunction (Peterson et al 2001, Spinale et al 1999), may be activated by  $\beta$ -adrenergic receptor stimulation and attenuated by mineralocorticoid receptor blockade. In addition the role of sympathetic activation and mineralocorticoid receptor blockade in modulating the phenotypic characteristics of myocardial collagen also requires assessment. In this regard, decreases in myocardial collagen cross-linking or increases in myocardial type III collagen may play an important role in mediating cardiac dilatation and LV systolic chamber dysfunction (Woodiwiss et al 2001). Moreover, the role of cardiomyocyte apoptosis requires consideration. In this regard, the role of cardiomyocyte apoptosis in heart failure (Foo et al 2005, Wencker et al 2003) and  $\beta$ -adrenoreceptor activation as a stimulus for cardiomyocyte apoptosis (Singh et al 2001) are well documented and adrenergic-induced

cardiomyocyte apoptosis may accompany initial, but not later, periods of adrenergic stimulation (Osadchii et al 2007b). Last, the impact of mineralocorticoid receptor blockade on cardiomyocyte length, an acknowledged mediator of cardiac dilatation and heart failure in hypertension (Tamura et al 1998), requires further investigation.

The aim of the present study was therefore to determine whether the beneficial effects of mineralocorticoid receptor blockade on the transition from hypertensive LVH to LV dilatation and consequently systolic chamber dysfunction induced by  $\beta$ -adrenergic receptor activation are associated with alterations in myocardial MMP activation, changes in the phenotypic characteristics of myocardial collagen, cardiomyocyte apoptosis and/or cardiomyocyte lengthening.

### **3.2 Methods.**

All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institute of Health, publication no. 86-23, 1996). The Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand approved the present studies (AESC approval numbers: 99:01:2b, 2002:37:5, 2002:39:5 and 2006:37:4).

### 3.2.1 Groups studied.

Long-term study. In chapter 2, the changes in cardiac function and chamber remodelling associated with chronic  $\beta$ -adrenoreceptor activation and aldosterone receptor blockade were described. The same 14-to-18.5 month old rats described in chapter 2 were therefore employed to determine the impact of chronic mineralocorticoid receptor blockade on interstitial myocardial changes, necrosis, apoptosis and cardiomyocyte dimensions and to assess whether these changes are commensurate with the effects on cardiac remodelling. Importantly, the possibility that changes associated with mineralocorticoid receptor blockade occur secondarily to improvements in LV systolic chamber function cannot be excluded in this study.

Short-term study. The impact of short-term isoproterenol (ISO) and spironolactone (SPIRO) on SHR was studied in order to assess the effects of mineralocorticoid receptor blockade on cardiac MMP activity, MMP and TIMP expression and cardiomyocyte apoptosis independent of improvements in pump function. In this study ~12 month old SHR and normotensive Wistar Kyoto (WKY) rats were assigned to the following experimental groups: SHR receiving either ISO or vehicle and either SPIRO suspended in a gelatine-meat extract or the gelatine-meat extract alone. The ISO and SPIRO doses were identical to those described in chapter 2, and were administered for 4-5 days. I adopted this approach because other groups have demonstrated that  $\beta$ -adrenoreceptor activation

directly induces MMP activation and expression in cardiomyocytes (Menon et al 2005) and cardiomyocyte apoptosis (Singh et al 2001), and neurohormones which are elevated in response to  $\beta$ -adrenoreceptor activation also increase MMP activity in cardiomyocytes (Coker et al 2001) *in vitro*. Hence I reasoned that similar short-term increases should be noted *in vivo*. Furthermore, if mineralocorticoid receptor blockade abrogates cardiac dilatation via direct actions on MMP activation or expression, or cardiomyocyte apoptosis, then these effects should be noted prior to remodelling occurring.

### **3.2.2 Myocardial collagen.**

To characterize the role of the interstitial matrix in mediating the beneficial effects of mineralocorticoid receptor blockade on adverse cardiac remodelling, samples of the left ventricular myocardium were collected for tissue analysis from the aforementioned 14-to-18.5 month old rats. Tissue used to assess the characteristics of the myocardial matrix was obtained from the antero-lateral LV wall in the mid-portion of the longitudinal axis. In order to preserve the integrity of the samples, they were flash frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  prior to analysis. The concentration of myocardial hydroxyproline ([HPRO]) was determined after acid (HCl) hydrolysis using previously described methods (Stegemann and Stalder 1967) which have been extensively employed by

our group (Badenhorst et al 2003a, Norton et al 1997, Norton et al 2002, Tsotetsi et al 2001, Woodiwiss et al 2001).

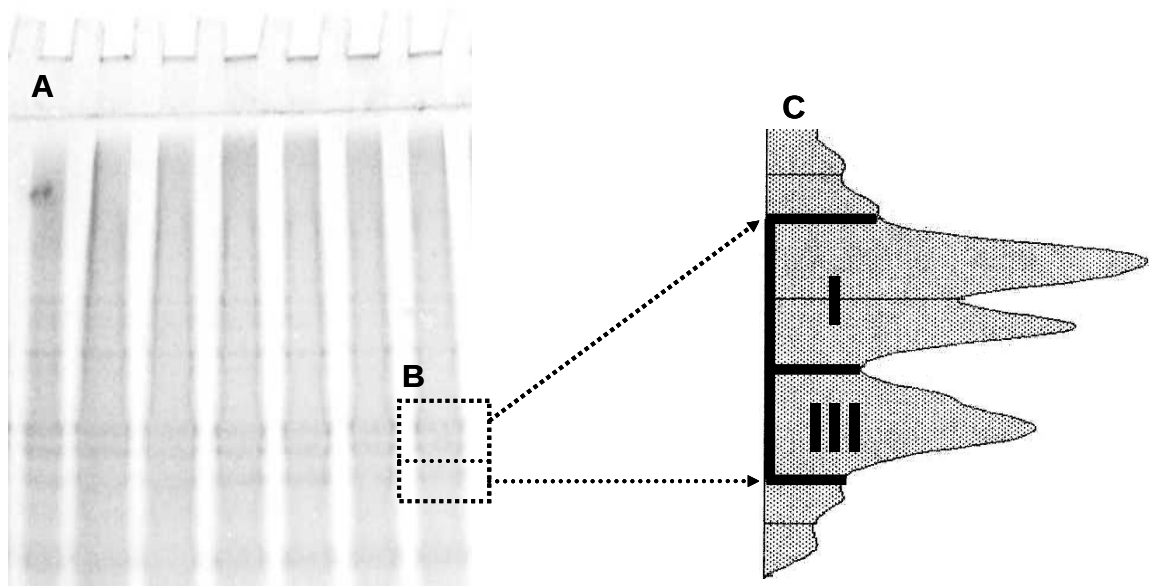
Myocardial collagen was also extracted and digested with cyanogen bromide (CNBr) (Badenhorst et al 2003a, Norton et al 1997, Tsotetsi et al 2001, Woodiwiss et al 2001). Thereafter a portion of the CNBr digested collagen sample was subjected to acid hydrolysis and [HPRO] determination. The amounts of cross-linked (insoluble) and non-cross-linked (soluble) collagen in the myocardium were ascertained based on the relative solubility of myocardial collagen to CNBr digestion as compared to HCl hydrolysis (Badenhorst et al 2003a, Norton et al 1997, Tsotetsi et al 2001, Woodiwiss et al 2001).

Vertical polyacrylamide gel electrophoresis was subsequently performed on the remaining portion of the CNBr digested sample, with stacking and separation gel concentrations of 3% and 12.5% respectively, as previously described (Laurent et al 1981, Mukherjee and Sen 1990, Mukherjee and Sen 1993). On the electrophoretic patterns of myocardial collagen extracts, bands of collagen type I and III were identified using standards of collagen type I (Sigma) and type III (Calbiochem). These bands correspond to bands G [ $\alpha 1(I)$ -CB-8] and H [ $\alpha 2(I)$ -CB-3] (collagen type I) (Laurent et al 1981) and band M [ $\alpha 1(III)$ -CB-5 plus  $\alpha 1(III)$ -CB-9] (collagen type III) (Laurent et al 1981, Mukherjee and Sen 1990). Bands G, H and M were chosen because they contain very little interference from co-migrating peptides of other collagen types. The relative amounts of type I and III collagen were determined from the relationship between the

quantity of collagen applied to the gel and the relative area under the densitometry curve (Norton et al 1997, Tsotetsi et al 2001, Woodiwiss et al 2001). The concentrations of myocardial type I and III collagen were assessed from type I-to-III ratios and the [HPRO] in myocardial tissue (Norton et al 1997, Tsotetsi et al 2001, Woodiwiss et al 2001). A representative example of the polyacrylamide electrophoretic gels used to determine cardiac collagen type I-to-III ratios is illustrated in Figure 3.1.

### **3.2.3 Pathological score.**

To establish whether mineralocorticoid receptor blockade influences the extent of tissue necrosis, thus contributing towards a reduction in cardiac dilatation, samples of the left ventricular myocardium were collected for histological analysis from all rats studied from 14-to-18.5 months of age. Prior to isolating and freezing myocardial tissue for collagen assessment, a longitudinal slice of the LV free wall from the apex to the base was dissected out and stored in buffered formalin. Using routine processing techniques for light microscopy, left ventricular tissue was embedded in paraffin wax, from which 5 µm-thick sections of the long axis circumference were cut through the full thickness of the LV wall, from endocardium to epicardium. The sections were stained with van Gieson's stain, which stains collagen a pink colour. Slides were randomised and a pathological score was assigned to each slide in a blinded analysis. Thereafter the mean score for each rat was calculated. Scores were

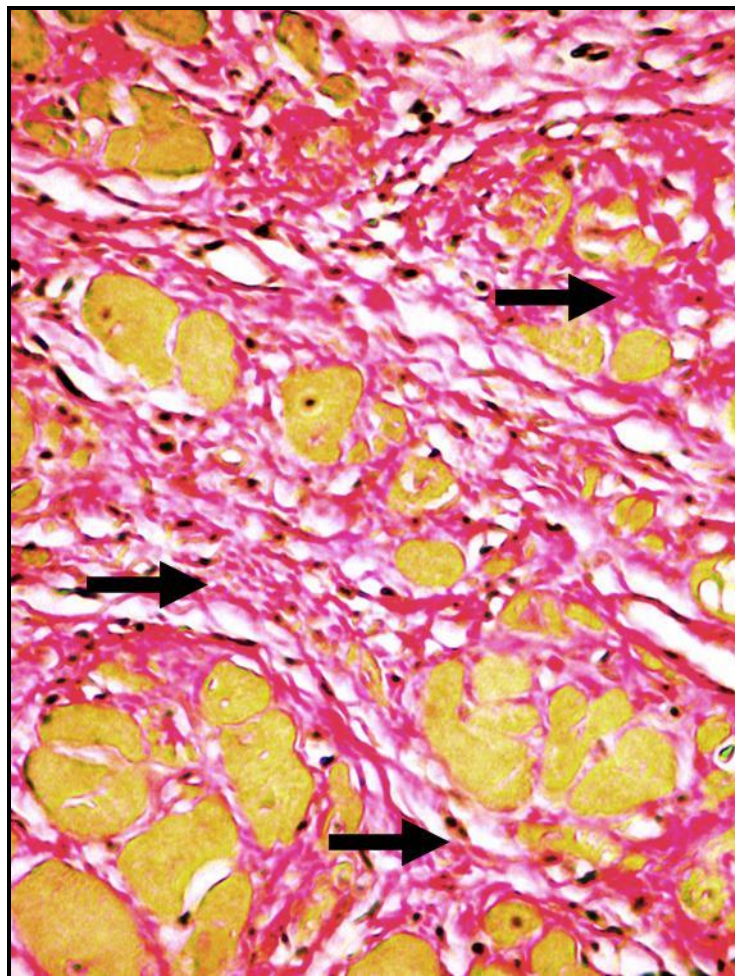


**Figure 3.1.** Polyacrylamide gel electrophoresis showing the typical banding pattern of myocardial collagen (A). Box highlights area containing collagen type I and III bands (B), and densitometry curve of collagen type I and III bands (C). See text for explanation.

defined as follows: 0 indicates no damage; 1 and 2, patchy fibrosis in less than or more than 20% of the total field respectively; 3 and 4, diffuse contiguous subendocardial fibrosis in less than or more than half the circumference respectively and 5 and 6, full thickness fibrosis in less than or more half the circumference respectively (Teerlink et al 1994, Woodiwiss et al 2001). Figure 3.2 shows a histologically fibrotic section of the myocardium.

#### **3.2.4 Cardiomyocyte apoptosis.**

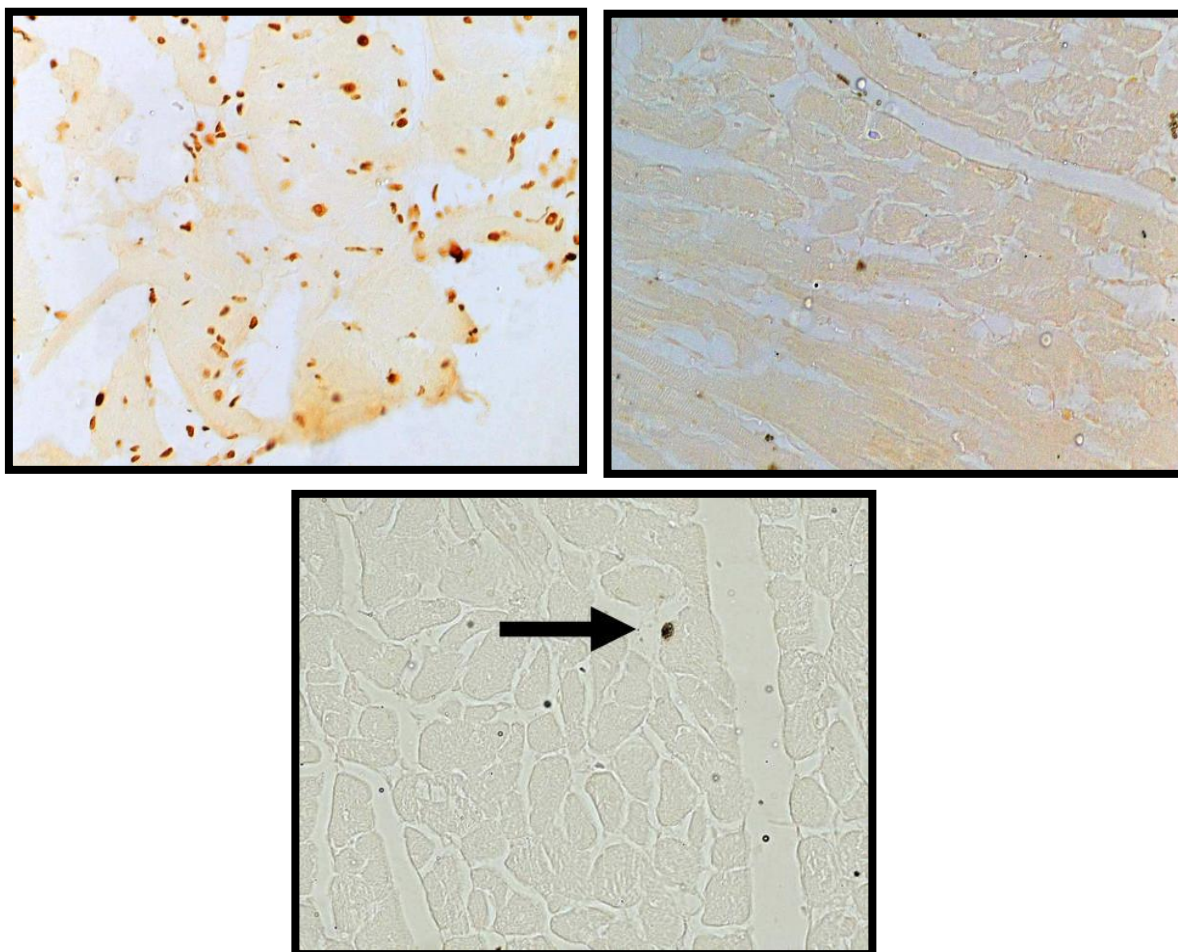
In rats studied from 14-to-18.5 months of age, and in rats receiving ISO and/or SPIRO for 4-5 days, the extent of cardiomyocyte apoptosis was assessed in order to determine whether mineralocorticoid receptor blockade modulates apoptosis thus attenuating adverse cardiac remodelling. From the same wax embedded myocardial tissue blocks used to assess the pathological score 5 µm thick histological sections were sliced, stained, and the extent of cardiomyocyte apoptosis enumerated. More specifically, overhanging 3'-OH ends of fragmented nuclear deoxyribonucleic acid (DNA) in tissue sections were detected using a histological, *in situ*, non-radioactive, apoptotic cell death detection kit (DeadEnd™ Colorimetric TUNEL system, Promega, Madison, WI, USA). In this staining system terminal deoxynucleotidyl transferase (TdT) catalyses the incorporation of biotinylated nucleotides at the



**Figure 3.2.** Histological image photographed using light microscopy, of a cross-section of myocardial tissue stained with van Gieson's stain. The photograph shows evidence of tissue necrosis and diffuse fibrosis (arrows).

3'-OH DNA ends, onto which horseradish-peroxidase-labeled streptavidin is bound. Thereafter in the presence of hydrogen peroxide and diaminobenzidine a dark brown stain is generated (Agarwala and Kalil 1998). Control negative (no TdT added) and positive (DNase treated) tissue sections were included in each TUNEL staining assay. Furthermore for each rat a histological section obtained from the same tissue block was also stained with hematoxylin and eosin (H&E).

Ten evenly spaced 400 times magnification fields, from the apex to the base, were captured for each slide using a computer-based image acquisition and analysis system (Axiovision 3, Carl Zeiss, Gottingen, Germany). On these fields the number of apoptotic cardiomyocyte nuclei (TUNEL) and the total number of cardiomyocyte nuclei (H&E) were counted, and the percentage of the total number of cardiomyocyte nuclei that were apoptotic was calculated. Prior to image analysis, sections were randomized and coded. A single observer, blinded to the identity of each image, recorded the number of apoptotic and total number of cardiac myocyte nuclei. A representative example of a histological section stained with TUNEL used in quantifying apoptosis is shown together with a positive and negative control in Figure 3.3.



**Figure 3.3.** Histological detection of apoptotic nuclei in the myocardium. The upper left and right panels demonstrate images of positive and negative control TUNEL stained sections respectively. The lower panel depicts a section of myocardium containing one apoptotic cardiomyocyte nucleus (arrow). Note the numerous stained nuclei in the positive control section.

### 3.2.5 Assessments of isolated cardiomyocyte morphometry.

To assess the contribution of cardiomyocyte morphometry to the favourable effects of mineralocorticoid receptor blockade on cardiac remodelling, the dimensions of isolated cardiomyocytes were measured on myocardial tissue obtained from rats studied from 14-to-18.5 months of age. In order to isolate individual cardiomyocytes, 100 mg of homogenised left ventricular tissue was placed in a tissue culture flask containing 10 ml of incubation solution (125 mg of bovine serum albumin [Fraction V, Sigma Chemical Co., St. Louis, MO], 15 mg Collagenase Type 2 [Worthington Biochemical Corporation, Lakewood NJ, USA] [317 U/mg], 14mg of Hyaluronidase [Worthington Biochemical Corporation, Lakewood NJ, USA] [581 U/mg] and 22.7  $\mu$ l of 110 mM  $\text{CaCl}_2$  dissolved in 50 ml of calcium free physiological saline solution [PSS]). The PSS consisted of (in mM) NaCl 120, taurine 20, KCl 10, glucose 10, pyruvate 6.2, HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid hemisodium salt) 4.8,  $\text{MgCl}_2$  2.6 and  $\text{KH}_2\text{PO}_4$  1.2 (all from Sigma Chemical Co., St. Louis, MO), dissolved in distilled water, final pH 7.2~7.3. Tissue samples were incubated for two 10 minute and a third 15 minute period in an oscillating water bath which was preheated to 37 °C. The incubation solution which was constantly gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , was aspirated and replaced with 10 ml of fresh incubation solution after each 10 minute incubation period. The frequency of the oscillating water bath was 120 cycles per minute for each of the 10 minute incubations and 215 cycles

per minute for the 15 minute incubation.

After the third oscillation cycle two thirds of the incubation solution was aspirated from the tissue culture flask. The remaining incubation solution, which contained the digested tissue and tissue precipitants, was passed through a 250  $\mu$ m nylon mesh gauze into a 15 ml polypropylene test tube. Thereafter approximately 10 ml of wash solution (0.045 ml of 110 mM  $\text{CaCl}_2$  and 250 mg of bovine serum albumin) was used to flush the remaining tissue through the mesh gauze into the test tube. After allowing the sample to settle in the test tube for 15 minutes, the upper two thirds of the supernatant was aspirated and replaced with fresh wash solution. Following a further 15 minute settling period the supernatant was completely aspirated without disturbing the pellet. The cell pellet was then diluted in PSS containing 0.01% bromophenyl blue (The Coleman and Bell Co., USA), a protein stain, which enables the easy identification of cardiomyocyte sarcomere bands.

Aliquots of the cell suspension were placed in tissue culture dishes, and consistently upon initial examination, it was found that 60-80% of the isolated cardiomyocytes were rod-shaped. Cardiomyocytes were viewed at 400X magnification through an inverted light microscope and images of individual cardiomyocytes were captured using a digital camera (Nikon Digital Sight DS-U1 & DS-5M, Nikon Corporation, Japan) when clearly visible sarcomeres could be identified along the entire length of the cell (Figure 3.4). Good quality images of between 30 and 35 randomly selected cardiomyocytes were obtained from each LV sample, and stored



**Figure 3.4.** Image of an isolated cardiomyocyte, captured at 400X magnification, with sarcomeres clearly visible. Digital image analysis techniques were used to count the number of sarcomeres and to measure the cell dimensions.

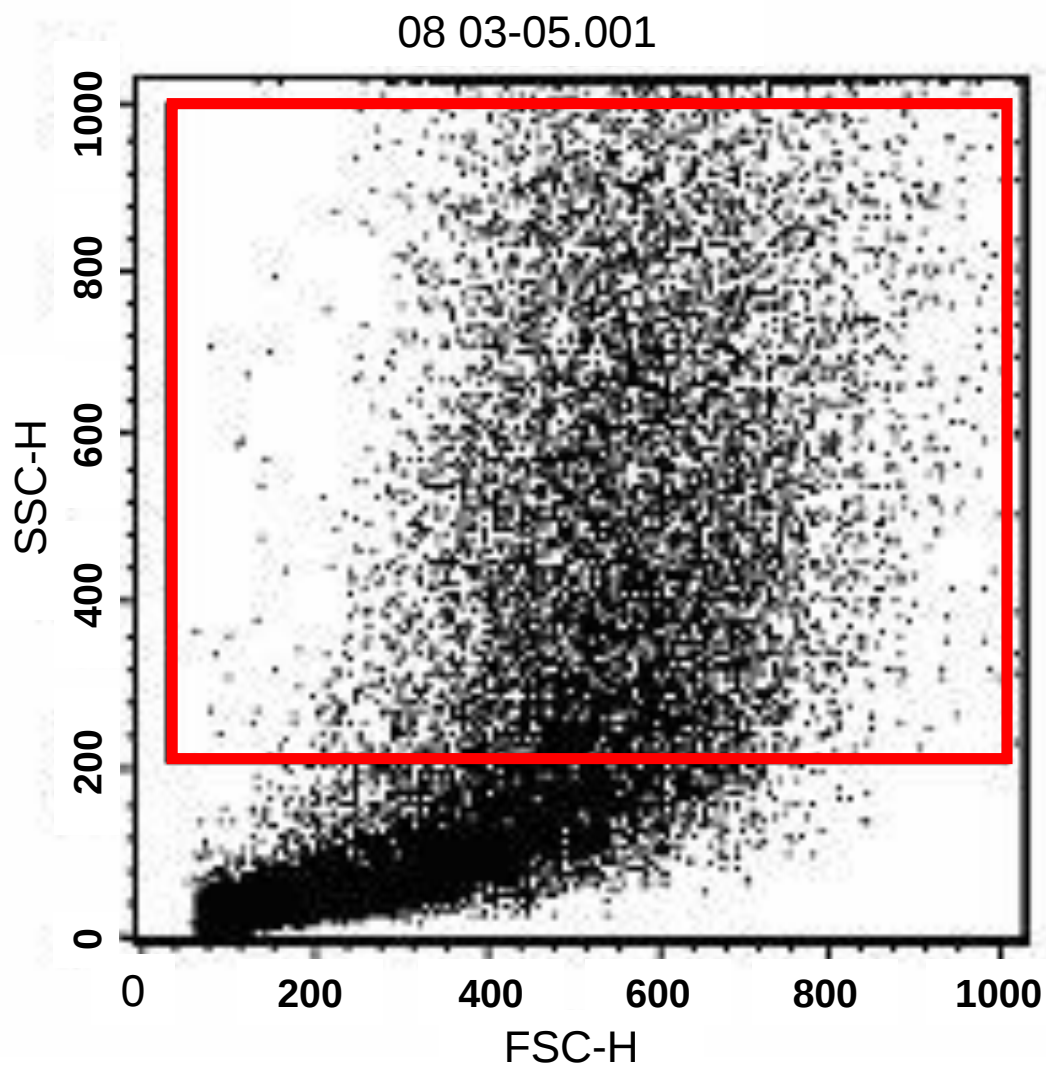
for later analysis. Image analysis of cell length and width was performed by a blinded observer, using Act2U Version 1.70 (Nikon Corporation, Japan) software that was calibrated using a 1 mm (100 X 0.01mm) graticule (Graticules Ltd, Kent, England).

Since histological image analysis provides very accurate indexes of cardiomyocyte dimensions but on only a limited number of cells, additional flow cytometric measurements of cardiomyocyte morphometry were performed as previously described (Diez and Simm 1998, Nash et al 1979). Since only cylindrical calibration beads are available for flow cytometry, absolute cell length could not be determined. However flow cytometry enables the measurement of relative cell size on thousands of cells per sample. The larger sample size generated thus compensates for the small sample of cardiomyocytes that can be evaluated using image analysis. Cardiomyocytes for flow cytometry were isolated in the same manner as described above for image analysis, and were resuspended in 1ml of PSS in Polystyrol tubes following which cell morphometry was quantified using a Becton-Dickinson flow cytometer (FACSCalibur®). Autofluorescence of cardiomyocytes at the single cell level was measured in the FL1 channel (excitation / emission wavelength: 488 / 530 nm) and cell length was determined from the time of flight (FL1-W). Data analysis, limited to 30 000 cells, was performed with Cellquest® version 3.3 (Becton Dickinson). Both forward and side scatter, which are measures of cell size and granularity respectively, were obtained and plotted (Figure 3.5). The scatter plot is used to gate data, allowing the exclusion of debris and

non-cardiomyocytes, which have low granularity, from analysis (Strijdom et al 2004).

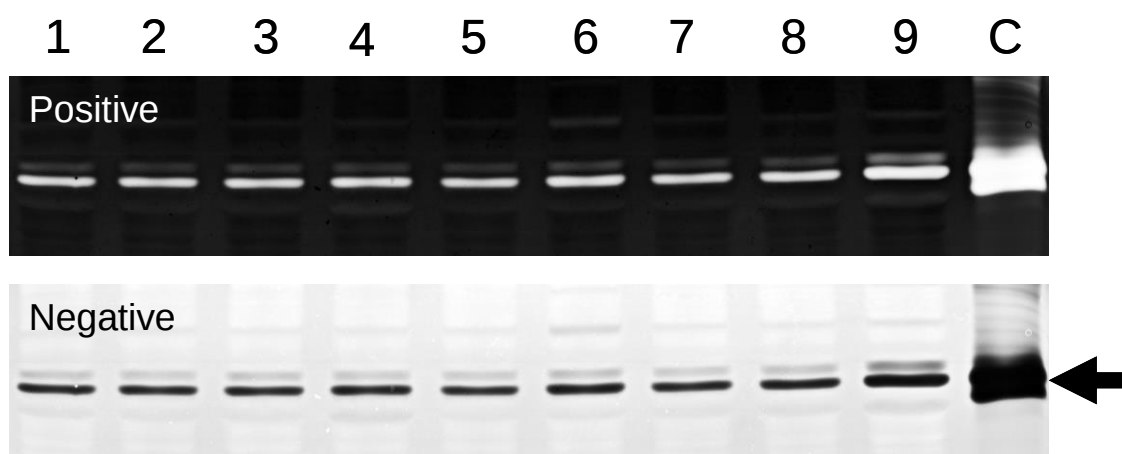
### **3.2.6 Matrix metalloproteinase activity.**

Gelatine zymography was performed on myocardial tissue as previously described (Tyagi et al 1993), to assess whether mineralocorticoid receptor blockade abrogates adverse cardiac remodelling induced by adrenergic activation in SHR, through short (4-5 days) or long-term (14-18.5 months) alterations of gelatinase (MMP-2 and MMP-9) activity. Within five minutes of removing hearts from the thoracic cavity (for the short term study) and within five minutes of completing haemodynamic measurements on the isolated perfused heart preparations (for the long term study), lateral LV tissue samples were dissected out, frozen in liquid nitrogen and stored for later analysis at -70°C. In order to extract cardiac tissue protein, myocardial samples were crushed to a powder in the presence of liquid nitrogen. The samples were then incubated for 18 h at 4°C in 100 µl extraction buffer (50 mM Tris, 0.1 % SDS) per 100 mg ground tissue powder. The samples were centrifuged for 10 minutes at 12 000 rpm following which the soluble protein containing supernatants were decanted and stored at -70°C until the protein concentrations were measured with a modified Lowry/Folin technique (Lowry et al 1951).



**Figure 3.5.** Dot-plot of cardiac myocyte side scatter height (SSC-H) and forward scatter height (FSC-H). The red box illustrated on the scatter plot defines the SSC-H gate above 200 which thus excludes debris and low granularity cells, which are not cardiomyocytes.

The relative activity of MMP-2 in each sample was determined by loading 20 µg of extracted protein into each well of a 10% polyacrylamide gel which contained 1mg.ml<sup>-1</sup> type A gelatin. The proteins were electrophoretically separated at a constant current of 30 mA over 1.5 hours. The gels were incubated in MMP zymogen activating substrate buffer (Tris 50 mM, CaCl<sub>2</sub> 5 mM, pH 8) overnight to promote the enzymatic degradation of gelatin. Coomassie blue which is a protein stain was applied to the gels, resulting in a gel with a dark blue background and lighter bands (Figure 3.6, upper panel), the translucency which correlates with MMP activity. Since MMP-2 but not MMP-9 is detectable in the myocardium of rats of this age, only MMP-2 standards (Sigma, purity >95% by SDS-PAGE visualized by silver staining) were included on gels. The position of the MMP-2 standard was used to locate cardiac MMP-2 bands after electrophoresis. A flat bed transmission scanner (Cano Scan 4200 F, Cannon Solutions, China) was used to scan the gels, and images were digitally inverted for densitometry analysis (Figure 3.6, lower panel). Digital densitometry using LabWorks Version 4.5 (UVP, Upland, USA) enabled the density of each MMP-2 band to be measured and compared to the density of a standard sample included on all gels. All samples were electrophoresed in duplicate, randomized on the gels and quantified in a blinded analysis.



**Figure 3.6.** Representative zymogram of myocardial tissue. The positive image of a scanned zymogram (top) is digitally inverted (bottom) to create a negative which can be analyzed by digital densitometry. Lanes 1-9 contain equal quantities of protein extract. Lane C contains MMP-2 standard. The arrow illustrates the position of the MMP-2 bands.

### **3.2.7 Matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase expression.**

In order to further define the role of the myocardial matrix in mediating anti-remodelling effects of mineralocorticoid receptor blockade on adrenergic induced cardiac dilatation in SHR, myocardial expression of MMP-2 and TIMP-2 was measured. Tissue was obtained from the lateral wall of the LV within five minutes of isolating the heart from the thoracic cavity and was immediately stored for messenger RNA (mRNA) extraction and analysis in RNeasy® (Ambion, The RNA Company, Applied Biosystems, USA). Alterations in myocardial MMP-2 and TIMP-2 mRNA expression occurring prior to cardiac dilatation (Polyakova et al 2004) are less likely than changes occurring post-cardiac dilatation to be confounded by the direct impact of cardiac pump dysfunction on their expression. Therefore tissue was only collected for mRNA analysis from 12 month old rats in all groups in the short term (4-5 days) study.

Myocardial mRNA was extracted with RNeasy Direct mRNA Mini Kits (Qiagen, Hilden, Germany). Thereafter complementary deoxyribonucleic acid (cDNA) was synthesized from extracted mRNA complexes using Transcriptor First Strand cDNA Synthesis Kits (Roche, Mannheim, Germany). Real-time quantitative polymerase chain reaction (RT-PCR) was carried out using a LightCycler 1.2 instrument (Roche, Germany) with LightCycler FastStart DNA Master<sup>PLUS</sup> SYBR Green I Kits

(Roche, Mannheim, Germany). Primers employed in the amplification of specific mRNA targets included the following:

GAPDH forward: 5' – CTC CCTCAAGATTGTCAGCAA - 3'

GAPDH reverse: 5' – GTCAGATCCACAACGGATACATT - 3'

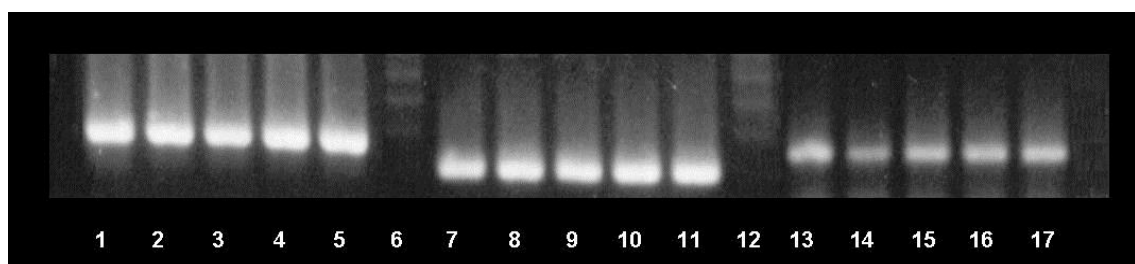
MMP-2 forward: 5' – CCTCCCCTGATGCTGATA - 3'

MMP-2 reverse: 5' – ATACACAGCGTCAATCTTTTC - 3'

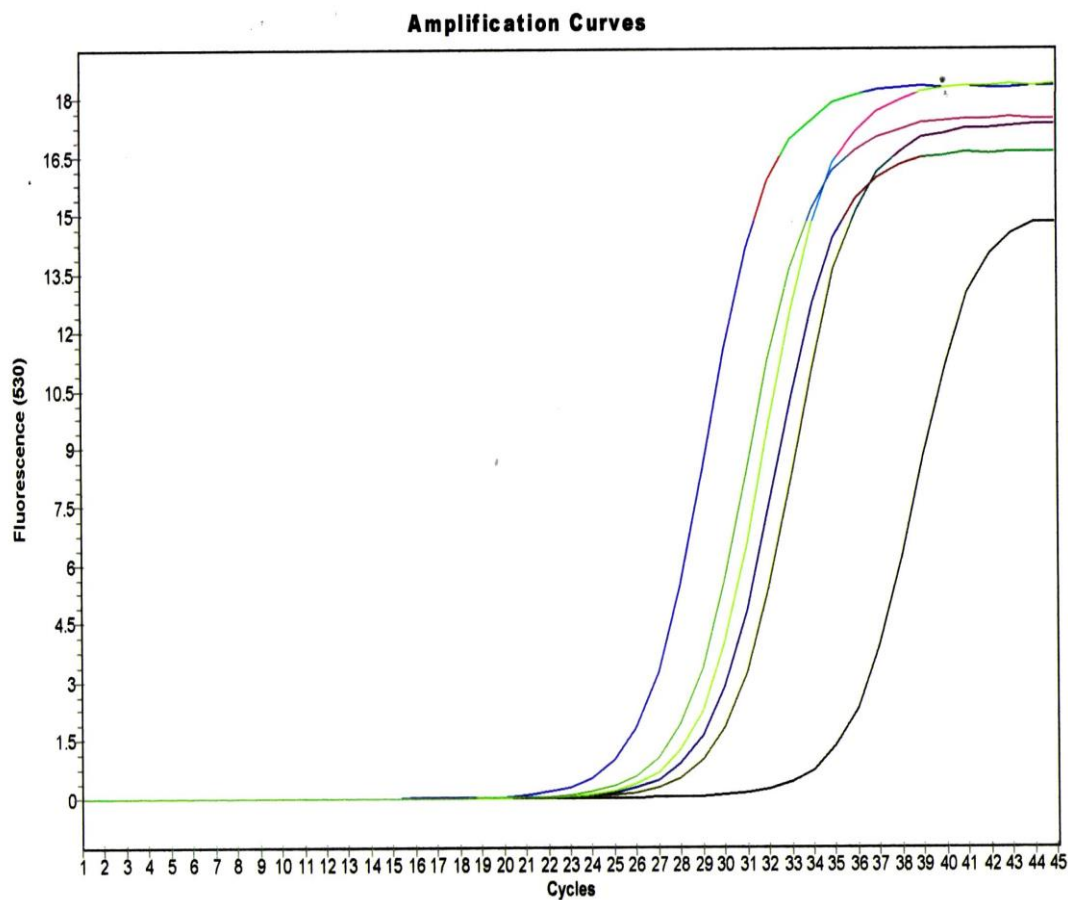
TIMP-2 forward: 5' – ATGAGATCAAGCAGATAAAGATGTT - 3'

TIMP-2 reverse: 5' – GATGCTAAGCGTGTCCC - 3'

Annealing temperatures for MMP-2, TIMP-2 and GAPDH were, 51°C, 53°C and 56°C respectively. The PCR for all targets included a single initial 10 minute 95°C cycle, followed by 40 amplification cycles consisting of 5 seconds at 95°C followed by 15 seconds at the appropriate annealing temperature and then 10 seconds to allow for extension at 72°C. Lightcycler Version 4.0 software (Roche, Germany) was used for data analysis. The expression of each mRNA target was quantified by expressing its production relative to that of the housekeeping gene, GAPDH, using a calibrator normalized method. Agarose gel electrophoresis was performed as illustrated in Figure 3.7 to demonstrate the cDNA amplification products of MMP-2, TIMP-2 and GAPDH. Figure 3.8 illustrates typical RT-PCR amplification curves of cardiac TIMP-2 cDNA.



**Figure 3.7.** Monochromatic photograph of an agarose gel showing complementary deoxyribonucleic acid (cDNA) amplification products for cardiac GAPDH, the housekeeping gene (289bp, lanes 1-5), tissue inhibitor of MMP type 2 (TIMP-2) (186bp, lanes 7-11) and matrix metalloproteinase-2 (MMP-2) (218bp, lanes 13-17). Lanes 1-5, 7-11, and 13-17 contain randomized cDNA from rats studied. Lanes 6 and 12 contain a molecular weight marker.



**Figure 3.8.** Typical example illustrating real time-polymerase chain reaction (RT-PCR) amplification curves of cardiac tissue inhibitor of matrix metalloproteinase type 2 (TIMP-2). Each line represents the accumulation of complementary deoxyribonucleic acid (cDNA) for a different rat sample. The earlier the turning point (i.e. curve inflection at a lower number of cycles) the greater the expression of TIMP-2.

### **3.2.8 Data analysis.**

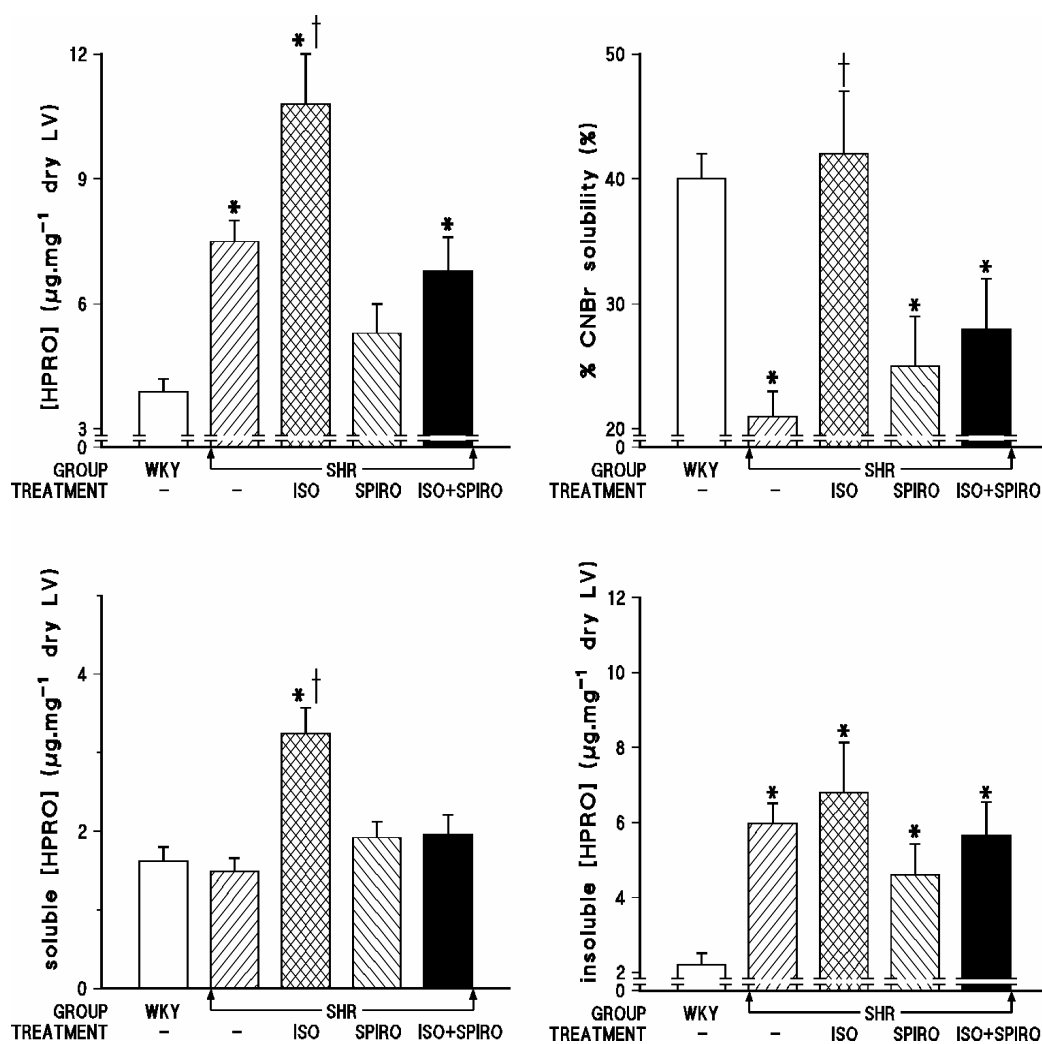
In order to identify differences between groups a one or two-way (where appropriate) ANOVA followed by a Tukey *post hoc* analysis was performed. Unless otherwise specified, all values represented in the text are mean  $\pm$  SEM.

## **3.3 Results.**

Importantly, the heart weight, LV dimension and functional data for rats assessed in the long-term study, have been described in chapter 2. Furthermore, LV weight and function were not determined in the short-term study since a pilot study demonstrated that ISO administration to SHR and normotensive WKY rats, for 5-7 days, does not alter LV weights or impair LV function.

### **3.3.1 Myocardial collagen.**

Figure 3.9 illustrates the myocardial collagen characteristics of SHR and WKY rats in response to ISO and SPIRO administration for 4.5 months. Myocardial [HPRO] was significantly elevated in untreated, 18.5 month old SHR, as compared to age-matched WKY. Of the increased total collagen content noted in SHR, only cross-linked collagen concentrations



**Figure 3.9.** Effect of chronic administration of isoproterenol (ISO) and spironolactone (SPIRO) on myocardial collagen characteristics in spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto controls; [HPRO], hydroxyproline concentrations; CNBr, cyanogen bromide. \*  $p < 0.01$  versus WKY group. †  $p < 0.01$  versus other SHR groups. Sample sizes are indicated in Table 3.1.

were increased, as evidenced by augmented concentrations of insoluble collagen. Not only did ISO administration amplify the increment in total myocardial [HPRO] noted in SHR, but it also increased the solubility of myocardial collagen to cyanogen bromide digestion thus signifying reduced myocardial collagen cross-linking commensurate with levels noted in WKY rats. ISO thus increased soluble, but not insoluble collagen concentrations. Co-administration of SPIRO prevented ISO-mediated increases in myocardial [HPRO], percent collagen solubility and soluble collagen concentrations.

On regression analysis of data obtained for all SHR, significant relations were demonstrated between myocardial soluble (non-cross-linked) collagen concentrations and LV  $V_0$  ( $r = 0.53$ ,  $p < 0.01$ ); soluble collagen and LVED  $r$  (e.g. at 2 mm Hg,  $r = 0.41$ ,  $p < 0.05$ ); total collagen and LV  $V_0$  ( $r = 0.45$ ,  $p < 0.02$ ); and total collagen and LVED  $r$  (e.g. at 2 mm Hg,  $r = 0.38$ ,  $p < 0.05$ ).

Table 3.1 shows myocardial collagen type I and III phenotypic characteristics in SHR and WKY rats in response to 4.5 months of ISO and SPIRO administration. In SHR, ISO augmented the increments of type I and III collagen concentrations, and this effect was prevented by SPIRO. Finally, although in all groups of SHR both the collagen type I and III concentrations were increased, the myocardial collagen type I-to-III ratios were similar to that of WKY.

**Table 3.1.** Effect of chronic administration of isoproterenol (ISO) and spironolactone (SPIRO) on myocardial necrosis and collagen characteristics in spontaneously hypertensive rats (SHR).

|                                                                | WKY       | SHR       | SHR+ISO    | SHR+SPIRO | SHR+ISO+SPIRO |
|----------------------------------------------------------------|-----------|-----------|------------|-----------|---------------|
| n=                                                             | 9         | 9         | 9          | 6         | 10            |
| Type I-to-III collagen ratio                                   | 2.89±0.23 | 3.06±0.13 | 3.02±0.19  | 2.92±0.20 | 2.61±0.21     |
| Collagen type I ( $\mu\text{g} \cdot \text{mg}^{-1}$ dry LV)   | 23±3      | 46±3*     | 65±10**†   | 32±8      | 40±5*         |
| Collagen type III ( $\mu\text{g} \cdot \text{mg}^{-1}$ dry LV) | 8±1       | 15±1*     | 21±2**†    | 11±3      | 16±2*         |
| Pathological score                                             | 1.00±0.19 | 1.90±0.32 | 3.10±0.06* | 2.33±0.33 | 2.67±0.44*    |

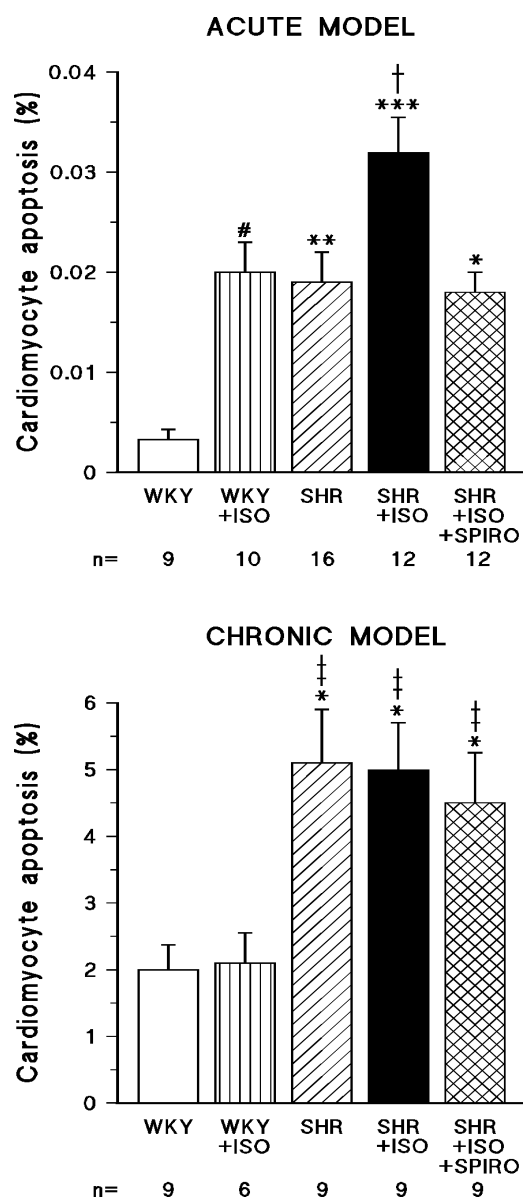
WKY, Wistar Kyoto control \* p<0.05, \*\* p<0.01 versus WKY group, † p<0.01 versus other SHR groups.

### **3.3.2 Pathological score.**

The impact of ISO and SPIRO administration for 4.5 months to SHR and WKY rats, on myocardial necrosis is shown in Table 3.1. In untreated SHR as compared to WKY controls, there was a trend for increased pathological score, and in SHR receiving ISO the effect achieved significance. SPIRO did not however modify the degree of myocyte necrosis.

### **3.3.3 Cardiomyocyte apoptosis.**

In both the short (acute) and long (chronic) term studies the percentage of TUNEL positive stained cardiomyocytes was elevated in all SHR groups as compared to WKY controls (Figure 3.10). Four-to-five days of ISO administration increased the percentage TUNEL positive-stained cardiomyocytes in both SHR and WKY rats, and SPIRO administration prevented the ISO-induced increase in cardiomyocyte apoptosis in SHR (Figure 3.10, upper panel). Although the percentage apoptosis remained significantly greater in the untreated SHR as compared with the WKY control rats, the impact of ISO on cardiomyocyte apoptosis noted in the acute model was not sustained after ISO administration for 4.5 months to SHR (Figure 3.10, lower panel). Moreover, SPIRO had no effect on cardiomyocyte apoptosis in SHR receiving ISO (Figure 3.10, lower panel).



**Figure 3.10.** Effect of 4-5 days (acute model) and of 4.5 months (chronic model) administration of isoproterenol (ISO) and spironolactone (SPIRO) on cardiomyocyte apoptosis in spontaneously hypertensive rats (SHR) and Wistar Kyoto controls (WKY). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus WKY group; †  $p < 0.05$  versus other SHR groups; #  $p < 0.05$  versus WKY and SHR + ISO group; ‡  $p < 0.05$  versus WKY + ISO group.

### **3.3.4 Cardiomyocyte morphology.**

Table 3.2 shows the effect of ISO and SPIRO administration for 4.5 months on cardiomyocyte morphology in SHR. The relaxed sarcomere lengths was greater in the SHR compared with the WKY rats. Cardiomyocyte length assessed using image analysis was correlated with cardiomyocyte length assessed using flow cytometry ( $r=0.61$ ,  $p<0.0001$ ). As compared to WKY rats at 18.5 months of age, cardiomyocyte length, determined either using image analysis or flow cytometry, was greater in age-matched SHR (Table 3.2). Although cardiomyocyte width tended to be greater in SHR, this failed to reach significance (Table 3.2). Neither ISO administered to SHR or WKY, nor ISO plus SPIRO administered to SHR modified cardiomyocyte morphology.

### **3.3.5 Matrix metalloproteinase activity and expression.**

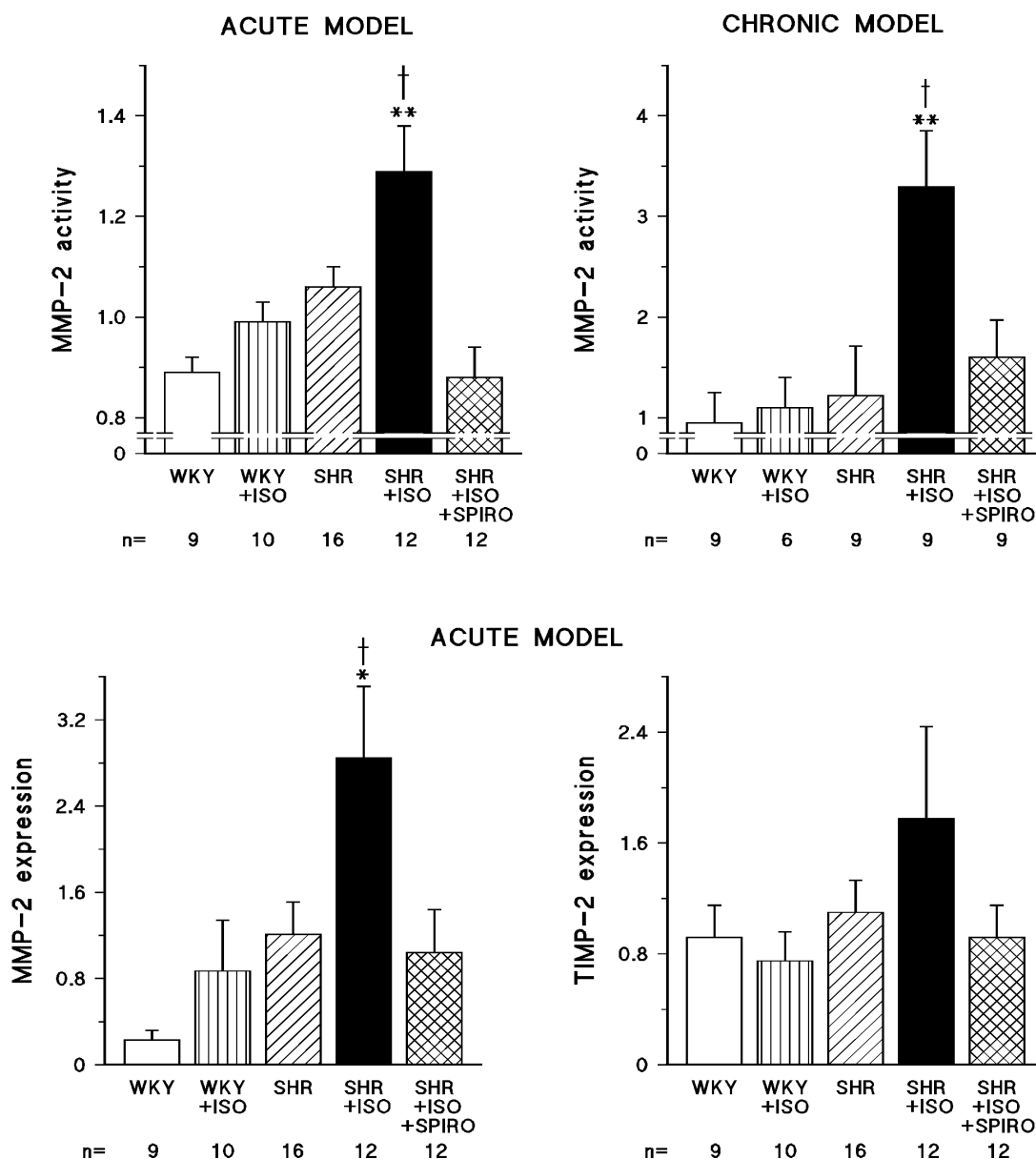
Figure 3.11 (upper panels) show the impact of 4-5 days and of 4.5 months of ISO and SPIRO administration on myocardial MMP-2 activity. Figure 3.11 (lower panels) show the impact of 4-5 days of ISO and SPIRO administration on myocardial MMP-2 and TIMP-2 expression in SHR and WKY rats. As compared to WKY control rats, age-matched SHR had a similar myocardial MMP-2 activity and expression. Four-to-five days of ISO produced a marked increase in myocardial MMP-2 activity and expression in SHR, but failed to produce similar effects in WKY rats.

**Table 3.2.** Effects of chronic administration of isoproterenol (ISO) and spironolactone (SPIRO) on cardiomyocyte morphology in spontaneously hypertensive rats (SHR).

|                                        | WKY             | WKY+ISO         | SHR                | SHR+ISO           | SHR+ISO+SPIRO      |
|----------------------------------------|-----------------|-----------------|--------------------|-------------------|--------------------|
| n=                                     | (9)             | (6)             | (9)                | (9)               | (9)                |
| Cardiomyocyte length ( $\mu\text{m}$ ) | 75.7 $\pm$ 1.5  | 74.9 $\pm$ 1.6  | 87.1 $\pm$ 2.9*    | 92.1 $\pm$ 1.6*** | 86.7 $\pm$ 1.6**   |
| Cardiomyocyte width ( $\mu\text{m}$ )  | 24.7 $\pm$ 1.7  | 26.2 $\pm$ 1.5  | 28.3 $\pm$ 0.7     | 28.8 $\pm$ 0.8    | 28.4 $\pm$ 0.6     |
| FL1-W#                                 | 85.8 $\pm$ 12.2 | 81.2 $\pm$ 7.3  | 179.9 $\pm$ 6.9*** | 182.2 $\pm$ 8.5** | 188.4 $\pm$ 6.7*** |
| Sarcomere length ( $\mu\text{m}$ )     | 1.87 $\pm$ 0.01 | 1.89 $\pm$ 0.02 | 2.21 $\pm$ 0.02    | 2.27 $\pm$ 0.01   | 2.20 $\pm$ 0.01    |

# FL1-W indicates time of cell in path of laser beam. WKY, Wistar Kyoto control; LV, left ventricle. \* p<0.05,

\*\* p<0.01, \*\*\* p<0.001 versus WKY and WKY+ISO groups.



**Figure 3.11.** Effects of 4-5 days (acute model) and of 4.5 months (chronic model) of isoproterenol (ISO) and spironolactone (SPIRO) administration on myocardial matrix metalloproteinase-2 (MMP-2) activity (gelatinase activity) (upper panels), and of 4-5 days (acute model) of ISO and SPIRO administration on MMP-2 expression and tissue inhibitor of MMP-2 (TIMP-2) expression (lower panels) in spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) control rats. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus WKY and WKY+ISO groups; †  $p < 0.05$  versus other SHR groups.

The ISO-induced increase in MMP-2 activity and expression noted in SHR was prevented by SPIRO administration. Changes in MMP-2 activity in response to either ISO or SPIRO in SHR were not paralleled by changes in TIMP-2 expression. Similarly, 4.5 months of ISO produced a marked increase in myocardial MMP-2 activity in SHR; an effect which was not observed in WKY rats. In addition, the ISO-induced increase in MMP-2 activity noted in SHR was prevented by SPIRO administration.

### **3.4 Discussion.**

The main finding of the present study is that independent of blood pressure changes, mineralocorticoid receptor blockade prevented the adverse effects of  $\beta$ -adrenoreceptor activation on the hypertensive heart. In this respect aldosterone receptor blockade abolished adrenergic-induced increases in MMP-2 expression and activity, the accumulation of myocardial collagen of the non-cross-linked form, and cardiomyocyte apoptosis.

By promoting tears in myocardial collagen and hence encouraging side-to-side cardiomyocyte slippage, myocardial MMP activation may play a key role in mediating cardiac dilatation (King et al 2003, Peterson et al 2001, Spinale et al 1999). The present study provides the first evidence to suggest that the hypertrophic myocardium is susceptible to  $\beta$ -adrenergic-mediated increases in MMP-2 expression and activation, which in-turn may contribute toward an enhanced susceptibility in pressure-overload

states to cardiac dilatation and hence LV systolic chamber dysfunction. The present study also suggests that these effects are in-part dependent on mineralocorticoid receptor activation and hence can be prevented by mineralocorticoid receptor blockade. Prior studies have demonstrated a beneficial effect of mineralocorticoid receptor blockade on myocardial MMP-2 activity (Fraccarollo et al 2011, Kuster et al 2005, Suzuki et al 2002). In contrast however, despite producing beneficial effects on LV cavity dimensions, aldosterone synthase inhibition or mineralocorticoid receptor blockade are unable to attenuate the increased MMP activity noted in viable myocardium post-myocardial infarction (Mulder et al 2008). Furthermore, mineralocorticoid receptor blockade, although reducing infarct expansion post-myocardial infarction, was unable to modify MMP expression in the infarct zone (Fraccarollo et al 2008). Therefore, it is important to consider whether beneficial effects of mineralocorticoid receptor blockade on myocardial MMP-2 activity in some studies (Fraccarollo et al 2011, Kuster et al 2005, Suzuki et al 2002) were a cause or a consequence of an improved LV systolic chamber function. In this regard, an important aspect of the present study is that the ability of spironolactone to prevent  $\beta$ -adrenergic-mediated increases in myocardial MMP-2 expression and activation occurred well before the development of cardiac dilatation and LV systolic chamber dysfunction. Thus, the present study also provides the evidence to suggest that mineralocorticoid receptor activation causes increases in myocardial MMP-2 expression and that blockade of this effect may prevent cardiac dilatation and LV systolic

chamber dysfunction. In this regard, these data are supported by the evidence that aldosterone stimulates MMP release in isolated cardiomyocytes (Rude et al 2005). Moreover, the role of mineralocorticoid receptors on macrophages may result in the release of metalloproteinases (Marney and Brown 2007) which could also contribute toward mineralocorticoid receptor-mediated cardiac dilatation.

As previously demonstrated (Badenhorst et al 2003b, Veliotis et al 2005), the present study also suggests that the hypertensive hypertrophic heart is susceptible to adrenergic-induced increases in myocardial collagen of the non-cross-linked form and that this effect is in-part dependent on aldosterone receptor activation. In this regard, excessive non-cross-linked myocardial collagen may promote cardiac dilatation by increasing the susceptibility of the collagen to degradation by collagenases such as MMP-2 (Badenhorst et al 2003b). Although previous studies have provided evidence to show that the ability of mineralocorticoid receptor blockade or inactivation to attenuate the development of cardiac dilatation and LV systolic chamber dysfunction is associated with a reduced myocardial fibrosis (Fraccarollo et al 2005, Fraccarollo et al 2011, Kuster et al 2005, Mulder et al 2008, Suzuki et al 2002, Wang et al 2004), the ability of conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene to protect against the development of LV systolic chamber decompensation produced by pressure-overload is not associated with a reduced myocardial fibrosis (Lothar et al 2011). However, in that study (Lothar et al

2011), the cross-linked properties of myocardial collagen were not evaluated and as suggested from the present study this is an essential interstitial change that may contribute toward LV dilatation and systolic chamber decompensation. Importantly, the role of interstitial changes in cardiac remodelling and the beneficial effects of mineralocorticoid receptor blockade is supported by clinical studies in heart failure (Tsutamoto et al 2001, Zannad et al 2000).

In the present study, the ability of  $\beta$ -adrenergic activation to increase myocardial MMP-2 activity in SHR and the beneficial action of mineralocorticoid receptor blockade on this effect were attributed to changes in myocardial MMP-2 but not TIMP-2 expression. Although I cannot exclude a possible role of other members of the TIMP family (1, 3 or 4), TIMP-2 has a high affinity for MMP-2 (Olson et al 1997) and hence is the most likely TIMP to modify MMP-2 activity. Although not evaluated in the present study,  $\beta$ -adrenoreceptor mediated increases in myocardial MMP-2 expression may be produced by an increased expression of an extracellular matrix metalloproteinase inducer (EMMPRIN) via a reactive oxygen species-dependent pathway (Siwik et al 2008). An additional mechanism that could contribute to changes in myocardial MMP-2 activity in the present study may be through a direct action of non-cross-linked collagen. Indeed, the presence or addition of soluble collagen in vitro may stimulate MMP activity (Ruangpanit et al 2001).

In contrast to the increases in myocardial MMP-2 expression and/or activity that have been reported to occur in Dahl salt-sensitive rats (Sakata

et al 2004) and in humans with aortic stenosis (Polyakova et al 2004) prior to the development of pump dysfunction, in the present study SHR not receiving ISO had similar levels of myocardial MMP-2 activity and expression as compared to normotensive controls. However, the data obtained from the present study are indeed consistent with a normal myocardial MMP-2 activity reported to occur in SHR prior to the development of heart failure as previously described (Mujumdar and Tyagi 1999).

The present study employed the technique of zymography to assess the activity of MMP 2 and MMP 9, the cardiac gelatinases. Zymography is unable to adequately assess the activity of other MMPs which have been found to be present in the myocardium in small quantities including MMP 1, MMP 3, MMP 13 and MMP 14 (Spinale 2002). These MMPs may be dysregulated in adverse cardiac remodeling and thus should be considered candidates for future study as techniques to improve assessments of their activity improve.

Although  $\beta$ -adrenergic activation is a recognized stimulus for cardiomyocyte apoptosis (Singh et al 2001) and mineralocorticoid receptor blockade has been shown to prevent apoptosis in cardiac disease (Fraccarollo et al 2011, Kuster et al 2005, Suzuki et al 2002), the ability of conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene to protect against the development of LV systolic chamber decompensation produced by pressure-overload is not associated with a reduced myocardial apoptosis (Lothar et al 2011).

Hence, whether a reduction of myocardial apoptosis is an important mechanism explaining the benefits of mineralocorticoid receptor inactivation on LV chamber dimensions and systolic chamber function is still uncertain. In this regard, the present study is the first to show that adrenergic-induced increases in cardiomyocyte apoptosis in hypertensive hypertrophy is in-part dependent on mineralocorticoid receptor activation and hence is prevented by mineralocorticoid receptor blockade. In keeping with previous studies (Osadchii et al 2007b), in the present study  $\beta$ -adrenoreceptor activation promoted cardiomyocyte apoptosis in the initial period of ISO administration, but this effect was not sustained. Moreover, adrenergic activation induced the same degree of cardiomyocyte apoptosis in normotensive control rats, but this did not translate into cardiac dilatation and pump dysfunction. Hence, although cardiomyocyte apoptosis is an acknowledged cause of heart failure (Foo et al 2005, Wencker et al 2003), its role in promoting adrenergic-induced pump dysfunction in the present study may be questioned. Nevertheless, even a relatively brief period of cell death produced by ISO, together with the sustained increases in cardiomyocyte apoptosis noted in SHR as compared to WKY rats irrespective of whether they were receiving ISO, may have contributed toward myocardial damage and dysfunction. The higher percentage apoptosis in SHR as compared to WKY is consistent with prior studies (Fortuno et al 1999) and the differences in percentage apoptosis noted in the present study between the acute and chronic

models are likely to reflect previously reported age associated changes in apoptosis (Kajstura et al 1996).

Since the cellular myocardium is composed of cardiomyocytes, fibroblasts, immune cells, smooth muscle cells and endothelial cells of the vasculature we must bear in mind that these cells could contribute towards LVH and may through direct or indirect means contribute to LV remodeling. Hence in future studies it may be useful to assess the morphology and rate of apoptosis within these various cell populations.

Cardiac chamber dilatation may be the consequence of a greater increase in myocyte length than width (Gerdes et al 1992, Tamura et al 1998). In the present study, using both image analysis as well as flow cytometry, although I was able to show that SHR had a considerably enhanced cardiomyocyte length as compared to WKY rats, neither chronic  $\beta$ -adrenoreceptor activation, nor mineralocorticoid receptor blockade modified these changes, despite influencing cardiac cavity dimensions. Thus, the present study suggests that alterations in cardiomyocyte length are unlikely to contribute to the susceptibility of the hypertrophic heart to adrenergic-induced cardiac dilatation in male SHR. A potential reason for the inconsistency between the present and a previous study (Tamura et al 1998) is that this previous study was conducted in a model of hypertension in female rats, whereas ours was performed in males.

In conclusion, the present study suggests that mineralocorticoid receptor blockade may abolish the adverse effects of  $\beta$ -adrenoreceptor-mediated deleterious cellular and molecular effects on the hypertensive

heart, including activation of MMPs, cardiomyocyte apoptosis and interstitial changes. These results may explain the ability of a mineralocorticoid receptor blocker to prevent the transition from compensated LVH to cardiac dilatation and LV systolic chamber dysfunction induced by chronic  $\beta$ -adrenoreceptor-mediated stimulation. The results of the present study therefore provide further support for the use of an alternative option (mineralocorticoid receptor blockade) in hypertensive patients at a high risk of developing heart failure (LVH with eccentric LV remodelling) and in whom  $\beta$ -adrenoreceptor blockers could have special benefits, but in whom these benefits are offset by the potential of harmful or inadequate beneficial actions of this class of agents as compared to other antihypertensive agents (Bakris and Sowers 2004, Dahlof et al 2002, Dahlof et al 2005, Devereux et al 2004a, Williams et al 2006). Clinical studies are therefore required to evaluate whether mineralocorticoid receptor blockers may be especially advantageous in those with hypertensive LVH and asymptomatic left ventricular systolic dysfunction.

## Chapter 4

### **Susceptibility of Chronically Infarcted Spontaneously Hypertensive Rat Myocardium to Systolic Dysfunction.**

Norton GR, Veliotis DGA, Osadchii O, Woodiwiss AJ and Thomas DP. Susceptibility to systolic dysfunction in the myocardium from chronically infarcted spontaneously hypertensive rats. *Am J Physiol Heart and Circ Physiol.* 2008;294:H372-H378.

**ABSTRACT**

I explored whether the hypertensive heart is susceptible to myocardial dysfunction in viable non-infarcted tissue post-myocardial infarction (MI), the potential mechanisms thereof, and the impact of these changes on pump function. Six-to-seven months after ligation of the left anterior descending coronary artery, left ventricular (LV) myocardial systolic function as assessed from % shortening of the non-infarcted lateral wall segmental length determined over a range of filling pressures (ultrasonic transducers placed in the lateral wall in anaesthetized, open-chest, ventilated rats) and % thickening of the posterior wall (echocardiography) was reduced in infarcted spontaneous hypertensive rats (SHR-MI) ( $p < 0.05$ ), but not in normotensive Wistar Kyoto (WKY-MI) animals as compared to corresponding controls (SHR-Sham, WKY-Sham). This change in regional myocardial function in SHR-MI, but not in WKY-MI, occurred despite a similar degree of LV dilatation (increased LV end-diastolic dimensions and volume intercept of the LV end-diastolic pressure-volume relation) in SHR-MI and WKY-MI rats and a lack of difference in LV relative wall thinning, LV wall stress, apoptosis (TUNEL) or necrosis (pathological score) between SHR-MI and WKY-MI rats. Although the change in regional myocardial function in the SHR-MI group was not associated with a greater reduction in baseline global LV chamber systolic function (endocardial fractional shortening- $FS_{end}$  and end-systolic elastance [ $LV E_{es}$ ] determined in the absence of an adrenergic stimulus), in the presence of an isoproterenol challenge, non-infarct zone LV systolic

myocardial dysfunction manifested in a significant reduction in LV  $E_{es}$  in SHR-MI compared to WKY-MI and SHR and WKY-Sham rats ( $p < 0.04$ ). In conclusion, these data suggest that with chronic MI, the hypertensive heart is susceptible to development of myocardial dysfunction, a change which cannot be attributed to excessive chamber dilatation, apoptosis or necrosis, but which in-turn, contributes toward a reduced cardiac adrenergic-inotropic reserve.

#### **4.1 Introduction.**

Hypertension induces the development of cardiac hypertrophy (Levy et al 1988), which is an acknowledged independent risk factor for the occurrence of a myocardial infarction (Kannel et al 1961, Levy et al 1988, Okin et al 2004). Reductions in pump function post-myocardial infarction may be exaggerated by hypertensive cardiac hypertrophy (Fletcher et al 1982, Fletcher et al 1986, Itter et al 2004, Jain et al 2002, Nass et al 2002, Nishikimi et al 1995). The excess pump dysfunction post-myocardial infarction that may be associated with hypertensive cardiac hypertrophy could be explained in-part through the adverse effects of afterload on infarct size (Nolan et al 1988, Pierard et al 1987) or through an augmented adverse remodelling process of non-infarcted cardiac tissue (Itter et al 2004, Jain et al 2002, Jilaihawi et al 2003). However, what has not been explored is whether the chronically infarcted hypertensive heart is susceptible to a decrease in systolic function in the remaining non-infarcted and chronically remodeled viable myocardium.

Some studies conducted in normotensive animals have established the role of a decreased regional myocardial systolic function in dilated, non-infarcted, but viable myocardial tissue post myocardial infarction (Cheung et al 1994, Davidoff et al 2004, Li et al 1995, Litwin et al 1992, Litwin et al 1994, Mill et al 1998, van der Velden et al 2004, Zhang et al 1999). These changes in regional myocardial systolic function may be caused through a number of mechanisms involving alterations in

cardiomyocyte calcium handling, apoptosis, myosin isoforms, morphometry, cytoskeleton proteins and ion transport systems as well as myocardial interstitial remodelling and non-myocyte factors (Gupta et al 2000, van der Velden et al 2004). Because hypertensive compared to normotensive hearts are more susceptible to alterations in viable tissue adrenergic signaling post myocardial infarction (Kouchi et al 2000) and to apoptosis in general (Liu et al 2000), this may increase the chances of myocardial systolic dysfunction occurring in remote cardiac tissue. The aim of the present study was therefore to assess whether the hypertensive as compared to the normotensive heart is more susceptible to the development of myocardial dysfunction in viable cardiac tissue post myocardial infarction, the potential mechanisms thereof, and whether this effect contributes toward global pump dysfunction.

#### **4.2 Methods.**

The study was conducted in accordance with the Guidelines on the Care and Use of Laboratory Animals (National Institute of Health, publication no. 86-23, 1996). The Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand approved this study (AESC Approval numbers: 2004:43:4 and 2004:59:4).

#### **4.2.1 Models and groups.**

Rats were housed at the Central Animal Services of the University of the Witwatersrand Medical School. Young (50-100g) post-pubescent male Spontaneously Hypertensive Rats (SHR) and normotensive control Wistar Kyoto rats (WKY) were subject to surgically-induced myocardial Infarctions (MI) by coronary artery ligation (McCormick et al 1994, Thomas et al 1998, Thomas et al 1999, Zimmerman et al 2001). Sham-operations were also performed in separate groups of SHR (SHR-Sham) and WKY (WKY-Sham). In order to optimize survival of the rats post-surgery several modifications of the previously described approach (McCormick et al 1994, Thomas et al 1998, Thomas et al 1999, Zimmerman et al 2001) were applied (see subsequent descriptions). Prior experience with the infarct model in normotensive rats has established that homogeneous infarct sizes may be produced thus allowing small groups of 5-6 rats to be studied at a time (Zimmerman et al 2001). However, as prior to the present study I was uncertain as to whether similarly homogeneous myocardial infarcts would occur in SHR, I therefore studied a larger sample of SHR than that of WKY.

##### **4.2.1.1 Surgery to induce myocardial infarction.**

Surgical procedures were performed in a surgical theatre under sterile conditions at the Central Animal Services, University of the

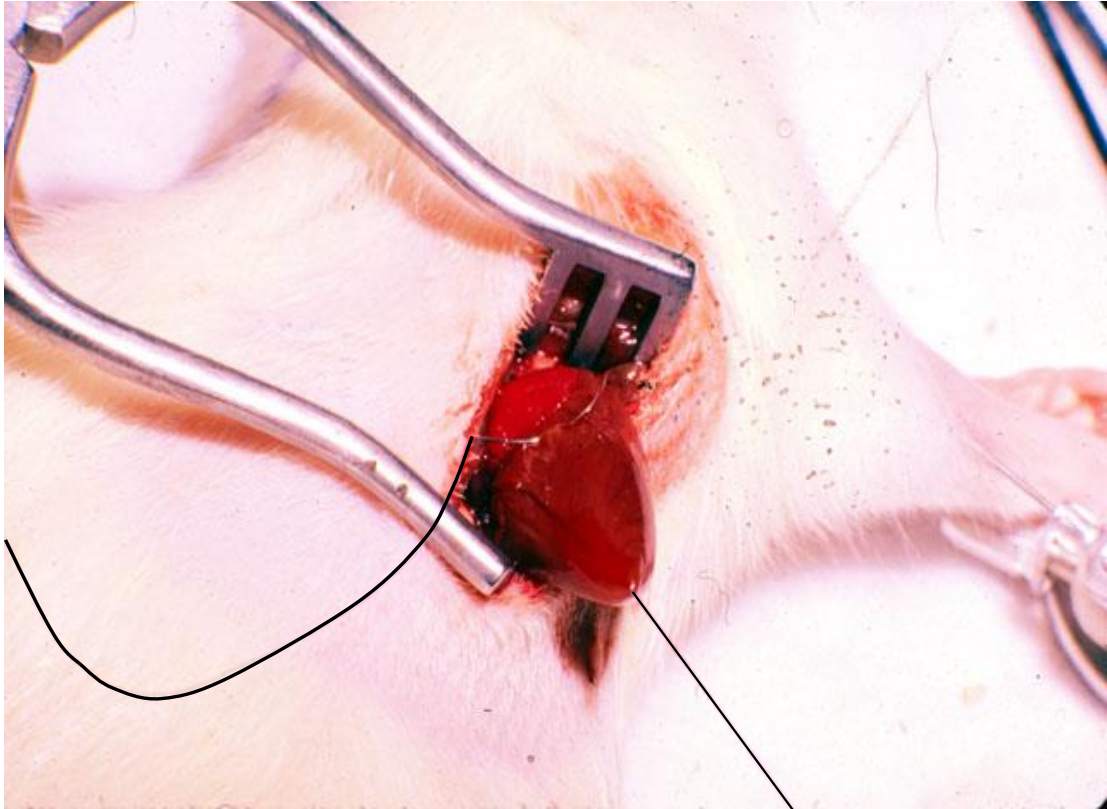
Witwatersrand. In brief, rats were anaesthetised with intraperitoneal injections of ketamine (50 mg/kg) and metomidine HCl (0.25 mg/kg). Under direct visualization of the vocal cords, using an especially designed modification to a neonatal laryngoscope, an appropriately sized uncuffed endotracheal tube was used to intubate the rats, following which the tube was connected to a rodent respirator (model 683, Harvard). Rats were ventilated at a respiratory rate of approximately 50-to-90 breaths per minute, with tidal volumes of approximately 2.5-to-4.5 mls and at pressures not greater than 20 cm H<sub>2</sub>O. Clinical criteria were used to judge the presence of appropriate and effective respiration including identifying the presence of appropriate chest expansion; the absence of cyanosis; and restitution post-surgery. Throughout each procedure the ventilation rate and volumes were altered in order to maintain stability of clinical signs.

To expose the heart, a 15 mm thoracotomy was incised on the left anterolateral thorax between the fifth and sixth ribs and a pericardial window was opened. To stabilize the exposed hearts for further manipulation and procedures a 6-0 silk suture tie was passed superficially through the cardiac apex and secured with minimal tension in a position that exposed the left coronary artery. Once the left main coronary artery was clearly visualized a second 6-0 silk suture was passed through the myocardium under the left main coronary artery, 3-4 mm caudal to its emergence inferior to the left main atrial appendage (Figure 4.1). In order

to produce myocardial infarcts through coronary ligation, the sutures around the coronary artery were tied in the SHR-MI and WKY-MI groups.

Ligation at this level consistently induced medium sized myocardial infarctions. Alternatively, in control operations in the SHR-Sham and WKY-Sham groups the suture was left untied *in situ*. The surgical incision was closed in three separate stages; the intercostal muscle layer was opposed first with a continuous 3-0 chromic suture, while ensuring that the lungs were fully expanded; the second muscular layer was opposed with a continuous 3-0 vicryl suture; and lastly the skin was opposed with a 3-0 silk purse string suture. Anaesthesia was reversed with the  $\alpha_2$ -adrenoreceptor antagonist (atipamezole HCl at 0.1 ml/100 g) following which the rats were extubated once spontaneous breathing ensued. Immediately post-surgery, to assist recovery from the anaesthetic, rats were placed in a perspex container containing 95% O<sub>2</sub> and 5% CO<sub>2</sub>, until they became mobile.

Thereafter, in order to reduce post-operative mortality several steps were taken. During recovery, while the rats were housed at 36°C, 5 mg of the antibiotic enrofloxacin, dissolved in 1 ml of Ringer's lactate solution, was injected subcutaneously. To minimize pain, two intraperitoneal injections of the opiate analgesic buprenorphine (10 µg/kg) were administered, the first when the rats started moving and the second after 8-to-12 hours. For one week post-surgery, rats were fed a nutritionally enriched supplement (Ensure) to guarantee adequate nutritional intake. Under these conditions, during the first week post-coronary ligation or



**Figure 4.1.** Photograph illustrating the surgical procedure used to pass a suture under the left anterior descending coronary artery through a left lateral thoracotomy in anaesthetised rats. The suture was either tied or left loose *in situ* depending on whether the operation was to be a coronary ligation or a control procedure respectively. The tie holding the heart in place is clearly visible at the cardiac apex.

sham surgery, the post-operative mortality was zero. All sham-operated rats and only those rats with distinct evidence of myocardial ischaemia noted immediately post-coronary ligation, were followed up over a period of six-to-seven months. Signs used to confirm the presence of myocardial ischaemia post-coronary artery ligation included pallor of the myocardium distal to the ligated artery as well as akinesis and “ballooning” of the LV free wall.

Only one rat (SHR-MI) died during the follow-up period of six-to-seven months. Additionally, at the end of the study, one rat from the SHR-MI and another from the WKY-MI group were excluded from the statistical analysis because the surgically-induced infarct scars in these rats were identified on visual inspection to be non-transmural.

#### **4.2.2 Echocardiography.**

Six-to-seven months post-surgery, echocardiography was employed to evaluate LV remodelling, systolic chamber and myocardial function, as well as LV wall stress *in vivo*. Two-dimensional targeted M-mode echocardiography, in conjunction with simultaneous invasive carotid blood pressure assessments, was performed on rats anaesthetized with ketamine and xylazine as previously described (chapter 2, Chung et al 1998, Norton et al 2002). A 7.5 MHz transducer probe coupled to a Hewlett Packard Sonos 2500 sector scanner was used to measure LV end-diastolic (LVED) and LV end systolic (LVES) internal diameters

(LVEDD and LVESD respectively) and both the LVED and LVES posterior wall thickness (PWT). All measurements were performed as described in detail in chapter 2, section 2.2.3 (Chung et al 1998, Norton et al 2002). The simultaneous carotid artery blood pressure measurements were determined by means of a previously described fluid-filled catheter system connected to a pressure transducer (chapter 2, Norton et al 1996).

From the echocardiographic measurements several further indices of cardiac performance and remodelling were calculated from standard equations. Left ventricular endocardial fractional shortening ( $FS_{end}$ ), an *in vivo* assessment of global systolic chamber function, was determined as described in chapter 2 (Chung et al 1998, Norton et al 2002). In addition, left ventricular end diastolic (LVED) relative wall thickness (RWT) (which indicates the degree of hypertrophy of viable myocardium) was calculated (Cheung et al 1994) from the equation  $RWT = \frac{LVEDD}{LVEDD} \times \frac{LVEDD}{LVEDD}$ , where  $LVEDD$  = LV end diastolic diameter (LVEDD) and  $LVEDD$  = LV end diastolic diameter (LVEDD).

An *in vivo* assessment of regional myocardial systolic function in myocardial tissue remote from the infarct scar (as compared to the global systolic chamber function described above) was determined from the percentage thickening (change in or  $\Delta$ ) of the LV posterior wall from end diastole to peak systole ( $\Delta PWT$ ) (Litwin et al 1994). This was calculated from the equation  $([LVES\ PWT - LVED\ PWT] / LVED\ PWT) \times 100$ , where LVES is LV end systole. Circumferential LV systolic wall stress was calculated as previously described (Shimizu et al 1991) as:

$$\frac{SBP (0.5 LVIDs)^2 [1+ \{(0.5 LVIDs + PWTs)^2/(0.5 LVIDs + 0.5 PWTs)^2\}]}{(0.5 LVIDs + PWTs)^2 - (0.5 LVIDs)^2}$$

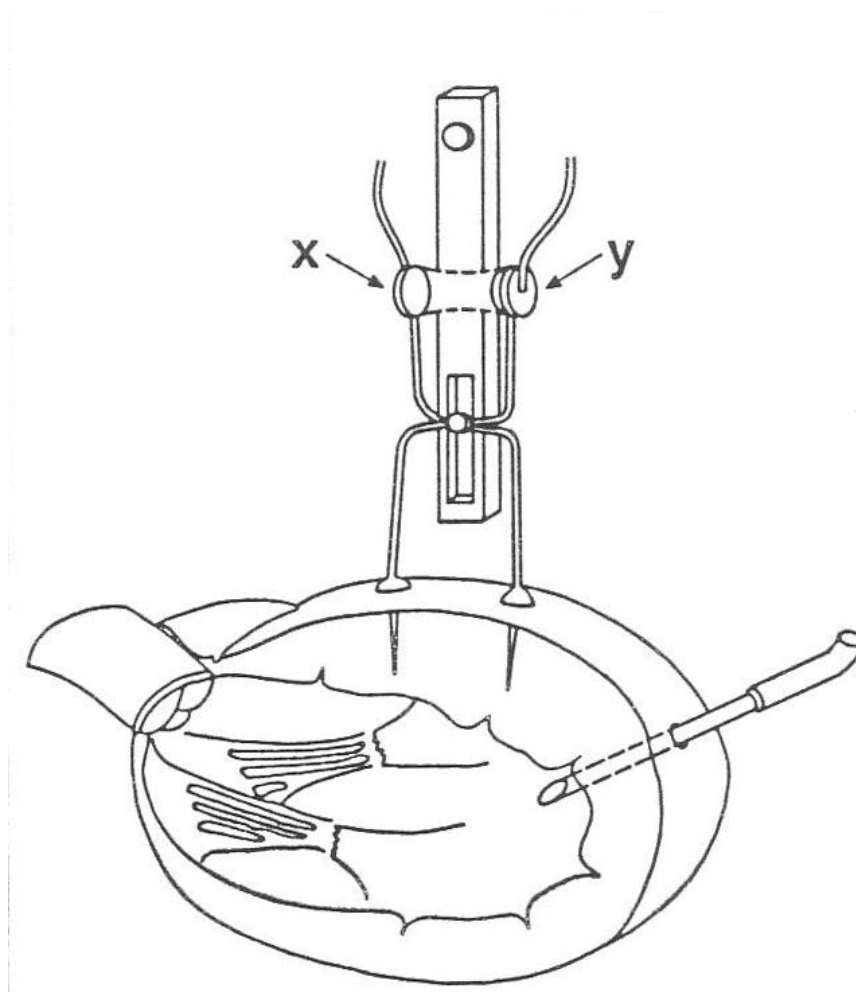
where SBP is systolic blood pressure, LVIDs is LV internal diameter in systole and PWTs is posterior wall thickness in systole.

#### **4.2.3 Regional myocardial systolic function.**

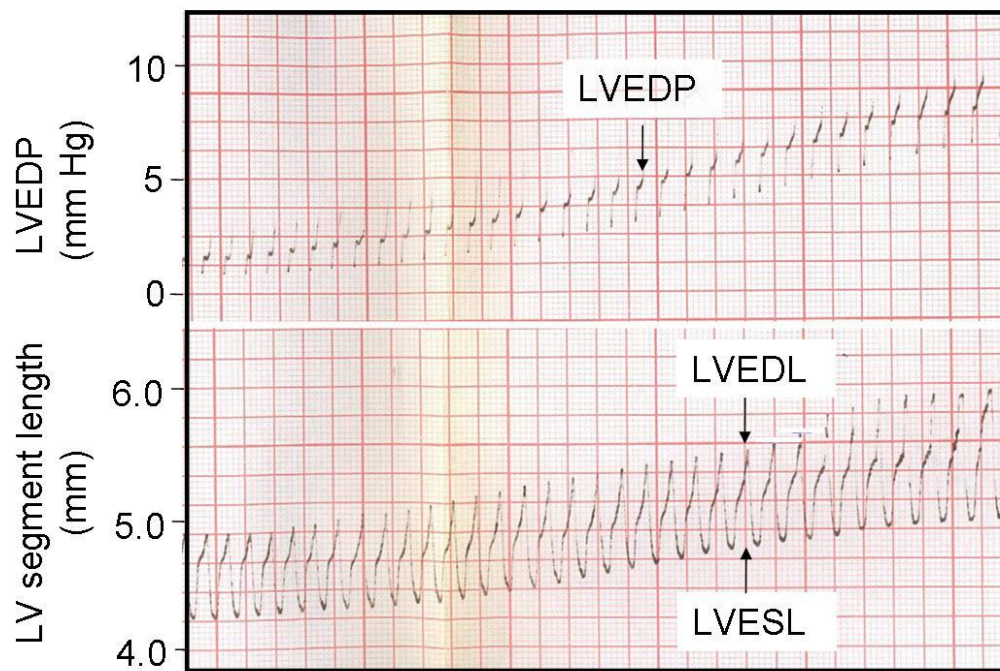
Techniques described and validated previously (Trifunovic et al 1995, Woodiwiss et al 1995) were employed to assess regional myocardial systolic function in segments of viable non-infarcted myocardium, over a range of LV filling pressures. Procedures were performed on hearts exposed through midline thoracotomies in ventilated rats anaesthetized with ketamine and xylazine (Trifunovic et al 1995, Woodiwiss et al 1995). In chapter 2, section 2.2.5, of the present thesis, the general measurement approach employed is described. However, myocardial segmental length changes rather than short axis diameter changes were quantified for the current assessment of regional myocardial systolic function. Briefly, piezoelectric ultrasonic transducers attached to the distal arms of two 30 gauge needles, which pivot around a central hinge and which were inserted to equal depths into the myocardium (Figure 4.2), were used to measure myocardial segmental length changes (Trifunovic et al 1995, Woodiwiss et al 1995). The 30 gauge needles were inserted into myocardial tissue without visible scar tissue either present at the point of insertion or between the points of insertion. The absence of scar tissue in

these areas was verified post-mortem. In order to allow for signal transmission between the ultrasonic transducers an ultrasonic medium was suspended between the transmitting and receiving transducers. The signals thus obtained were recorded and calibrated in order to measure the distance between the tips of the needles inserted in the myocardium (Trifunovic et al 1995, Woodiwiss et al 1995). Left ventricular end diastolic pressure (LVEDP) was measured using a fluid-filled catheter system connected to both a cannula inserted into the LV and a pressure transducer with an amplitude-frequency response uniform to 10 Hz.

In order to assess regional myocardial systolic function over a range of preloads, percentage shortening of the LV lateral wall long axis segmental length was calculated as  $(\text{LVED length} - \text{LVES length})/\text{LVED length} \times 100$  from LVED and LVES length measurements obtained over a range of LVEDP values (Figure 4.3) (Trifunovic et al 1995, Woodiwiss et al 1995). To modify preloads LV filling pressures were first gradually elevated by injecting an isotonic, iso-oncotic solution into a carotid artery cannula (Dextran) (Figure 4.3) and then slowly reduced by occluding the inferior vena cava. In three rats (2 SHR-Sham and 1 WKY-MI) percentage shortening could not be obtained at LVEDP values below 4 mm Hg because of the occurrence of numerous spontaneous extrasystolic contractions. Hence data are only presented for measurements made at LVEDP values greater than 4 mm Hg.



**Figure 4.2.** Diagrammatic representation of the experimental approach used to measure LV viable myocardium segmental length changes in rats. The transmitting and receiving piezoelectric transducers (X and Y respectively) are shown. A cannula inserted into the left ventricular chamber (shown on the bottom right) was coupled to a pressure transducer to measure LV filling pressures.



**Figure 4.3.** Representative data obtained from the experimental approach illustrated in Figure 4.2. LVEDL, left ventricular end diastolic length; LVESL, left ventricular end systolic length; LVEDP, LVED pressure.

#### 4.2.4 Isolated, perfused, heart preparations.

In addition to the preceding *in vivo* assessments of haemodynamic status, *ex vivo* measurements of LV systolic chamber function and LV remodelling were determined at controlled preloads, afterloads and heart rate in isolated, constant retrograde flow perfused, isovolumic heart preparations as described in chapter 2, section 2.2.6 (Norton et al 2002, Woodiwiss et al 2001). Briefly, a fluid-filled balloon-tipped catheter, connected via a three way tap to a pressure transducer and a micromanipulator, was inserted through the mitral valve into the LV thus enabling the measurement of developed and end diastolic LV pressures over a range of filling volumes. As described in chapter 2, the micromanipulator was used to increase the LV balloon volumes at 0.005-to-0.01 ml increments and at each filling volume LV pressures were measured (Norton et al 2002, Woodiwiss et al 2001). These measurements were made in the absence and then again in the presence of the inotrope, isoproterenol ( $10^{-8}$ M, Sigma). Isoproterenol, a non-selective  $\beta$ -adrenoreceptor agonist was specifically selected as an inotrope for the present study in order to evaluate contractile effects induced by both  $\beta_1$  and  $\beta_2$ -adrenoreceptor activation. In this regard, we have previously demonstrated that both  $\beta_1$  and  $\beta_2$ -adrenoreceptor-mediated inotropic effects occur in isolated, perfused rat heart preparations (Osadchii et al 2007a, Osadchii et al 2007b).

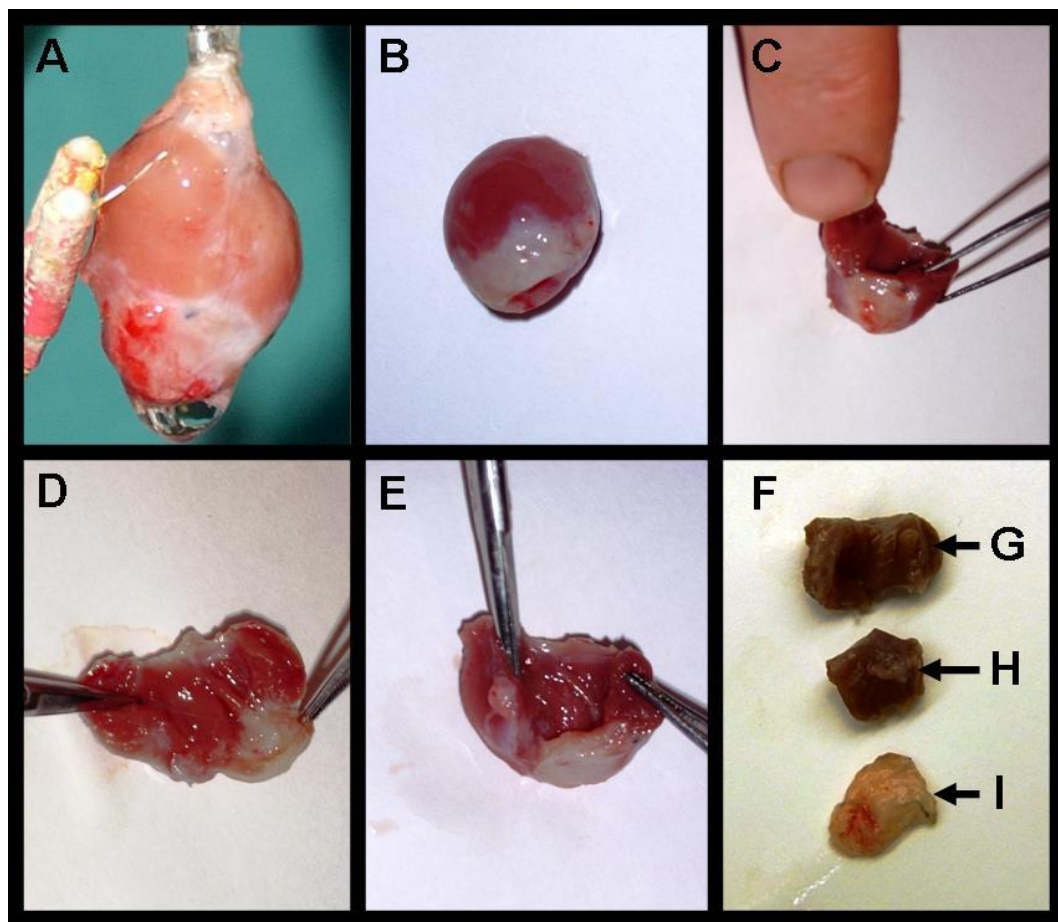
Left ventricular end diastolic (LVED) pressure-volume and left ventricular end systolic (LVES) pressure-volume relations were constructed in order to assess LV remodelling and LV systolic chamber function respectively (see chapter 2, section 2.2.6). To assess systolic chamber function left ventricular end systolic elastance (LV  $E_{es}$ ) was compared between groups (Norton et al 2002, Woodiwiss et al 2001). The  $\beta$ -coefficients of the relations which best fit LVES pressure-volume relationships, as described in chapter 2, section 2.2.6, were used to determine LV  $E_{es}$  (Osadchii et al 2007a). Since LV  $E_{es}$  is both preload and afterload-independent it strongly reflects contractile function, and was thus used in preference to other indices of LV systolic chamber function in the present study (Norton et al 2002, Sagawa et al 1988). In order to account for differences in body size between SHR and WKY rats and the impact of body mass on cardiac filling volumes, LV volumes were expressed as both an absolute value as well as an index per 100 g body weight (Zdorjewski et al 2002). For statistical comparisons of the degree of MI-induced LV dilatation (LV remodelling), the volume intercepts at a LVEDP of 0 mm Hg (LV  $V_0$ ) of the LVED pressure-volume relationships, were compared both with and without adjustments for body size differences (Norton et al 2002, Osadchii et al 2007a, Woodiwiss et al 2001).

#### **4.2.5 Scar tissue dimensions.**

After having obtained data from isolated, perfused heart preparations, the hearts were arrested in diastole by infusing a solution containing a high  $K^+$  concentration ( $8 \text{ mmol.l}^{-1}$ ) and no  $Ca^{2+}$ . When the hearts were examined a clearly demarcated area of scar tissue was evident which thus allowed for easy dissection of the scar tissue from the non-infarcted viable left ventricle. The viable left ventricular tissue was then further dissected into the left ventricular free wall and the inter-ventricular septum (Figure 4.4). The scar tissue and the viable LV myocardium were placed on a flat surface and the outer borders of both were delineated. Using planimetry the areas contained within the borders were quantified. The size of the infarct scar tissue was expressed both as the absolute area and as a percentage of the area of the endocardial surface of viable myocardium.

#### **4.2.6 Myocardial necrosis and apoptosis.**

A longitudinal slice of viable LV posterior wall tissue from the cardiac apex to the base, was obtained from all rats for histological analysis. The tissue was stored, embedded in paraffin wax blocks, and cut into a series of  $5 \text{ }\mu\text{m}$  thick sections as described in chapter 3, section 3.2.3. Sections were stained with van Gieson's stain and a pathological



**Figure 4.4.** Example of a heart, with a myocardial infarction scar, illustrating the dissection procedures used to separate viable myocardium from scar tissue. A, Isolated perfused heart with pacing electrodes attached prior to dissection; B, heart with infarct scar clearly visible; C, D, E, progressive dissection of myocardium from infarct scar; F, completed dissection with; G, LV free wall; H, inter-ventricular septum; I, myocardial infarct scar.

score was assigned as described in chapter 3, section 3.2.3. On myocardial tissue sections conjoining those used to assess the pathological score, the extent of cardiomyocyte apoptosis was also quantified as described in detail in chapter 3, section 3.2.4. Apoptosis was expressed as the percentage of cardiomyocyte nuclei undergoing apoptosis.

#### **4.2.7 Data analysis.**

A two-way ANOVA was used to determine differences and interactions between groups. The lines of best fit for cardiac function and other relations were determined by regression analysis. In the text, all values are represented as mean  $\pm$  SEM, and for these studies statistical significance was set at  $p < 0.05$ .

### **4.3 Results.**

#### **4.3.1 Cardiac structural characteristics.**

Measurements of viable LV and right ventricular weights, body weight and scar tissue size are summarized in Table 4.1. Both LV weight and LV weight indexed to 100g body weight were increased in Sham-operated SHR as compared to sham-operated WKY controls (Table 4.1). Scar tissue dimensions were similar between SHR-MI and WKY-MI groups

**Table 4.1.** Left (LV) and right (RV) ventricular characteristics of normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) 6-7 months after myocardial infarction (MI) or sham operations (Sham).

|                                 | WKY Sham<br>(n=7) | WKY MI<br>(n=6) | SHR Sham<br>(n=11) | SHR MI<br>(n=10) |
|---------------------------------|-------------------|-----------------|--------------------|------------------|
| Body weight (BW) (g)            | 400±17            | 436±11          | 360±5*             | 360±7.5*         |
| Viable LV weight (g)            | 0.94±0.03         | 0.86±0.05       | 1.20±0.03*         | 1.01±0.04*†      |
| Viable LV weight/BW (%)         | 0.24±0.007        | 0.20±0.009†     | 0.33±0.009*        | 0.28±0.01*†      |
| RV weight (g)                   | 0.30±0.02         | 0.30±0.03       | 0.32±0.02          | 0.34±0.02        |
| Scar tissue weight (g)          | -                 | 0.17±0.02       | -                  | 0.17±0.02        |
| % Scar tissue area <sup>a</sup> | -                 | 25±1            | -                  | 26±2             |

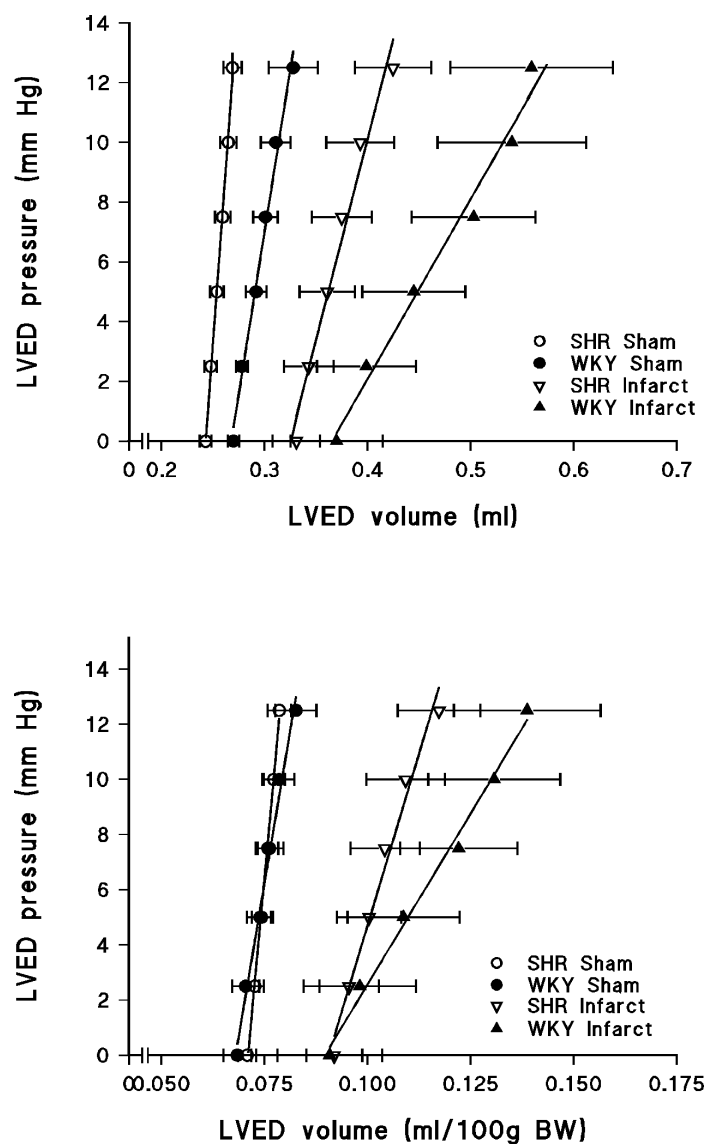
a, Scar tissue area/viable tissue area.\* p<0.05 versus WKY groups, † p<0.05 versus WKY or SHR Sham.

when comparing either the wet weight of scar tissue or the percentage endocardial area of scar tissue versus that of the viable myocardium (Table 4.1). Post-myocardial infarction, the viable LV myocardial weight expressed as grams myocardium per 100 grams body weight, was reduced in both SHR and WKY groups (Table 4.1). Nevertheless, post-MI the viable LV myocardial tissue weight was still greater in SHR than in WKY (Table 4.1).

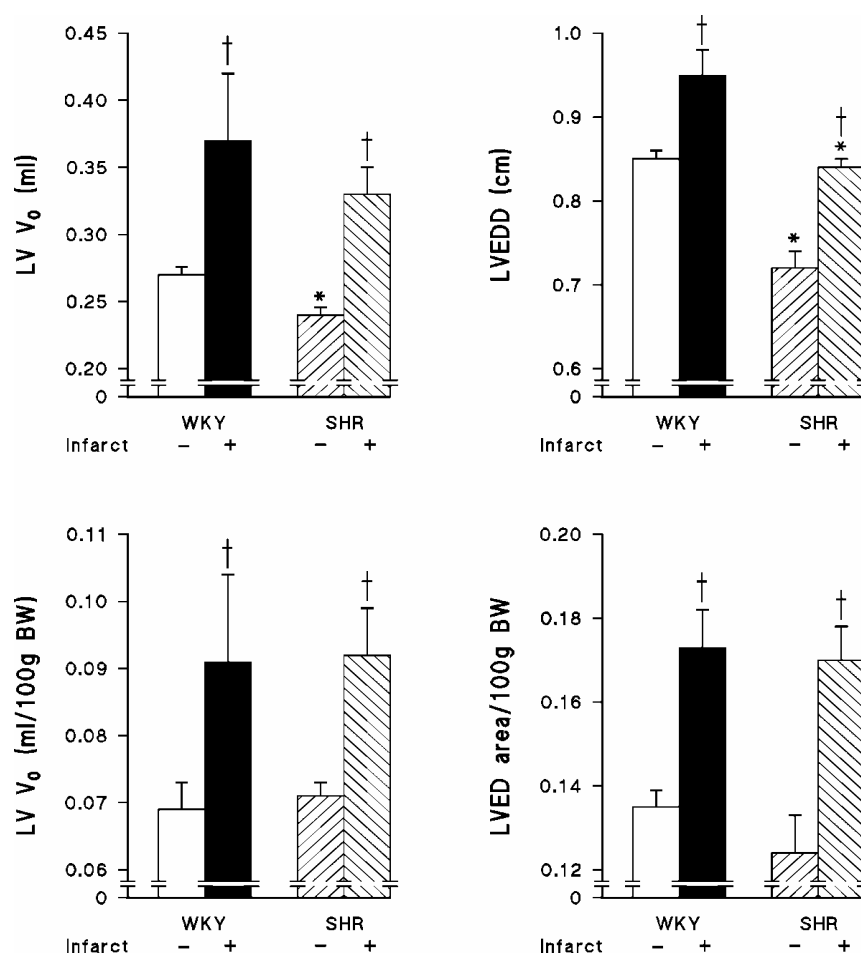
#### **4.3.2 LV remodelling.**

The impact of MI on LV dimensions and LVED pressure-volume relations in SHR and WKY rats is shown in Table 4.2 and Figures 4.5 and 4.6. Sham-operated SHR as compared to sham-operated WKY rats had left shifted LVED pressure-volume relations (Figure 4.5) with lower LV  $V_0$ , as well as LVEDD and LVESD values (Table 4.1 and Figure 4.6). However, as SHR had a reduced body mass this may account for the differences in cardiac dimensions. To account for differences in body size, LV dimensions and volumes were therefore expressed per 100 grams body weight. In this regard LV cross-sectional area (Figure 4.6) and LV volumes (Figures 4.5, lower panel and Figure 4.6 for LV  $V_0$ ) indexed to body weight were similar between SHR-Sham and WKY-Sham groups.

Six-to-seven months post-coronary artery ligation, LVED pressure-volume relations were substantially right shifted (Figure 4.5) and marked increases in LV  $V_0$  (Figure 4.6), LV dimensions (Figure 4.6 and Table 4.2)



**Figure 4.5.** Left ventricular end diastolic (LVED) pressure-volume relations obtained six-to-seven months post-myocardial infarction (MI) or sham-operation (Sham) in spontaneously hypertensive rats (SHR) and normotensive control Wistar Kyoto rats (WKY). Upper panel shows relations with actual LV volumes and the lower panel shows the relations with LV volume indexed to body weight (BW). Table 4.1 gives sample sizes and Figure 4.7 compares volume intercepts at 0 mm Hg (LV  $V_0$ ).



**Figure 4.6.** Left ventricular volume intercepts (LV  $V_0$ ) and body weight (BW) indexed LV  $V_0$  of the LV end diastolic (LVED) pressure-volume relations, the LVED diameters (LVEDD) and LVED areas indexed to BW, obtained six-to-seven months post-myocardial infarction (MI) or sham-operation (Sham), in spontaneously hypertensive rats (SHR) and normotensive control Wistar Kyoto rats (WKY). Table 4.1 gives sample sizes. \*  $p < 0.05$  versus WKY groups, †  $p < 0.05$  versus WKY or SHR Sham.

**Table 4.2.** Left ventricular (LV) characteristics, blood pressures and LV wall stress of normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) 6-7 months after myocardial infarction (MI) or sham operations (Sham).

|                                  | WKY Sham<br>(n=7) | WKY MI<br>(n=6) | SHR Sham<br>(n=11) | SHR MI<br>(n=10) |
|----------------------------------|-------------------|-----------------|--------------------|------------------|
| LVESD (cm)                       | 0.47±0.01         | 0.66±0.04†      | 0.34±0.02*         | 0.54±0.02*†      |
| LVED h/r <sup>a</sup>            | 0.36±0.02         | 0.34±0.04       | 0.54±0.04*         | 0.57±0.03*       |
| SBP/DBP (mm Hg)                  | 120±12/91±9       | 141±5/102±5     | 181±4/120±4*       | 161±6/117±3*     |
| LVES stress (g/cm <sup>2</sup> ) | 43±4              | 63±2†           | 42±3               | 62±5†            |

a, LVED wall thickness (h)-to-radius (r) ratio from echocardiography. LVED, LV end diastolic; LVES, LV end systolic; LVESD, LVES diameter; SBP, systolic blood pressure; DBP, diastolic BP. \* p<0.05 versus WKY groups, † p<0.05 versus WKY or SHR Sham.

and LV cross-sectional area (Figure 4.6) were evident in both the WKY-MI and SHR-MI groups. Notably, LV diastolic pressure-volume relations (Figure 4.5, lower panel), and LV cross-sectional area and LV  $V_0$  (Figure 4.6) were similar in SHR-MI and WKY-MI groups after normalizing the volumes to body weight. Although post-coronary ligation the internal LV diameters in both SHR-MI and WKY-MI were increased (Figure 4.6 and Table 4.2), the relative wall thickness as expressed as the ratio of the LVED posterior wall thickness to the internal radius remained unchanged in both groups (Table 4.2).

#### **4.3.3 LV wall stress.**

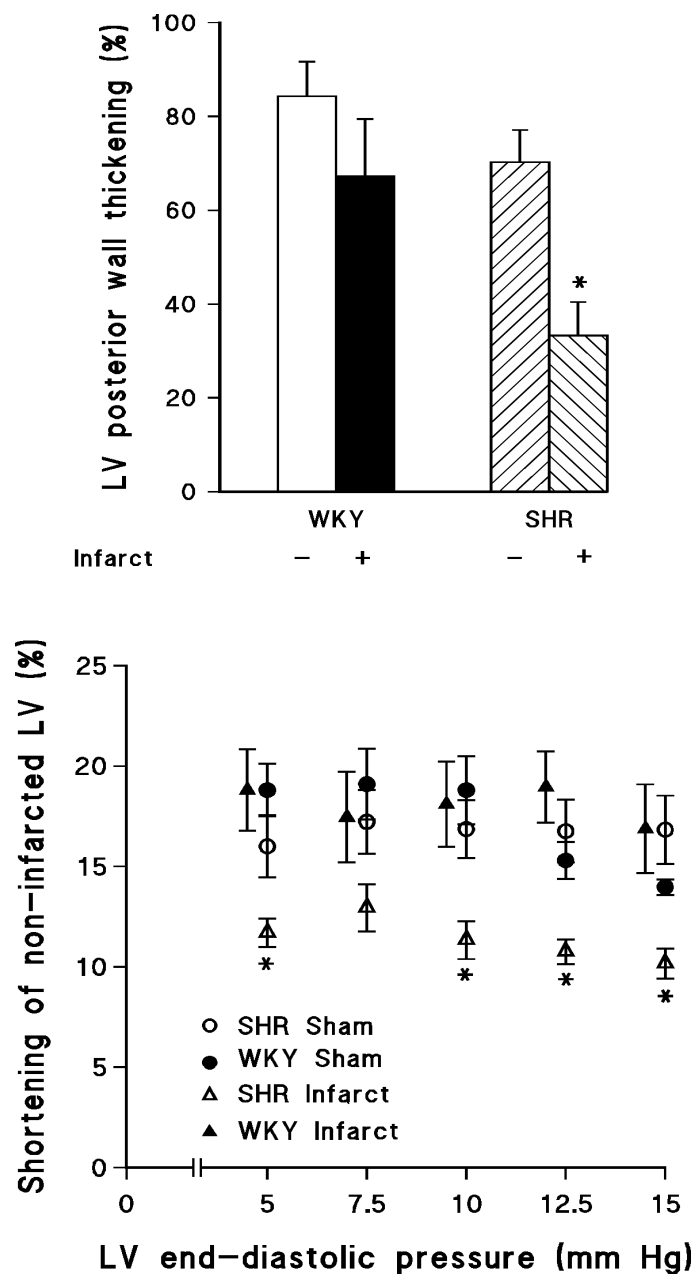
Coronary artery ligation resulted in an equivalent increase in LVES posterior wall stress in both SHR-MI and WKY-MI as compared to the corresponding SHR-Sham and WKY-Sham operation control groups (Table 4.2). The elevated LVES wall stress may be accounted for by an increased LVES radius (see increased LVESD in Table 4.2), without a proportionate parallel increase in LVES wall thickness (data not shown). Importantly, these differences cannot be attributed to an effect on structural remodelling of viable tissue as the LVED relative wall thickness values were unchanged post-myocardial infarction (Table 4.2). Rather, the effect of MI on LVES wall stress are attributed to decreases in LV systolic chamber function with associated increases in LVES diameters (Table 4.2).

#### 4.3.4 Regional systolic function of viable myocardium.

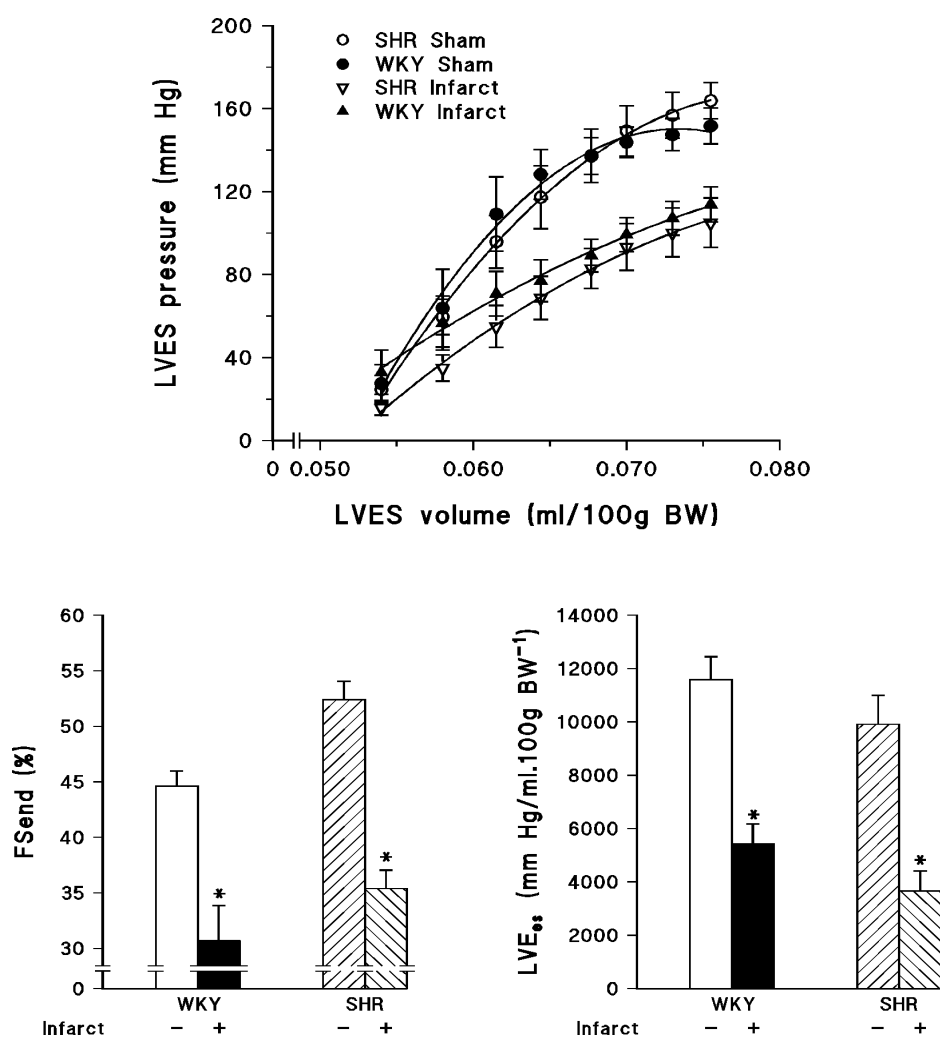
The impact of myocardial infarction, assessed six-to-seven months post-coronary artery ligation, on viable myocardial tissue systolic function, in regions distant to the infarct scar is shown in Figure 4.7. Systolic function as assessed both non-invasively using echocardiographic measurements of LV systolic posterior wall thickening and invasively using *in vivo* assessments of percentage segmental shortening of viable myocardial tissue, in the LV lateral wall, determined over a range of filling pressures, were similar in SHR-Sham and WKY-Sham operated groups. Although WKY rats still had a preserved systolic function in the posterior and lateral walls of the left ventricle six-to-seven months post-myocardial infarction, SHR had a reduced LV viable myocardial tissue systolic function six-to-seven months post-myocardial infarction (Figure 4.7).

#### 4.3.5 Systolic chamber function.

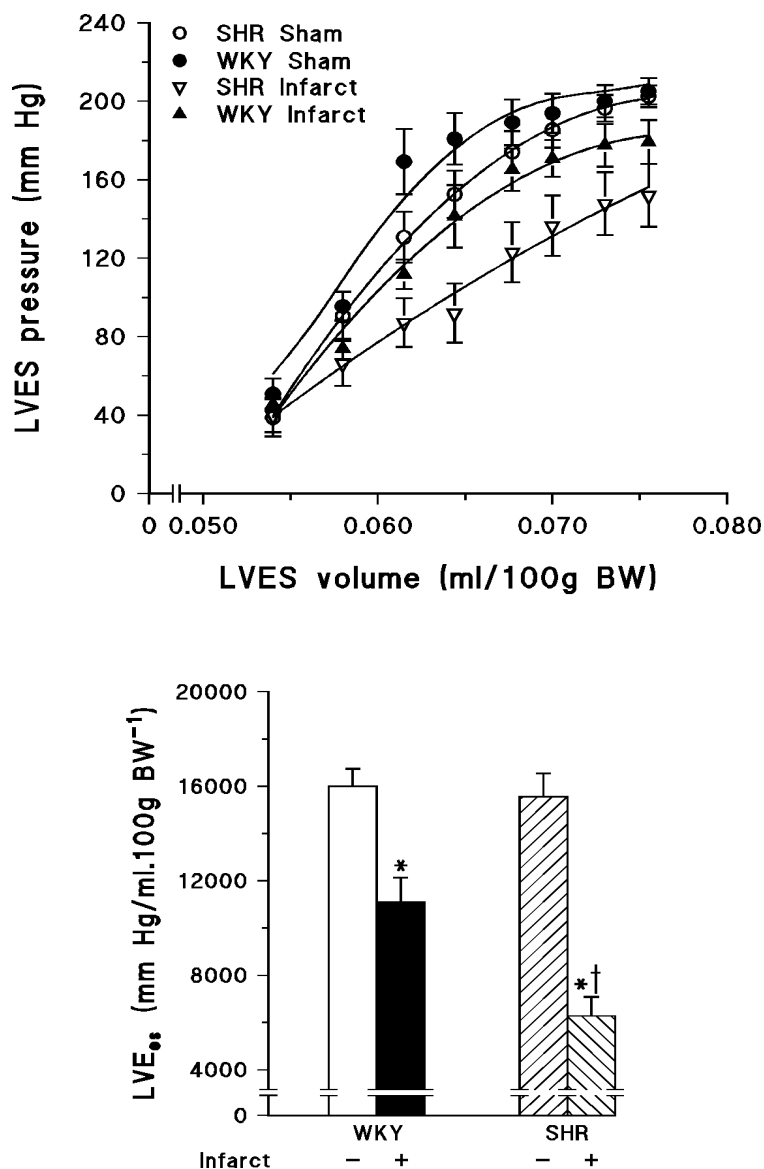
The impact of myocardial infarction assessed six-to-seven months post coronary artery ligation on global LV chamber systolic function in SHR and WKY rats, at baseline and in the presence of a  $\beta$ -adrenergic-inotropic stimulus, is shown in Figures 4.8 and Figure 4.9 respectively. Global LV systolic chamber function was assessed *in vivo* from FSend and *ex vivo* from LV  $E_{es}$  assessments.



**Figure 4.7.** Effect of myocardial infarction (MI) on regional, left ventricular (LV) non-infarcted viable myocardial tissue systolic function in spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats as compared to sham-operated (Sham) controls. Posterior wall thickening is illustrated in the upper panel, and percentage shortening of the lateral LV wall over a range of LVEDP is shown in the lower panel. Sample sizes are as given in Table 4.1. \*  $p < 0.05$  versus other groups.



**Figure 4.8.** Left ventricular (LV) systolic chamber function in the absence of an inotropic stimulus, six-to-seven months post-coronary artery ligation (Infarct) or sham-operations, in Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Regression coefficients of the LV end systolic (LVES) pressure-volume relations, illustrated in the top panel, were used to determine LV systolic elastance (LV  $E_{es}$ ) shown in the lower right panel. For the LVES pressure-volume relations LV volume was indexed to body weight (BW). Fractional shortening determined at the endocardial surface (FSend) is illustrated on the lower left panel. Sample sizes are as given in Table 4.1. \*  $p < 0.01$  compared to respective sham-operated group.



**Figure 4.9.** Left ventricular (LV) systolic chamber function in Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats, six-to-seven months after coronary artery ligation (Infarct) or sham-operations, in the presence of the inotropic stimulus isoproterenol  $10^{-8}$  M (ISO). LV end systolic (LVES) pressures determined over a range of volumes indexed to body weight (BW) are shown in the upper panel. Regression coefficients of the LVES pressure-volume relations were used to obtain LVES elastance (LV  $E_{es}$ ) shown in the lower panel. Sample sizes in as given in Table 4.1. \* $p < 0.01$  versus respective sham-operated groups, †  $p < 0.04$  versus WKY-MI group.

Baseline measurements of systolic chamber function were similarly reduced in both the SHR-MI and WKY-MI groups as compared to their respective sham-operated controls (Figure 4.8). However, in the presence of a  $\beta$ -adrenergic- inotropic stimulus, global LV systolic chamber function (LV  $E_{es}$ ), assessed six-to-seven months post-coronary artery ligation, was reduced to a greater extent in SHR-MI as compared to the WKY-MI rats (Figure 4.9).

#### **4.3.6 Apoptosis and necrosis of non-infarcted myocardial tissue.**

The percentage of cardiomyocytes staining positive with the TUNEL stain and the average pathological scores, in contiguous histological sections of non-infarcted myocardium, in both SHR and WKY, six-to-seven months post-MI or sham operations, are shown in Table 4.3. In SHR-Sham, as compared to WKY-Sham rats of this age, neither the percentage cardiomyocyte apoptosis nor pathological score were increased. Furthermore, in both SHR and WKY, both the percentage cardiomyocyte apoptosis assessed by TUNEL and the pathological score in non-infarcted LV tissue, were unaffected by MI as compared to sham operated groups.

**Table 4.3.** Left ventricular (LV) pathological and apoptotic scores of normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) 6-7 months after myocardial infarction (MI) or sham operations (Sham).

|                                    | WKY Sham<br>(n=7) | WKY MI<br>(n=6) | SHR Sham<br>(n=11) | SHR MI<br>(n=10) |
|------------------------------------|-------------------|-----------------|--------------------|------------------|
| LV pathological score              | 1.10±0.37         | 1.54±0.16       | 1.63±0.24          | 1.71±0.21        |
| % LV apoptotic nuclei <sup>a</sup> | 0.11±0.03         | 0.14±0.09       | 0.13±0.02          | 0.12±0.02        |

a, apoptotic nuclei expressed as a % of the number of normal nuclei. No differences were noted between the groups.

#### 4.4 Discussion.

The main findings of the present study are as follows: First, six-to-seven months after a moderate-sized LV antero-lateral wall myocardial infarction (~25% of the LV), SHR, but not WKY rats had reduced regional myocardial systolic function in both the posterior (echocardiography) and the lateral (over a range of filling pressures as determined with ultrasonic transducers) walls of viable tissue of the LV. Second, regional myocardial systolic abnormalities in SHR did not translate into a reduced global LV systolic chamber function at baseline, but could explain a reduced global systolic chamber function in the presence of an adrenergic-inotropic stimulus. Third, regional myocardial systolic abnormalities in SHR-MI rats were not associated with an increased scar tissue-to-viable tissue surface area, an enhanced degree of LV dilatation or relative wall thinning, alterations in viable tissue wall stress, or excessive apoptosis or necrosis in viable tissue.

The present study is the first to explore whether the hypertensive heart is susceptible to alterations in regional myocardial systolic function in tissue adjacent and remote from an infarct. In this regard, previous studies have focused on the role of regional myocardial systolic dysfunction remote from an infarct in the normotensive heart after a myocardial infarct. Although some studies have reported on a decreased systolic function in isolated cardiomyocytes (Cheung et al 1994, Li et al 1995, Van der Velden et al 2004, Zhang et al 1999), papillary muscle (Litwin and Morgan 1992,

Mill et al 1998), isolated trabeculae (Davidoff et al 2004), and regional tissue (Litwin et al 1994) from the viable myocardium remote from infarcted tissue in normotensive hearts after a myocardial infarct, in keeping with the present study, not all studies have reproduced these findings in normotensive animals (Anand 2002, Anand et al 1997, Gupta et al 2000, Kim et al 2002, Kramer et al 1993, Lefroy et al 1996, Mellilo et al 1996, Prahash et al 2000). A number of factors could explain the discrepancies between studies including the size of the myocardial infarct produced, where a myocardial infarct that is larger may result in regional viable tissue systolic abnormalities (Litwin et al 1994) as compared to a smaller myocardial infarct where viable tissue systolic abnormalities are not noted (Gupta et al 2000, Kim et al 2002), or species differences between studies (Anand et al 1997, Kim et al 2002, Van der Velden et al 2004). Irrespective of discrepancies in normotensive hearts, the present study provides clear evidence that the hypertensive heart is susceptible to decreases in regional myocardial systolic function in tissue remote from an infarct post-myocardial infarction when studied six-to-seven months after a myocardial infarction. These data are in keeping with a reduced adrenergic-inotropic response of papillary muscle in SHR after an MI (Kouchi et al 2000).

In the present study a number of mechanisms were explored to attempt to explain an increased susceptibility of the hypertensive heart to decreases in regional myocardial systolic function in tissue remote from an infarct post-myocardial infarction. In this regard, although the

measurements of LV regional myocardial systolic function are load-dependent, the reduction in posterior wall systolic function in SHR after a myocardial infarction was associated with an estimated LVES wall stress comparable with WKY rats with a myocardial infarct. Further, the reduction in LV lateral wall systolic function in SHR after a myocardial infarct occurred over a range of filling pressures. Thus, the susceptibility of the hypertensive heart to decreases in LV regional myocardial systolic function in tissue adjacent to, or remote from, an infarct post-myocardial infarction, does not appear to be either afterload- or preload-dependent. However, these results do not exclude the possibility that complex interactions between the LV and large vessels may not contribute toward decreases in regional myocardial systolic function in tissue adjacent to or remote from an infarct post-myocardial infarction in SHR.

Alterations in LV regional myocardial systolic function in non-infarcted tissue after an myocardial infarction have previously been attributed to cardiac dilatation (Litwin et al 1994), producing an increase in LV wall stress and hence myocardial dysfunction. In this regard a similar degree of LV dilatation and increases in LVES wall stress were noted in both SHR and WKY infarcted groups. Thus, excessive cardiac dilatation is unlikely to contribute toward the increased susceptibility of SHR to developing LV regional myocardial systolic dysfunction in non-infarcted tissue after a myocardial infarction as compared to WKY rats.

Viable myocardium from SHR, but not WKY rats with a myocardial infarction has previously been reported to develop upregulated Gi

proteins, a change which may translate into a reduced contractile response to an adrenergic stimulus (Kouchi et al 2000). This is consistent with the finding in the present study of a reduced LV systolic chamber function noted after a myocardial infarction in SHR as compared to WKY rats only in the presence of an adrenergic stimulus. Importantly, in contrast to a previous study where it was unclear whether similar changes occurred in WKY rats (Kouchi et al 2000), the present study suggests that systolic function in the presence of an adrenergic stimulus is reduced in SHR as compared to WKY rats post-myocardial infarction. Moreover, in a previous study showing a reduction in systolic function in the presence of an adrenergic stimulus in SHR after myocardial infarction, the reduction in systolic cardiac function may have been attributed to an enhanced degree of cardiac dilatation (Kouchi et al 2000). In contrast, in the present study, the reduced LV systolic chamber function noted in the presence of an adrenergic stimulus in SHR-MI rats, was not associated with an enhanced degree of cardiac dilatation, and hence is more likely to be attributed to a depressed systolic function of viable myocardium.

After myocardial infarction, an enhanced myocardial expression of inducible nitric oxide synthase (iNOS) occurs in stroke-prone SHR as compared to WKY, and blockade of iNOS is associated with an attenuated decline in systolic cardiac function post-myocardial infarction in stroke prone SHR (Abe et al 2001). Thus, changes in NO, in-part through reactive oxygen species, could also explain the regional myocardial systolic abnormalities noted in SHR, but not WKY in the present study.

However, it is unclear whether iNOS-induced systolic functional abnormalities post-myocardial infarction in stroke prone SHR are through an impact on infarct size, increases in chamber dimensions and hence wall stress, or through decreases in myocardial function in viable tissue (Abe et al 2001).

Although upregulated Gi proteins (Kouchi et al 2000) or an increased iNOS (Abe et al 2001) could explain the reduced myocardial systolic function in remote viable tissue in SHR after an myocardial infarction, alternative changes have been described in remote tissue of normotensive hearts after myocardial infarction which could also explain these findings. These include alterations in  $\text{Ca}^{2+}$  handling, SERCA expression and the degree of troponin I phosphorylation or degradation (Van der Velden et al 2004). These potential mechanisms previously described (Van der Velden et al 2004) may be enhanced in the hypertensive heart post-myocardial infarction and this hypothesis requires further study. Moreover, an increased cardiomyocyte apoptosis has previously been shown to occur in viable tissue of rats with a myocardial infarction (Gupta et al 2000), a change that may promote regional myocardial systolic dysfunction. However, in the present study, neither excessive apoptosis nor necrosis was associated with regional myocardial dysfunction in SHR with a myocardial infarction.

An inability of hypertension and cardiac hypertrophy to increase the susceptibility of the myocardium to infarct expansion and cardiac dilatation post-myocardial infarction in the present study is in apparent contrast to

previous studies (Itter et al 2004, Jain et al 2002, Jilaihawi et al 2003, Nass et al 2002, Nishikimi et al 1995, Nolan et al 1988, Pierard et al 1987). Importantly cardiac dilatation was assessed six-to-seven months after myocardial infarction in the present study, a time period over which maximal LV dilatation is expected to occur. Moreover, not all studies have demonstrated an increased susceptibility to infarct expansion and cardiac dilatation post-MI in the hypertensive heart (Morita et al 1996, Zdorjewski et al 2002) and some have even shown a protective effect of hypertensive hypertrophy (Morita et al 1996). A potential explanation for these apparent discrepancies may be related to the size of the infarct studied or differences between studies in the time period after coronary artery occlusion when cardiac dimensions were assessed. Previous studies have demonstrated that the hypertensive heart does not increase the degree of adverse chamber remodelling if infarct sizes are similar to that reported on in the present study (Nishikimi et al 1995). However, with much larger infarcts of ~50% of the LV, the hypertensive heart is more susceptible to adverse LV remodelling (Nishikimi et al 1995).

Subsequent to the publication of the present study (Norton, Veliotes et al 2008), to the best of my knowledge two clinical studies have appeared which report on the impact of pre-existing LVH on post myocardial infarction LV remodelling and LV systolic chamber function (Galiuto et al 2010, Malek et al 2012). In keeping with the results of the present study, in neither study was pre-existing LVH associated with a reduced resting LV systolic chamber function post myocardial infarction

(Galiuto et al 2010, Malek et al 2012). Moreover, also in-keeping with the results of the present study, LV end diastolic chamber dimensions were no different post myocardial infarction in patients either with or without LVH (Galiuto et al 2010). However, in neither of these studies was the impact of exercise or a pharmacological inotropic challenge on LV systolic chamber function assessed. In this regard, it is possible that as noted in the present study, decreases in viable tissue myocardial systolic function could translate into a reduced LV systolic chamber function when cardiac work increases.

In conclusion, the present study suggests that, as assessed after an extended period post-myocardial infarction, the hypertensive heart is more susceptible to a depressed LV regional myocardial systolic function in both the adjacent and remote non-infarcted LV myocardium subsequent to a left anterior descending coronary artery occlusion, but that these changes cannot be explained by excessive LV dilatation, relative wall thinning, an excessive increase in wall stress, apoptosis (TUNEL) or necrosis. Although these LV regional myocardial systolic abnormalities in hypertension may not manifest as alterations in LV global dysfunction under baseline conditions, such as at rest, they could contribute toward a reduced global LV systolic chamber function in the presence of an adrenergic-inotropic stimulus, such as occurs with exercise. These findings lend further insight into the mechanisms of LV systolic chamber decompensation after myocardial infarction in the hypertensive heart.

## **Chapter 5**

### **Summary and Conclusions**

In the present thesis I aimed to advance our current knowledge of the progression from compensated hypertensive LVH to left ventricular systolic chamber decompensation. In this regard, I focused my efforts on two broad aspects of LVH which had not at the time of conducting the present thesis been appropriately addressed. I first evaluated whether mineralocorticoid receptor activation is critical in mediating the transition from compensated hypertensive LVH to cardiac dilatation and LV systolic chamber dysfunction induced by  $\beta$ -adrenoreceptor activation. As I was able to show a marked beneficial effect of mineralocorticoid receptor blockade on the progression from compensated hypertensive LVH to cardiac dilatation and LV systolic chamber dysfunction induced by  $\beta$ -adrenoreceptor activation, I subsequently evaluated the possible mechanisms of this effect. Second, I examined whether pre-existing hypertensive LVH exacerbates decrements of intrinsic myocardial systolic function, in viable myocardium, post myocardial infarction, the possible impact of these changes on LV systolic chamber function and the potential pathophysiological mechanisms thereof. The work described in the present thesis has been published in international peer-reviewed journals (Norton, Veliotes et al 2008, Veliotes et al 2005, Veliotes et al 2010). In the present chapter I will summarise the findings of the present thesis in the context of our existing understanding of the progression of compensated LVH to left ventricular systolic chamber dysfunction and the development of heart failure.

### **5.1 Does the evidence support a cause-effect relationship between LVH and systolic chamber decompensation?**

As summarised in chapter 1, a number of clinical studies have demonstrated that LVH is an independent predictor of the development of heart failure (Aurigemma 2001, Casale et al 1986, Drazner et al 2004, Gardin et al 2001, Gottdiener et al 2000, Levy et al 1990, Levy et al 1996). However, whether LVH *per se* is responsible for the transition from compensated LVH to systolic chamber decompensation is not entirely clear. In this regard, although there are innumerable pre-clinical studies that have demonstrated that the development of LVH is associated with LV systolic chamber dysfunction and that regression of LVH is associated with improvements in systolic function, few of these studies have been able to segregate the adverse effects of BP or cardiac loads on the heart from those potentially produced by LVH *per se*. One pre-clinical study in which genetic modifications targeting molecules that influence sympathetic nervous system activation, and that reduce the LV hypertrophic response to a pressure overload state in mice, was noted to attenuate the development of LV systolic chamber decompensation (Esposito et al 2002). However, in that study (Esposito et al 2002) a reduced sympathetic nervous system activity, rather than a decrease in LVH, may have been protective. In contrast, in rats following chronic pressure-overload, the extent of LVH may be equivalent in animals with, as opposed to those without, evidence of left heart failure and reductions in systolic chamber

function (Norton et al 2002). Moreover, the progression from compensated LVH to systolic chamber decompensation and cardiac dilatation can be attenuated by antihypertensive agents that do not regress LVH (Tsotetsi et al 2001).

With respect to clinical studies that have provided insight into the role of LVH as a determinant of LV systolic chamber decompensation, an increased LV mass has been demonstrated to be associated with an unchanged ejection fraction (EF) (Aurigemma et al 1995, de Simone et al 1994, Shimizu et al 1991). Moreover, LVH may even be associated with an enhanced EF for that predicted by wall stress (Hartford et al 1985). In contrast however, independent of traditional risk factors, including conventional BP measurements, LVH has been shown to be associated with the development of a decreased LV EF (Drazner et al 2004). Nevertheless, on-treatment decreases in LV mass have been shown to be associated with an unchanged EF (Perlini et al 2001) and in the LIFE study, on-treatment decreases in LV mass were related to reductions rather than increases in indices of LV systolic chamber function, despite improvements in indices of myocardial systolic function (Wachtell et al 2002). However, in the Heart Outcomes Prevention Evaluation (HOPE) trial, angiotensin-converting enzyme inhibition both reduced LV mass and improved LV systolic chamber function beyond BP control (Lonn et al 2004), providing some additional evidence that LVH may be causally related to LV chamber dysfunction. Despite the inconsistencies in the evidence for inverse relationships between LV mass and systolic chamber

function, strong inverse relationships between LVH in excess of that predicted from stroke work and EF independent of LV mass or LV mass index and additional confounders have recently been reported on (Libhaber et al 2013). Moreover, strong relationships between antihypertensive treatment-induced decreases in LVH in excess of that predicted from stroke work and increases in EF independent of LV mass or LV mass index have also been noted (Woodiwiss et al 2012). Thus LVH may indeed be the cause of LV systolic chamber decompensation, but this relationship may only occur when LVH exceeds that predicted from workload (Libhaber et al 2013, Woodiwiss et al 2012). Hence, there are data that both support and refute a causal role for LVH in mediating LV systolic chamber dysfunction. As a consequence of these disparities, in the present thesis I have pursued the notion that neurohumoral activation associated with LVH may play more of an important role in explaining the transition process. What is this evidence, and how has the current thesis contributed toward our understanding of this process?

## **5.2 Mineralocorticoid receptor activation mediates the transition from LVH to adrenergic-induced systolic chamber dysfunction.**

As the development of systolic chamber decompensation, cardiac dilatation and heart failure in response to pressure-overload states is significantly diminished in transgenic animals with a decreased adrenergic activity (Esposito et al 2002), an important question which has arisen is

whether sympathetic overactivation, which accompanies hypertensive LVH (Agabiti-Rosei et al 1987, Kelm et al 1996, Schlaich et al 2003), is responsible for systolic chamber decompensation. Indeed, in a hypertensive rat model, compensated LVH prematurely progresses to chamber dilatation and LV systolic chamber dysfunction in response to chronic  $\beta$ -adrenoreceptor activation (Badenhorst et al 2003b) and  $\beta$ -adrenoreceptor blockade, without altering BP, is able to prevent the transition from compensated hypertensive LVH to LV systolic chamber dysfunction (Chan et al 2004).

In the present thesis I hypothesized that because sympathetic activation stimulates the renin-angiotensin-aldosterone system (Saxena 1992, van Zwieten and de Jonge 1986), one potential downstream or parallel target of  $\beta$ -adrenoreceptors which may mediate the transition from compensated hypertensive LVH to systolic chamber decompensation is through the effects of aldosterone acting on myocardial mineralocorticoid receptors. In this regard, excess cardiac mineralocorticoid receptor activation in a transgenic mice model, results in the development of LV systolic chamber dysfunction through BP-independent mechanisms (Qin et al 2003). Moreover, mineralocorticoid receptor blockade or inactivation has been established to attenuate the development of cardiac dilatation and LV systolic chamber dysfunction in an animal model of chronic pressure-overload hypertrophy (Kuster et al 2005, Lothar et al 2011). However, in the present thesis I provide the first evidence to suggest a potential role for mineralocorticoid receptor activation as an intermediary in the transition

from compensated hypertensive LVH to LV systolic dysfunction induced by adrenergic stimulation (Veliotis et al 2005). Moreover, in contrast to acute models of pressure-overload hypertrophy previously studied (Qin et al 2003, Kuster et al 2005, Lother et al 2011) which often produce LV decompensation through mechanisms that don't necessarily reflect what occurs in systemic hypertension (Norton et al 2002), I provide evidence from a chronic model of systemic hypertension for a key role of mineralocorticoid receptors in mediating the transition from LVH to LV systolic decompensation. What are the implications of these data?

### **5.3 Mineralocorticoid or $\beta$ -adrenergic receptor blockade to prevent the transition from LVH to systolic chamber dysfunction?**

As pointed out in chapter 1, despite the aforementioned accumulating evidence to demonstrate that elevated sympathetic activation induces the transition from compensated hypertensive LVH to systolic chamber decompensation, there is unlikely to be a shift to significantly increase the use of  $\beta$ -adrenoreceptor blockers in hypertension. The reasons not to increase the use of  $\beta$ -adrenoreceptor blockers include their ability to increase the probability of users developing new onset diabetes mellitus (Bakris and Sowers 2004). Furthermore,  $\beta$ -adrenoreceptor blockers may not regress LVH (Devereux et al 2004a) or decrease central aortic BP (Williams et al 2006) as effectively as alternative antihypertensive agents. As a consequence, the use of agents

that block the renin-angiotensin system or calcium channel blockers have a more pronounced benefit on cardiovascular events in comparison to the impact of  $\beta$ -adrenoreceptor blocker-based therapy (Dahlof et al 2002, Dahlof et al 2005). Therefore it is obvious that the potential benefits of  $\beta$ -adrenoreceptor blockers in preventing the transition to systolic chamber dysfunction in compensated LVH could be counteracted by potentially harmful adverse effects. Hence, in the absence of evidence of the availability of  $\beta$ -adrenoreceptor blockers that do not induce these potentially adverse effects, alternative pharmacological agents must be identified. In this regard, in the present thesis I provide clear evidence to show that mineralocorticoid receptor blockade prevents the transition from compensated LVH to adrenergic-induced cardiac dilatation and LV systolic chamber dysfunction. Thus, the present thesis provides strong evidence to suggest an approach to managing hypertension other than employing  $\beta$ -adrenoreceptor blockers, that may prevent the transition from hypertensive LVH to LV systolic chamber decompensation. Clinical studies are therefore required to evaluate whether mineralocorticoid receptor blockers may be especially advantageous in those with hypertensive LVH and asymptomatic left ventricular systolic dysfunction.

#### **5.4 Does mineralocorticoid receptor activation promote systolic chamber dysfunction primarily through adverse LV remodelling?**

In none of the studies that have evaluated the role of mineralocorticoid receptor activation or inactivation in promoting LV systolic decompensation conducted in either animal models (Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Kuster et al 2005, Lothar et al 2011, Mulder et al 2008, Qin et al 2003, Suzuki et al 2002, Wang et al 2004), or in humans (Kasama et al 2003, Pitt et al 1999, Pitt et al 2003, Tsutamoto et al 2001), were the authors able to identify whether the primary effect of mineralocorticoid receptor activation or inactivation was on myocardial contractile disturbances with subsequent changes in cardiac chamber dimensions, or whether the primary effect was indeed on cardiac dilatation, which as a consequence, modified LV systolic chamber dysfunction. This question is of particular importance considering the fact that mineralocorticoid receptor blockade in patients with mild-to-moderate heart failure is not associated with reverse remodelling (Udelson et al 2010), despite the improved outcomes in this group of patients (Zannad et al 2011).

In the present thesis, I have provided strong evidence to support the view that the beneficial effect of mineralocorticoid receptor blockade is primarily on the adverse chamber remodelling process. In this regard, I show that adrenergic stimulation in SHR with compensated LVH,

developed LV systolic chamber dysfunction in association with LV dilatation, but not with decreases in intrinsic myocardial contractility (LV En), and that this effect on LV dilatation was prevented by mineralocorticoid receptor blockade. Importantly, prior studies failed to assess LV chamber dimensions at controlled filling pressures, and only reported on end diastolic diameters *in vivo* (Fraccarollo et al 2005, Fraccarollo et al 2008, Fraccarollo et al 2011, Kasama et al 2003, Kuster et al 2005, Lothar et al 2011, Mulder et al 2008, Pitt et al 1999, Pitt et al 2003, Qin et al 2003, Suzuki et al 2002, Tsutamoto et al 2001, Wang et al 2004), which may be influenced by mineralocorticoid receptor-mediated changes in blood volume. In contrast, in the present study I show that mineralocorticoid receptor blockade prevented right shifts in the LV diastolic pressure-volume relationship, a relationship which accounts for differences in volume preloads. Moreover, these effects could not be attributed to the impact of variations in blood volume and hence preload as short-term mineralocorticoid receptor blockade failed to influence LV diameters. How does one then interpret the evidence to show that mineralocorticoid receptor blockade in patients with mild-to-moderate heart failure is not associated with reverse remodelling (Udelson et al 2010)? In this regard, because in the present study, the adverse effects of  $\beta$ -adrenergic receptor activation are indeed dependent on mineralocorticoid receptor activation, adding mineralocorticoid receptor blockers to patients already receiving background  $\beta$ -adrenergic receptor blocker therapy may produce no additional benefits to the LV chamber remodelling process.

Indeed, in that recent study where mineralocorticoid receptor blockade in patients with mild-to-moderate heart failure failed to reverse remodel the heart, between 90-100% of patients were receiving background  $\beta$ -adrenergic receptor blocker therapy (Udelson et al 2010).

### **5.5 Mechanisms of the effect of mineralocorticoid receptor blockade on adverse LV chamber remodelling.**

The results of the present thesis also provide clarity on a number of possible mechanisms responsible for the ability of mineralocorticoid receptor blockade to prevent the transition from LVH to LV dilatation and consequently LV systolic chamber dysfunction. How has the present thesis expanded our knowledge of these mechanisms?

Cardiac chamber dilatation could be the consequence of an inappropriate hypertrophic process occurring in cardiomyocytes, where increments in myocyte length result in chamber dilation (Gerdes et al 2002). This view is supported by the results of studies performed in various experimental models of cardiac remodelling and failure, including data obtained in ischaemic dilated cardiomyopathy (Anand et al 1997, Gerdes et al 1992, Gerdes and Capasso 1995, Zimmer et al 1990), hypertensive heart failure (Tamura et al 1998) and pacing-induced heart failure (Spinale et al 1991a). Currently, there are no studies that have explored the impact of mineralocorticoid receptor blockade or inactivation on cardiomyocyte length-to-width ratios. In the present thesis, however, I

show that mineralocorticoid receptor blockade is unable to modify the ratio between cell length and width, despite being able to prevent the transition from LVH to LV dilatation. These data suggest that therapeutic approaches designed to modify cardiomyocyte remodelling *per se* may be ineffective at preventing LV dilatation. What alternative potential mechanisms could therefore explain the ability of mineralocorticoid receptor blockade to prevent adverse remodelling in LVH?

Side-to-side slippage of cardiomyocytes has also been postulated as a mechanism resulting in cardiac chamber dilatation (Beltrami et al 1995, Linzbach 1960, Olivetti et al 1990) and this may be mediated by activation of matrix metalloproteinases (MMPs) or collagenases (King et al 2003, Li et al 2001, Mujumdar et al 1999, Mukherjee et al 2003, Peterson et al 2001, Polyakova et al 2004, Reddy et al 2004, Rohde et al 1999, Roten et al 2000, Sakata et al 2004, Spinale et al 1998, Spinale et al 1999, Spinale et al 2000, Spinale 2002). In this regard, blockade of mineralocorticoid receptors reduces the augmented MMP activity which accompanies chronic cardiac dysfunction (Fraccarollo et al 2011, Kuster et al 2005, Suzuki et al 2002). In contrast however, despite producing beneficial effects on LV cavity dimensions, aldosterone synthase inhibition or mineralocorticoid receptor blockade are unable to attenuate the increased MMP activity noted in viable myocardium post-myocardial infarction (Mulder et al 2008). Furthermore, mineralocorticoid receptor blockade, although reducing infarct expansion post-myocardial infarction, was unable to modify MMP expression in the infarct zone (Fraccarollo et al

2008). Therefore, it is important to consider whether beneficial effects of mineralocorticoid receptor blockade on myocardial MMP-2 activity in some studies (Fraccarollo et al 2011, Kuster et al 2005, Suzuki et al 2002) were a cause or a consequence of an improved LV systolic chamber function. A direct effect is indeed possible, as aldosterone stimulates MMP release in isolated cardiomyocytes (Rude et al 2005) and mineralocorticoid receptor activation in macrophages increases the release of metalloproteinases (Marney and Brown 2007). In this regard, an important aspect of the present study is that the ability of spironolactone to prevent  $\beta$ -adrenergic-mediated increases in myocardial MMP-2 expression and activation occurred well before the development of cardiac dilatation and LV systolic chamber dysfunction. Thus, the present study provides the first *in vivo* data to suggest that mineralocorticoid receptor activation mediates myocardial MMP activity prior to the onset of LV dilatation and LV systolic chamber decompensation. Moreover, the present thesis provides the evidence to suggest that mineralocorticoid receptor activation is central to adrenergic-induced myocardial MMP activity and expression. Is MMP activation the only potential mechanism through which mineralocorticoid receptors mediate adverse LV remodelling?

Although there is substantial evidence to show that mineralocorticoid receptor activation promotes myocardial fibrosis, whether this effect could contribute toward LV dilatation has been a matter of considerable uncertainty. In this regard, following the use of LV assist devices, a reduction in cardiac cavity dimensions is commonly coupled to

increases rather than decreases in total myocardial collagen concentrations (Li et al 2001, Madigan et al 2001, McCarthy et al 1995, Scheinin et al 1992). Furthermore, decreases as opposed to increases in total myocardial collagen concentrations accompany rapid pacing-induced cardiac dilatation (Spinale et al 1991b, Spinale et al 1998) and  $\beta$ -adrenergic-induced cardiac dilatation (Woodiwiss et al 2001). However, as our group have demonstrated, increased myocardial collagen synthesis may result in cardiac chamber dilatation if the phenotype of collagen produced is more susceptible to collagenase degradation (Badenhorst et al 2003a). Indeed, systolic dysfunction and cardiac dilatation are associated with collagen of the non-cross-linked phenotype (Badenhorst et al 2003a, Capasso et al 1989, Gunja-Smith et al 1996, Spinale et al 1996, Woodiwiss et al 2001). Furthermore, attenuation of pressure-overload-induced cardiac dilatation has been achieved by genetically reducing the susceptibility of collagen to MMP degradation, (Lindsay et al 2003). Does a similar controversy exist with respect to the relationship between mineralocorticoid receptor-mediated increases in myocardial fibrosis and effects on LV dilatation?

Although previous studies have provided evidence to show that the ability of mineralocorticoid receptor blockade or inactivation to attenuate the development of cardiac dilatation and LV systolic chamber dysfunction is associated with a reduced myocardial fibrosis (Fraccarollo et al 2005, Fraccarollo et al 2011, Kuster et al 2005, Mulder et al 2008, Suzuki et al 2002, Wang et al 2004), the ability of conditional, cardiomyocyte-specific

inactivation of the mouse mineralocorticoid receptor gene to protect against the development of LV systolic chamber decompensation produced by pressure-overload is not associated with a reduced myocardial fibrosis (Lothar et al 2011). However, in that study (Lothar et al 2011), the cross-linked properties of myocardial collagen were not evaluated. In the present thesis I provide clear evidence that mineralocorticoid receptor activation increases myocardial collagen concentrations, but that the phenotype that is principally expressed is that of the non-cross-linked form, and hence is likely to be susceptible to MMP degradation and hence the development of tears and cardiomyocyte side-to-side slippage. Moreover, the ability of mineralocorticoid receptor blockade to decrease myocardial collagen concentrations of the non-cross-linked form was associated with a marked attenuation of LV dilatation. The results of the present thesis therefore provide further evidence to support the importance of qualitative rather than quantitative changes in myocardial collagen as an important cause of adverse LV remodelling and subsequent LV systolic chamber decompensation. Are there other possible causes of LV dilatation that mineralocorticoid receptor activation could mediate?

Although the mechanisms of this effect are not entirely clear, cardiomyocyte cell death induced either through apoptosis or necrosis may provide an alternative explanation for cardiac dilatation. What is the effect of mineralocorticoid receptor activation on cardiomyocyte apoptosis? Mineralocorticoid receptor blockade has been shown to prevent apoptosis

in cardiac disease (Fraccarollo et al, 2011, Kuster et al 2005, Suzuki et al 2002). However, the ability of conditional, cardiomyocyte-specific inactivation of the mouse mineralocorticoid receptor gene to protect against the development of LV systolic chamber decompensation produced by pressure-overload is not associated with a reduced myocardial apoptosis (Lothar et al 2011). Hence, whether a reduction of myocardial apoptosis is an important mechanism explaining the benefits of mineralocorticoid receptor inactivation on LV chamber dimensions and systolic chamber function is still uncertain. In this regard, the question should be raised as to whether the favourable effect of mineralocorticoid receptor blockade on myocardial apoptosis are secondary to the beneficial effects on LV systolic chamber dysfunction. However, there is *in vitro* evidence to indicate that aldosterone mediates primary effects on cardiomyocyte apoptosis (De Angelis et al 2002, Sam et al 2004, Sohn et al 2010). In support of this notion that mineralocorticoid receptor activation mediates primary effects on myocardial apoptosis, in the present thesis I show that mineralocorticoid receptor blockade can attenuate adrenergic-induced myocardial apoptosis in hypertensive LVH well before the onset of LV systolic chamber dysfunction. Therefore, the present thesis provides the first *in vivo* data to suggest that mineralocorticoid receptor activation mediates myocardial apoptosis prior to the onset of LV dilatation and LV systolic chamber decompensation. Moreover, the present thesis provides the evidence to suggest that mineralocorticoid receptor activation is central to adrenergic-induced myocardial apoptotic activity.

## **5.6 Does pre-existing LVH predispose to systolic myocardial dysfunction in the viable myocardium, post-myocardial infarction?**

As indicated in the opening paragraph to this chapter, the second broad question that I addressed in the present thesis is whether pre-existing hypertensive LVH exacerbates decrements of intrinsic myocardial systolic function, in viable myocardium, post myocardial infarction; the possible impact of these changes on LV systolic chamber function; and the potential pathophysiological mechanisms thereof. This question was premised on the pre-clinical evidence to show that reductions in LV systolic chamber function post-myocardial infarction may be exaggerated by hypertensive cardiac hypertrophy (Fletcher et al 1982, Fletcher et al 1986, Itter et al 2004, Jain et al 2002, Nass et al 2002, Nishikimi et al 1995). The excess systolic chamber dysfunction post-myocardial infarction associated with hypertensive LVH could nevertheless be explained in-part through the adverse effects of afterload on infarct size. Indeed, in the presence of a higher BP, infarct size is increased (Nolan et al 1988, Pierard et al 1987). However, there is also evidence to suggest that the presence of LVH may augment the adverse remodelling process that occurs in non-infarcted cardiac tissue, the consequence being enhanced cardiac dilatation and subsequent worse systolic chamber function (Itter et al 2004, Jain et al 2002, Jilaihawi et al 2003). However, what had not been explored at the time of publication of the work on this topic in the present

thesis (Norton, Veliotes et al 2008) is whether the chronically infarcted hypertensive heart is susceptible to a decrease in systolic function in the remaining non-infarcted and chronically remodelled viable myocardium and whether this may also translate into systolic chamber decompensation. In this regard, studies conducted in normotensive animals had established the role of a decreased regional myocardial systolic function in dilated, non-infarcted, but viable myocardial tissue post myocardial infarction (Cheung et al 1994, Davidoff et al 2004, Li et al 1995, Litwin et al 1992, Litwin et al 1994, Mill et al 1998, van der Velden et al 2004, Zhang et al 1999). These changes in regional myocardial systolic function were attributed to a number of mechanisms involving changes in cardiomyocyte calcium handling, apoptosis, alterations in myosin isoforms, modifications in cardiomyocyte morphometry, cytoskeleton proteins and ion transport systems as well as changes in myocardial interstitial remodelling and non-myocyte factors (Gupta et al 2000, van der Velden et al 2004). However, whether these effects are exacerbated in LVH had not been evaluated. In this regard, as compared to normotensive hearts, hypertensive hearts are more susceptible to alterations in viable tissue adrenergic signaling post-myocardial infarction (Kouchi et al 2000) and apoptosis in general (Liu et al 2000). These changes (Kouchi et al 2000, Liu et al 2000) could therefore increase the chances of myocardial systolic dysfunction occurring in remote myocardial tissue post-myocardial infarction in the hypertrophied heart. Indeed, in the present thesis I provide evidence to show that as assessed after an extended period post-

myocardial infarction, the hypertensive heart with LVH is more susceptible to a depressed LV regional myocardial systolic function in both the adjacent and remote non-infarcted LV myocardium subsequent to left anterior descending coronary artery occlusion, but that these changes cannot be explained by an excessive increase in wall stress, apoptosis (TUNEL) or necrosis. What is the clinical significance of these findings?

### **5.7 Does pre-existing LVH exacerbate the progression to systolic chamber decompensation post-myocardial infarction?**

Although in the present thesis I was able to show that the hypertensive heart with LVH is more susceptible to a depressed LV regional myocardial systolic function in both the adjacent and remote non-infarcted LV myocardium subsequent to a left anterior descending myocardial infarction, I was unable to show that this translated into reductions in LV systolic chamber function at rest. These data are consistent with clinical studies published subsequent to the present study (Norton, Veliotis et al 2008), which report on the impact of pre-existing LVH on post myocardial infarction LV remodelling and LV systolic chamber function (Galiuto et al 2010, Malek et al 2012). In neither study was pre-existing LVH associated with a reduced resting LV systolic chamber function post myocardial infarction (Galiuto et al 2010, Malek et al 2012). Moreover, also in-keeping with the results of the present study, LV end diastolic chamber dimensions were no different post myocardial infarction

in patients either with or without LVH (Galiuto et al 2010). However, in neither of these studies was the impact of exercise or a pharmacological inotropic challenge on LV systolic chamber function assessed. In this regard, it is possible that, as noted in the present study, decreases in viable tissue myocardial systolic function could translate into a reduced LV systolic chamber function when cardiac work increases. Thus, further research is required to demonstrate whether at a clinical level, pre-existing LVH predisposes to the development of a reduced systolic chamber reserve during stress.

#### **5.8 Mechanisms of systolic myocardial dysfunction in viable tissue produced by pre-existing LVH, post-myocardial infarction.**

As pre-existing LVH predisposes to the development of a reduced systolic myocardial function in viable myocardium post myocardial infarction, and this translates into a reduced systolic chamber reserve during an adrenergic stimulus, it is important to understand the mechanisms responsible for these changes. In this regard, an increased cardiomyocyte apoptosis has previously been shown to occur in viable tissue of rats with a myocardial infarction (Gupta et al 2000), a change that may promote regional myocardial systolic dysfunction. However, in the present study, neither excessive apoptosis nor necrosis in the viable myocardium was associated with regional myocardial dysfunction in SHR

with a myocardial infarction. Alternatively, viable myocardium from SHR, but not WKY rats with a myocardial infarction, has previously been reported to develop upregulated Gi proteins, a change which may translate into a reduced contractile response to an adrenergic stimulation (Kouchi et al 2000). This is consistent with the finding in the present study of a reduced LV systolic chamber function noted after a myocardial infarction in SHR as compared to WKY rats only in the presence of an adrenergic stimulus. However, in the previous study showing a reduction in systolic function in the presence of an adrenergic stimulus in SHR after myocardial infarction, the reduction in systolic cardiac function may have been attributed to an enhanced degree of cardiac dilatation (Kouchi et al 2000). In contrast, in the present study, the reduced LV systolic chamber function noted in the presence of an adrenergic stimulus in SHR-MI rats, was not associated with an enhanced degree of cardiac dilatation, and hence is more likely to be attributed to a depressed systolic function of the viable myocardium. Are there additional mechanisms that may explain the ability of pre-existing LVH to predispose to the development of a reduced systolic myocardial function in viable myocardium post myocardial infarction?

After myocardial infarction, an enhanced myocardial expression of inducible nitric oxide synthase (iNOS) occurs in stroke-prone SHR as compared to WKY, and blockade of iNOS is associated with an attenuated decline in systolic cardiac function post-myocardial infarction in stroke prone SHR (Abe et al 2001). Thus, changes in NO, in-part through

reactive oxygen species, could also explain the regional myocardial systolic abnormalities noted in SHR, but not WKY in the present study. However, it is unclear whether iNOS-induced systolic functional abnormalities post-myocardial infarction in stroke prone SHR are through an impact on infarct size, increases in chamber dimensions and hence wall stress, or through decreases in myocardial function in viable tissue (Abe et al 2001). In addition, alterations in  $\text{Ca}^{2+}$  handling, SERCA expression and the degree of troponin I phosphorylation or degradation may occur in viable myocardium post-myocardial infarction (Van der Velden et al 2004). These potential mechanisms may be enhanced in the hypertensive heart post-myocardial infarction and this hypothesis requires further study.

## **5.9 Conclusions and clinical implications.**

In conclusion, the results of the present thesis expand on our current understanding of the transition from compensated hypertensive LVH to LV systolic decompensation. In this regard, the present thesis provides evidence first to show that mineralocorticoid receptor activation is a critical mediator of the adverse effects of chronic adrenergic stimulation on LV dilatation and consequently LV systolic chamber dysfunction. Importantly, the present thesis provides the first evidence to show that the beneficial effects of mineralocorticoid receptor blockade on LV systolic decompensation are largely through an impact on adverse LV remodelling

(LV dilatation and wall thinning) and not through an improvement in intrinsic myocardial systolic function. Second, the present thesis suggests that the mechanisms of this effect are through the beneficial actions on MMP activation, increased myocardial non-cross-linked collagen concentrations, and an increased cardiomyocyte apoptosis, but not through alterations in cardiomyocyte length-to-width ratio. Third, the present thesis provides evidence to show that pre-existing hypertensive LVH increases the susceptibility to a depressed LV regional myocardial systolic function in both the adjacent and remote non-infarcted LV myocardium subsequent to a left anterior descending coronary artery occlusion. Consistent with subsequent clinical studies assessing the impact of pre-existing LVH on LV systolic function, this does not translate into a decrease in resting LV systolic chamber function. However, it does translate into a decrease in adrenergic-stimulated LV systolic chamber function.

The clinical implications of the present thesis are first that in hypertensive patients with LVH who may be at risk of developing adrenergic-induced LV systolic chamber decompensation, but in whom the use of  $\beta$ -adrenergic receptor blockers may have deleterious consequences, mineralocorticoid receptor blockade may be an alternative clinical approach. Clinical studies are therefore required to evaluate whether mineralocorticoid receptor blockers may be especially advantageous in those with hypertensive LVH and asymptomatic left ventricular systolic dysfunction. Second, the results of the present study

also explain the recent clinical findings of a lack of benefit of mineralocorticoid receptor blocker therapy on LV cavity dimensions in patients with mild-to-moderate heart failure (Udelson et al 2010), despite the improvements in outcomes in this patient group (Zannad et al 2011). In the study that failed to show benefits on LV cavity dimensions between 90-100% of patients were receiving background  $\beta$ -adrenergic receptor blocker therapy (Udelson et al 2010). As indicated by the present study, the adverse effects of  $\beta$ -adrenergic receptor activation are dependent on mineralocorticoid receptor activation. Thus, adding mineralocorticoid receptor blockers to patients already receiving background  $\beta$ -adrenergic receptor blocker therapy may produce no additional benefits to the LV chamber remodelling process. Third, the results of the present thesis indicate that although in keeping with subsequent clinical studies, pre-existing hypertensive LVH does not exacerbate LV systolic chamber dysfunction post-myocardial infarction, that through a decreased myocardial systolic function in the viable myocardium, adrenergic-inotropic reserve of the LV is considerably reduced. Further studies are required to identify the mechanisms involved and to determine whether this contributes toward effort-related symptoms in patients post-myocardial infarction.

## **Chapter 6**

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