CHAPTER 1

Introduction

Sympathetic activation and heart failure

1. Introduction

Cardiac failure is a progressive condition that contributes to a substantial proportion of morbidity and mortality in any country (Cowie et al 2000; Mosterd et al 2001). Although much has been learned about heart failure, the condition still carries a poor prognosis. Indeed, from the time of diagnosis, survival rates are often comparable with those of the deadliest malignancies (Lenfant 1994; Stewart et al 2001; Hobbs 2004). Nevertheless, over the past twenty years considerable advances have been made in identifying the fundamental mechanisms responsible for disease progression. These advances have led to the introduction of a number of novel therapeutic approaches in heart failure, with the most successful being those that target neurohumoral systems, including the sympathetic nervous system (Hunt et al 2001).

1.1 Cardiovascular effects of the sympathetic nervous system

The sympathetic nervous system is well recognized as a system that contributes to short-term cardiovascular homeostasis. In response to abrupt physiological perturbations that reduce venous return to the heart, such as expiration, the valsalva maneuver, and postural changes, the sympathetic nervous system is activated (mainly via baroreceptor and the central nervous system ischaemic responses). Activation of the system increases cardiac contractility, relaxation, heart rate and conduction of cardiac action potentials, predominantly via stimulation of β_1-adrenoreceptors in the heart, but contractility and to some extent relaxation also increases through both β_2- and α-adrenoreceptor-induced effects. The consequence is an increase in stroke volume, cardiac output and hence blood pressure. The effects on the vasculature are predominantly to increase smooth muscle contraction via stimulation of α_1-

adrenoreceptors and hence to induce vasoconstriction, and an increase in total peripheral resistance and blood pressure. The sympathetic nervous system is also important in mediating the cardiovascular changes that occur during exercise, with similar effects noted as described for short-term physiological perturbations, but with the predominant vascular effect being a decrease in total peripheral resistance, mediated in-part through β_2-adrenoreceptors in vascular smooth muscle in skeletal muscle. Despite the bulk of evidence in favor of a cardio-stimulatory role of sympathetic activation on the heart, chronic (as opposed to short-term) sympathetic activation is now well recognized as mediating progressive cardiac dysfunction.

1.2 Sympathetic activation is a major determinant of progressive heart failure

Neurohumoral activation in heart failure, where increases in the activity of both the sympathetic nervous system and the renin-angiotensin-aldosterone system occur (Hasking et al 1986), is a major factor responsible for the progression of heart failure (Cohn et al 1984; Bristow 1997). With respect to the sympathetic nervous system, clinical evidence in favor of over-activation of this system contributing toward the progression of heart failure is summarized in Table 1. Briefly, plasma norepinephrine concentrations are increased in heart failure and predict the severity of heart failure, pump dysfunction in heart failure and mortality in heart failure (Table 1). Myocardial norepinephrine levels are enhanced in patients with chronic heart failure and predict mortality in heart failure (Table 1). Moreover, in randomized-controlled clinical trials, blockade of a major myocardial receptor target of the sympathetic nervous system, namely β-adrenergic receptors, improves pump function and decreases morbidity and mortality in patients with mild-to-moderate and severe heart failure (Table 1). Blockers of

Table 1. Evidence that implicates excessive sympathetic nervous system activation in progressive cardiac failure.

Pathophysiological change in heart failure or treatment	Positive association	Pharmacological agent	Reference
1. Plasma noradrenaline and adrenaline concentrations	Heart failure		(Kluger et al 1982)
	Cardiac dysfunction in heart failure		(Kluger et al 1982)
	Severity of heart failure (NYHA class)		(Sigurdsson et al 1994)
	Mortality in heart failure		(Cohn et al 1984), (Swedberg et al 1990), (Francis et al 1993), (Anand et al 2003),
2. Myocardial noradrenaline levels	Heart failure		(Esler et al 1997)
	Mortality in heart failure		(Kaye et al 1995)
3. Beta-adrenoreceptor blocker effects in heart failure	Pump function improves	Carvedilol	(Gerson et al 2002),(Packer et al 1996)
	Pump function improves	Carvedilol and metoprolol	(Toyama et al 2003)
	Pump function improves	Metoprolol	(Waagstein et al 1993; Waagstein et al 2003)
	Reduced mortality and hospitalization	Carvedilol	(Packer et al 1996)*, (Packer et al 2001), (Poole-Wilson et al 2003)
	Reduced mortality and hospitalization	Metoprolol	(MERIT-HF 1999)**
	Reduced mortality and hospitalization	Bisoprolol	(CIBIS-II 1999)*
	Reduced mortality and hospitalization	Nebivolol	(Flather et al 2005)

NYHA, New York Heart Association; * moderate-to-severe heart failure; ** mild-to-severe heart failure; *** heart failure in the elderly

neurohumoral systems, including the adrenergic receptor blockers, now form the cornerstone of heart failure therapy (Willenbrock et al 2000).

1.2 The natural history of heart failure: Does excess sympathetic activation contribute to more than the progression of the disease?

As sympathetic activation is so important in the progression of heart failure, an obvious question is whether sympathetic activation couldn't also contribute to the development of heart failure? From a causal perspective, there are a number of factors that mediate the clinical entity "heart failure". These include infective, autoimmune, congenital, genetic and malignant processes, coronary-vascular changes, and alterations in the peripheral vasculature (hypertension) or in endocrine function (e.g. diabetes or thyroid disease). From an anatomical perspective these diseases may produce endocardial, valvular, pericardial, and myocardial diseases. In contrast, other than unusual conditions such as phaeochromocytoma, which may cause a cardiomyopathy (Van Vliet et al 1966), as yet there is no direct clinical evidence that suggests that excess sympathetic activation causes heart failure. Yet there are many patients who carry a risk factor for heart failure, such as hypertension, diabetes mellitus, valvular lesions, etc (Figure 1), but who do not develop heart failure, whilst others carry the same risk factor and rapidly develop heart failure and the sequelae. This may of course be determined by the severity of the condition or its inappropriate management. However, it is unclear whether these factors are the only determinants of the development of heart failure in patients with known risk factors. Consequently, although we understand the risk factors (causes) of heart failure (Figure 1), and the factors that contribute toward the rapid progression of heart failure once it has occurred (Figure 1),

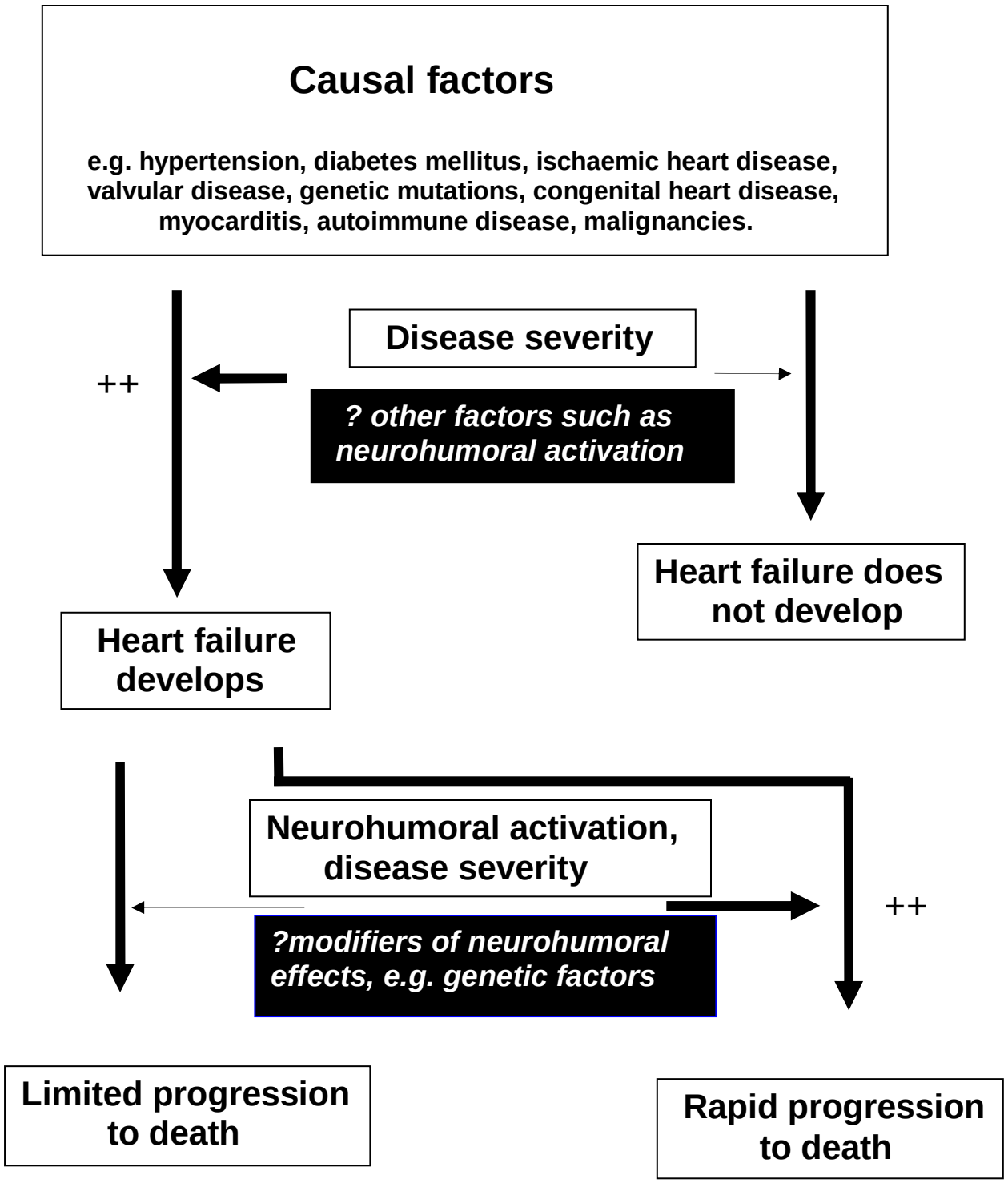


Figure 1. A proposed theoretical model of the known role and potential role of neurohumoral activation in the natural history of heart failure. Factors in dark squares indicate postulated effects studied in the present thesis. ++ indicates a major impact

we have little understanding of the factors that predict who develops heart failure in patients that carry the causal factors (Figure 1). In this regard, what may also contribute toward the development of heart failure in patients with risk factors is the degree of neurohumoral activation, in particular, sympathetic activation (Figure 1).

The role of sympathetic activation as a stimulus for the development of heart failure was in-part addressed in the present thesis. The data that implicate sympathetic effects in contributing toward the development of heart failure are discussed in section 2 below. The structural, functional, cellular and molecular mechanisms by which these effects may be mediated are highlighted as these changes were also evaluated as part of the present thesis.

1.3 Are the deleterious effects of sympathetic activation in progressive heart failure entirely predictable?

Although excessive sympathetic activation is a major determinant of disease progression in heart failure it is also not entirely clear why some patients respond particularly well to blockers of neurohumoral activation, whilst others progress rapidly to death. Undoubtedly, response to therapy is determined by the tolerable doses of pharmacological agents that patients receive and the severity of the disease (Figure 1). However, other factors may also contribute toward disease progression in heart failure. In particular, a recent hypothesis is that genetic variants that influence the proteins involved in mediating neurohumoral effects may modify the natural history of disease progression and development (Figure 1). In the present thesis, I also studied the role of gene variants that modify sympathetic effects as potential determinants of progressive heart failure or the development of heart failure. The data that implicate genetic variants

that control sympathetic effects as determinants of disease progression in heart failure or development of heart failure are therefore also discussed in this chapter (section 3).

2 Excess sympathetic activation may contribute toward the development of heart failure

In pressure overload states, it is not clear why some hearts, despite similar degrees of pressure overload, rapidly progress to cardiac decompensation, whilst others maintain normal or even enhanced function (Innes et al 1998; Norton et al 2002; Aiello et al 2004). Pressure overload states (such as hypertension or valvular diseases, such as aortic stenosis) as compared to other causes of heart failure are relatively more important causes of heart failure in developing countries such as South Africa, where the prevalence of ischaemic heart disease, although increasing, is thought to still be relatively low (Walker and Sareli 1997). In South Africa, hypertensive heart disease and valvular disease are therefore likely to contribute to a substantial portion of the heart failure burden. A potential explanation for the distinct progression to heart failure in some hearts, but not others in pressure overload states, could be through excess sympathetic activation. Indeed there is now increasing evidence in support of this notion. The following outlines this evidence.

First, increased myocardial norepinephrine concentrations are measured in the coronary sinus in patients with hypertensive hypertrophy prior to the development of heart failure (Agabiti-Rosei et al 1987; Kelm et al 1996; Schlaich et al 2003). The mechanism of this effect appears to be because of a reduced norepinephrine re-uptake as well as an increased sympathetic nervous system activity (Simpson et al 1991; Rumantir et al 2000). Second, transgenic animal models with decreased adrenergic activation are protected against the development of dilatation and heart failure when

exposed to pressure-overloads (Esposito et al 2002). Third, in compensated hypertensive hypertrophy excessive adrenergic activation is associated with downregulation of β-adrenergic systems (Limas and Limas 1978; Castellano et al 1993; Böhm et al 1994a; Böhm et al 1995), changes that could promote the development of contractile dysfunction. Indeed, the postulate that prolonged sympathetic activation could lead to β-adrenergic desensitization and hence promote the transition from cardiac hypertrophy to cardiac failure was hypothesized at least a decade ago (Böhm et al 1994a). Lastly, blockade of β-adrenoreceptors prevents the transition from cardiac hypertrophy to heart failure in hypertension independent of blood pressure effects (Chan et al 2004). These data obviously beg the question of the potential mechanisms involved.

2.1 How could excess sympathetic activation contribute toward the development of heart failure?

Conceptually, many scientists and clinicians grapple with the concept of sympathetic activation as a determinant of progressive cardiac dysfunction. The reason for this is that, as indicated in section 1.1 above, over relatively short periods, sympathetic activation increases, rather than decreases cardiac function, by promoting both increases in cardiac contractility and cardiac relaxation. However, heart function is far more complex than this and can be determined by a myriad of changes. From a pathophysiological perspective, irrespective of the cause of heart failure the following ultimately determine cardiac pump function and hence heart failure:

First, increases in cardiac loading conditions through increased chamber pressures or through alterations in the diastolic properties of the heart that lead to increments in chamber dimensions will reduce pump function. Second, alterations in

contractile properties produced by either cardiomyocyte dysfunction or cell death may reduce pump function. With respect to the long-term effects of sympathetic activation on the heart, the mechanisms of sympathetic-induced cardiac dysfunction may be through alterations in contractile properties produced by both cardiomyocyte dysfunction and death and through changes in the diastolic properties of the heart with subsequent increases in cardiac dimensions. Consequently, a brief overview of the impact of chronic sympathetic effects on cardiomyocyte contractile properties and survival will be provided. Further, the effect of chronic sympathetic activation on cardiac diastolic properties and chamber dimensions will also be provided. In these overviews, the structural, functional, cellular and molecular mechanisms of the deleterious actions of chronic sympathetic activation will be underscored.

2.1.1 Effects of chronic excess sympathetic activation on cardiac contraction and cell death.

Sympathetic activation of cardiac myocytes occurs via both β_1- β_2- and α-adrenoreceptor stimulation and subsequent signaling pathways. Moreover, sympathetic activity in the myocardium is modified by a number of pre-synaptic mechanisms that control either norepinephrine release or re-uptake. Norepinephrine effects on presynaptic release are mediated by α_2-adrenoreceptor stimulation. The presynaptic and post-synaptic effects on adrenoreceptors, the cellular pathways involved and the effects of the downstream targets on function are summarized in Figure 2. The signaling pathways involve guanosine (G) proteins, adenylyl cyclase (AC), cyclic adenosine monophosphate (cAMP), and cAMP-dependent protein kinases which phosphorylate several cellular proteins including calcium channels (Frey et al 2000; Molkentin and Dorn 2001).

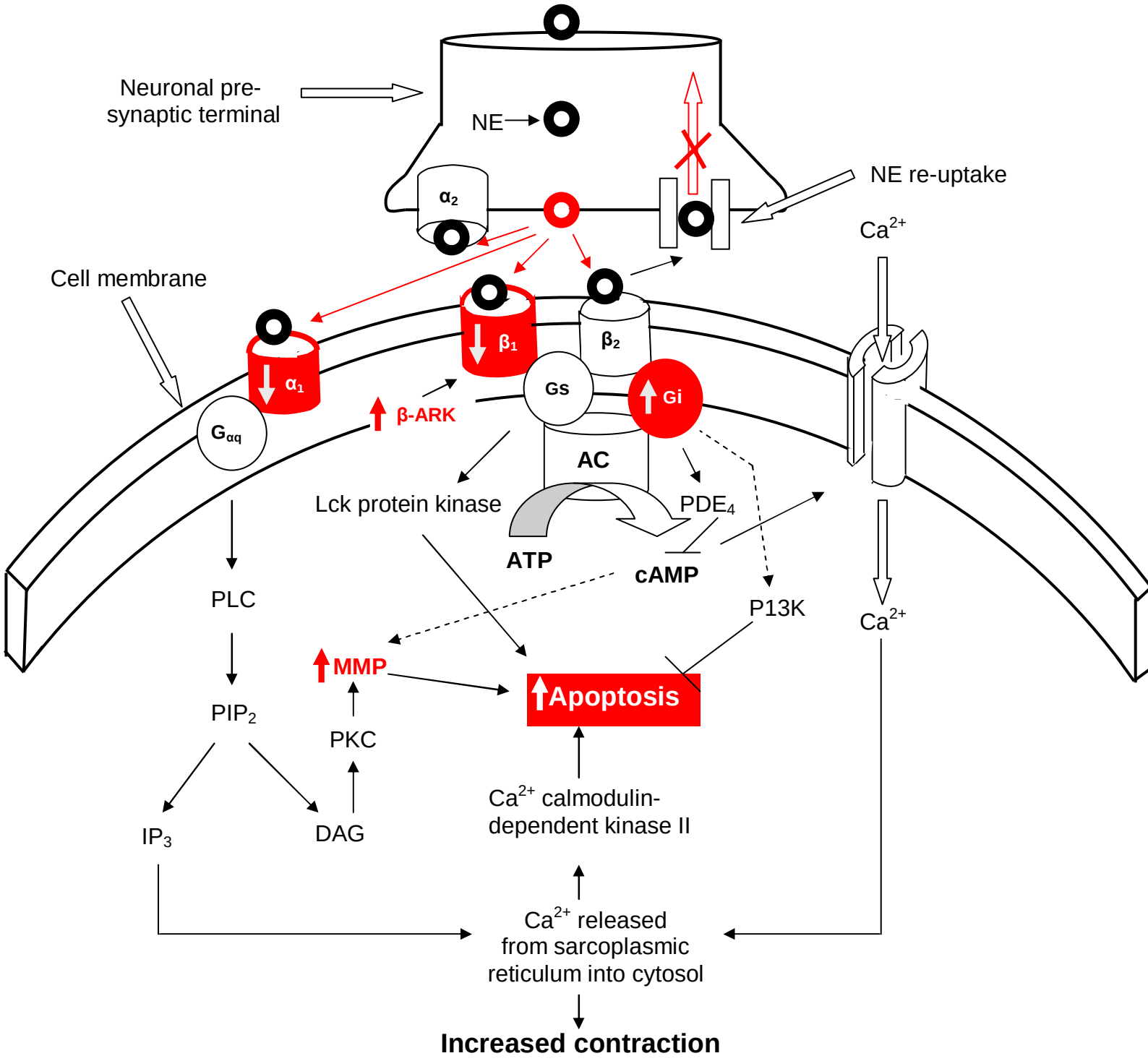


Figure 2. Cellular signaling systems mediated by sympathetic activation in cardiac myocytes. Alterations that may occur in heart failure are highlighted in red. NE, norepinephrine; α and β refer to adrenoreceptors; PDE, phosphodiesterase; G, guanosine triphosphate proteins; Gi, inhibitory G protein; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; PIP_2, phospho-inositol-bisphosphate; PLC, phopsholipase C; IP_3, inositol triphosphate; β-ARK, β-adrenoreceptor kinase; MMP, matrix metalloproteinase.

Calcium channels are also downstream targets for α-adrenoceptors (Bkaily et al 2003).

One potential mechanism by which chronic sympathetic activation is thought to promote progressive decreases in cardiac contraction, is through a reduced sensitivity of the system to agonist stimulation. A number of authors have demonstrated that in failing human hearts a decrease in β_1-adrenoreceptor density leads to subsensitivity of the β-adrenergic pathway and decreased β-adrenoreceptor-agonist-stimulated muscle contraction (Bristow et al 1982; Bristow et al 1986; Brodde et al 1986; Böhm et al 1988; Brodde et al 1989; Brodde 1991; Steinfath et al 1991; Schotten et al 2000; Tevaearai and Koch 2004) (Figure 2). β_1-adrenergic receptor desensitization is in-part through increases in the activity of β-adrenoreceptor kinase (β-ARK), an enzyme that inhibits the activated β-adrenoreceptor by phosphorylation (Feldman et al 1988; Neumann et al 1988; Böhm et al 1990; Eschenhagen et al 1992) (Figure 2).

Like the β_1-adrenoreceptor, the β_2-adrenoreceptor mediates contractile effects (Bristow 2000; Molenaar and Parsonage 2005), but in contrast to the β_1-adrenoreceptor where there is extensive evidence to support a role of this receptor in mediating heart failure, the β_2-adrenoreceptor does not appear to be involved. Indeed, in transgenic animals, although a 15-fold increase in β_1-adrenoreceptor expression results in heart failure (Molenaar and Parsonage 2005), a substantially greater increase (60-fold) in β_2-adrenoreceptor expression does not produce the same effects (Liggett et al 2000).

Changes in α_1-adrenoreceptor signaling may also play a role in the contractile dysfunction in the failing myocardium (Suematsu et al 2001). Indeed, α_1-adrenoreceptors may be downregulated in animal models of dilated cardiomyopathy (Yamada et al 1997). Skomedal et al (1997) have provided data to indicate that α_1-adrenoreceptor-mediated inotropic effects in the failing human myocardium are comparable to that of β-adrenoreceptor-mediated effects and thus play a functional role in the failing human

myocardium. Thus α_1-adrenoreceptor downregulation in heart failure (Yamada et al 1997) may have important functional consequences.

Signals downstream from β-adrenoreceptors (Figure 2) are also modified in heart failure, changes that could contribute to contractile dysfunction (Kacimi and Gerdes 2003). Indeed, in human end-stage heart failure, an increase in $G_{i\alpha}$ has been well documented (Feldman et al 1988; Böhm et al 1990; Böhm et al 1994b). As $G_{i\alpha}$ mediates decreases in adenylate cyclase activity (Figure 2), an increase in $G_{i\alpha}$ may also result in reduced β-adrenoreceptor-mediated contractile responses. Both $G_{\alpha q}$ and $G_{\alpha 12}$ protein levels and two isoforms of MAP kinase bioactivities also decrease in congestive heart failure whereas G_{β} and $G_{\alpha 13}$ protein content are upregulated (Kacimi and Gerdes 2003). This results in an increased contraction since chronic activation of $G_{\alpha q}$ decreases contractility.

A reduced cardiac contractility following excessive chronic sympathetic activation may occur not only through downregulation of adrenergic signaling pathways, but also through changes in cell survival. Via β_1-adrenoreceptors, chronic adrenergic stimulation may promote increases in cell death by activation of apoptotic signaling pathways (Singh et al 2001) (Figure 2). Apoptosis is a form of cell death that is distinct from necrotic cell death (Narula et al 2000). During heart failure sympathetic-induced upregulation of transcription factors induces myocyte hypertrophy and prepares the cell for entry into the cell cycle (McKinsey and Olson 2005). However, terminally differentiated myocytes cannot divide, and failing to divide they undergo apoptosis. The initiation of apoptosis is associated with activation of an upstream cascade including the release of cytochrome c from mitochondria into the cytoplasm and the processing of proteolytic caspases.

In contrast to the pro-apoptotic effect of β_1-adrenoreceptors, β_2-adrenoreceptors are thought to be anti-apoptotic (Communal et al 1999) (Figure 2). This may in-part explain the ability of a 15-fold increase in β_1-adrenoreceptor over-expression to cause

heart failure (Molenaar and Parsonage 2005), whilst a substantially greater increase (60-fold) in β_2-adrenoreceptor over-expression does not produce the same effects (Liggett et al 2000).

With respect to necrosis, possibly via an imbalance between oxygen demand-to-supply ratios and by direct effects on myocytes (Mann et al 1992) activation of the sympathetic nervous system may stimulate cardiomyocyte necrosis. As adult cardiac myocytes are largely terminally differentiated, replacement with new cells is unlikely. Necrosis would thus reduce the number of contractile units in the myocardium and contribute to pump dysfunction.

At a presynaptic level, alterations in cellular signaling systems may also contribute toward heart failure (Figure 2). As indicated in Table 1 there are data to support the notion that excessive myocardial norepinephrine synaptic concentrations (release) could contribute toward progressive heart failure. Although this is likely to involve alterations in synaptic norepinephrine re-uptake, it could also involve alterations in systems that modify presynaptic norepinephrine release. Importantly, presynaptic α_2-adrenoreceptor activation is an important mechanism responsible for preventing heart failure (Brede et al 2002) (Figure 2).

2.1.2 Effects of chronic excess sympathetic activation on cardiac diastolic properties

A major effect of excess sympathetic activation over chronic periods involves changes in the diastolic properties of the heart. This is a complex topic and hence, before describing these effects and the impact on cardiac function, a description of the normal diastolic properties of heart, and some of the changes that occur in heart failure is required. Moreover, the mechanisms responsible for these changes will be reviewed.

2.1.2.1 Normal cardiac diastolic properties and changes with cardiac hypertrophy

The diastolic properties of the heart are best described as a relationship between diastolic pressure (P) and volume (V). The diastolic P-V relationship is generally an exponential relationship in the heart where filling initially occurs under low pressures, but as it progresses, gradually changes to a point where even small changes in volume generate large pressures (Figure 3). Under resting conditions in a normal heart, peak filling volumes only occur at relatively low filling pressures (≈5-10 mm Hg). During exertion, filling volumes increase in the heart (as venous return increases following contraction of the skeletal muscle pump), but still, filling pressures only increase to a modest degree (Figure 3). If blood volume increases during fluid intake, filling volumes also increase in the heart, but filling pressures again only increase to a modest degree (Figure 3).

A common cardiac change associated with most cardiac diseases is cardiac hypertrophy. Cardiac hypertrophy is associated with alterations in diastolic P-V relations. In essence either a left or a right shift in diastolic P-V relations may occur with cardiac hypertrophy (Figure 4). If during the cardiac hypertrophic process in cardiac disease the tensile strength of the myocardium is increased or the cardiac wall increases in thickness, then the chamber of the heart will become stiffer or less compliant. The result is a left shift in the diastolic P-V relation (Figure 4). These changes are thought to be responsible for heart failure in patients with a preserved systolic function, but who do not have a high output state (Zile et al 2004). Conversely, if during the hypertrophic process in cardiac disease myocytes grow longer than they do wider, growth of the heart may only occur in an outward direction, a change that would increase cardiac cavity volumes and shift the diastolic P-V relation to the right (Figure 4, cardiomyocyte lengthening).

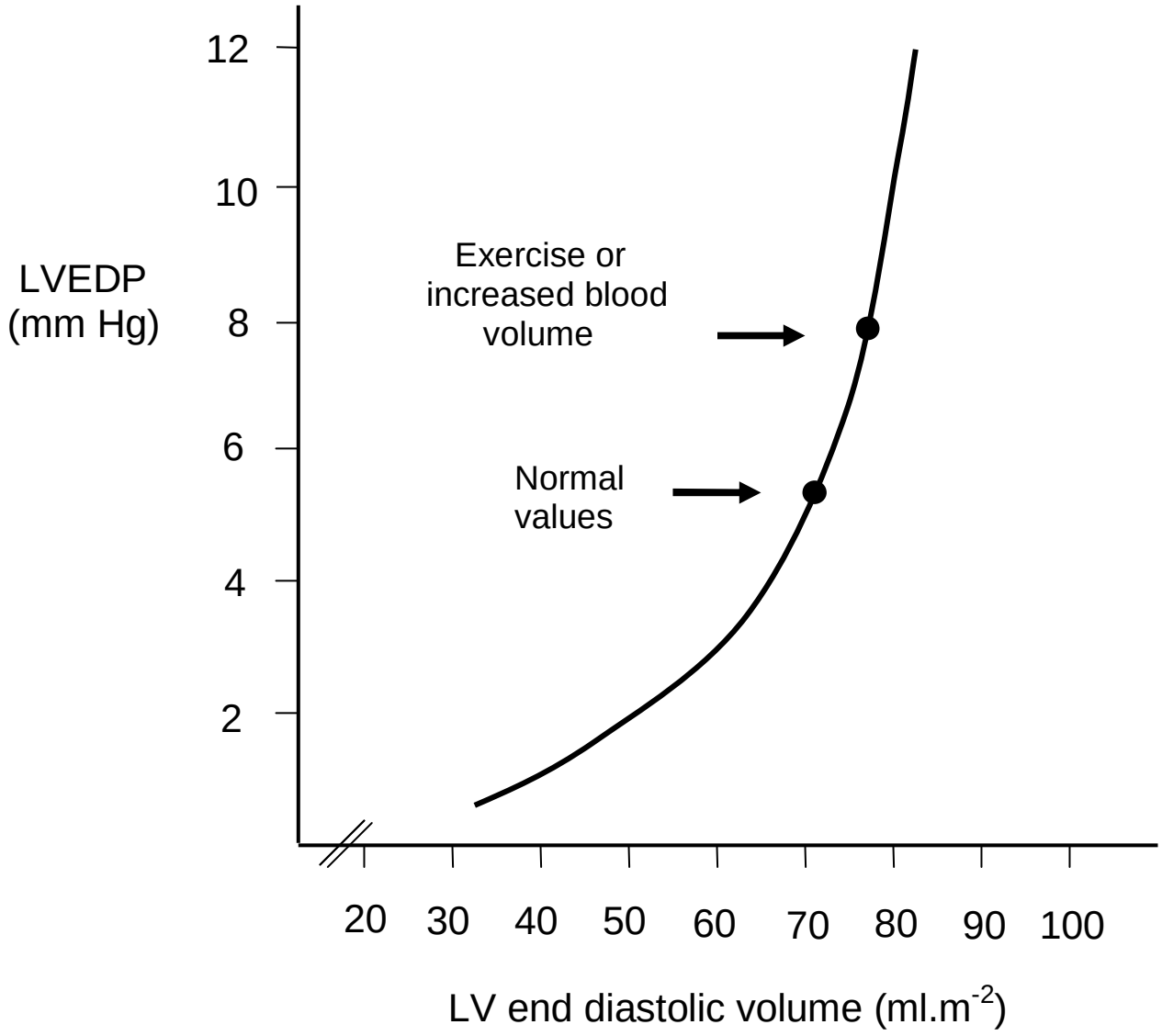


Figure 3. The normal left ventricular end diastolic pressure (LVEDP)-volume relationship and alterations that may occur with exercise or changes in blood volume.

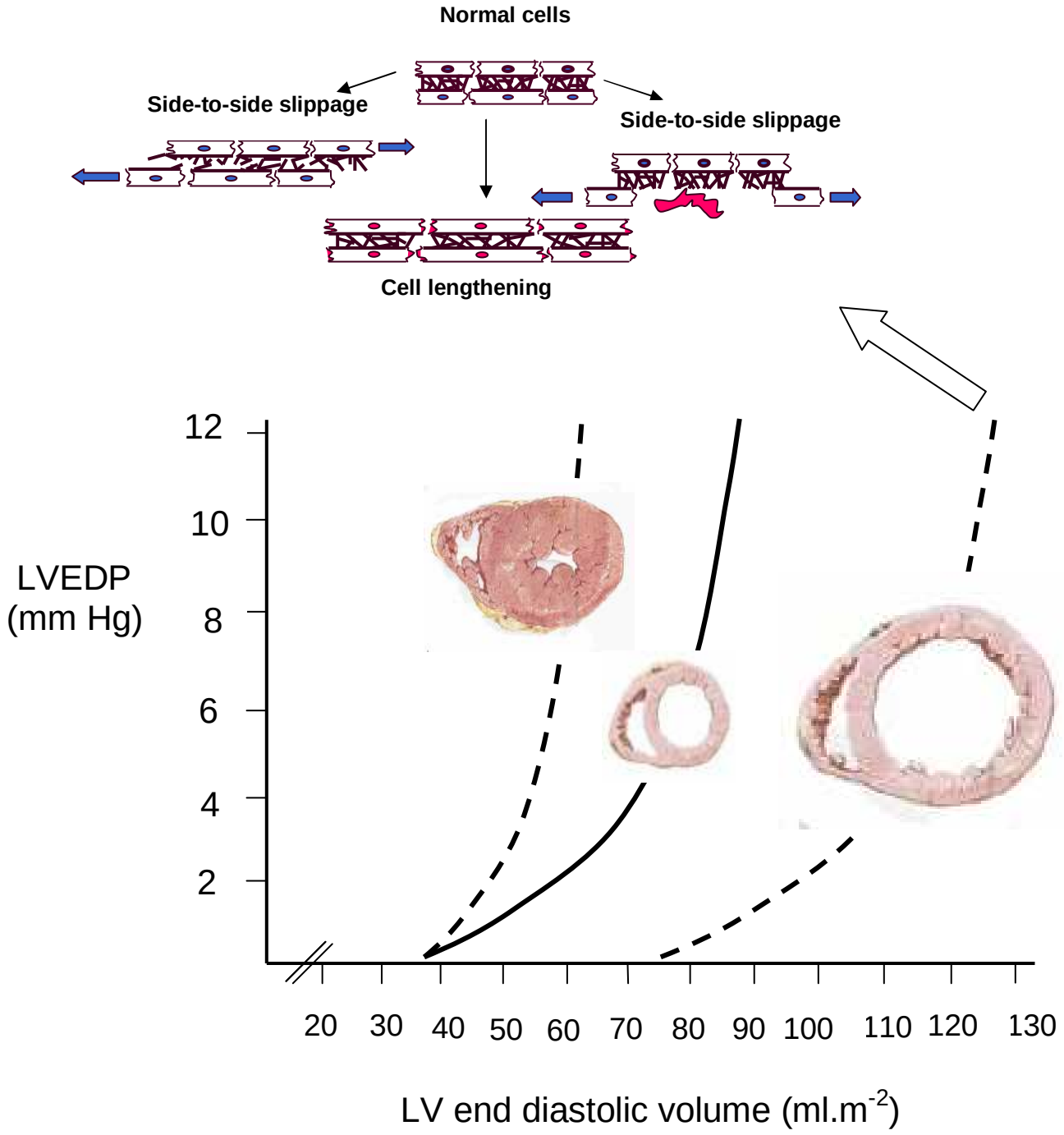


Figure 4. Potential alterations in left ventricular end diastolic pressure (LVEDP)-volume relations in cardiac hypertrophy (below) and the cellular changes that could produce the right shift in the relationship (above). See text for explanation.

Moreover, if either the interstitium changed or cells died, so that either connections between myocytes were weakened or myofibrils developed weak points because of cell death (Figure 4), myocytes may slip apart and the heart may also grow outward (cardiomyocyte side-to-side slippage). Again these changes would increase cardiac cavity volume and shift the diastolic P-V relation to the right (Figure 4). As will be discussed, right shifts in the chamber diastolic P-V relationship are an important determinant of heart failure associated with pump dysfunction (systolic heart failure).

2.1.2.2 Right shifts in diastolic pressure-volume relations

Right shifts in cardiac chamber diastolic P-V relations in cardiac disease are responsible for the enlarged cavity size that is frequently seen in advanced heart failure, a change that is referred to as cardiac “dilatation” or “adverse chamber remodeling” (Figure 4). Clinically, cardiac dilatation is determined using echocardiography, and diagnosed as an increase in cardiac internal diameters at end diastole above threshold values for normal hearts. In the left ventricle, an end diastolic internal diameter, as determined from M-mode echocardiography, that lies above 5.5 cm, is considered pathological (Abbasi et al 1973). This threshold for normality generally excludes increased cavity dimensions that are sensitive to preload changes (increased cavity volumes simply because the resting value has shifted up the normal curve in Figure 3). Indeed, as suggested by Figure 4, changes in preload, although altering cavity pressures, cannot significantly modify cavity volumes if a heart has a right shift in the diastolic P-V relation, unless preload is reduced to unusually low values.

2.1.2.3 Heart failure and cardiac dilatation

Cardiac dilatation was for many years perceived as a compensatory mechanism that occurs during heart failure (Ertl et al 1991). This is not surprising as in heart failure fluid accumulation and a reduced cardiac contractility will increase ventricular filling volumes (Patterson and Adams 1996) and thus enhance ejection volumes (stroke volume) through the Frank-Starling effect. The obvious negative effect of increased filling volumes is an increased filling pressure (Figure 3) with subsequent “backward” heart failure (if filling pressures in the left ventricle increase, pulmonary capillary hydrostatic pressures increase and pulmonary congestion occurs). To allow the heart to accommodate greater volumes at normal filling pressures it would obviously be useful to shift the diastolic P-V relation to the right, where a greater filling volume occurs at much lower filling pressure, thus relieving pulmonary congestion (Figure 4). Thus, cardiac dilatation was perceived as a useful compensatory change. Consequently the degree of dilatation was utilized as a clinical index of the degree of contractile dysfunction and fluid overload, rather than recognized as a process that worsens heart failure. Although these arguments are not necessarily incorrect, the more recent view of cardiac dilatation is that it is causally related to heart failure, and contributes toward mortality through an impact on wall stress and hence partly through further reductions in pump function. The following outlines these arguments.

Left ventricular dilatation is not only associated with heart failure, but plays a critical role in the development of heart failure. Indeed, there is now substantial evidence to indicate that cardiac dilatation is a precursor of left ventricular dysfunction and clinical heart failure (Gaudron et al 1993; Pfeffer et al 1993; Vasan et al 1997). Cardiac dilatation is also a major risk factor for mortality in heart failure (Nestico et al 1985; Foley et al 1995; Foley and Parfrey 1998). Indeed, Gadsboll and co-workers (1990) noted a

strong relationship between the degree of ventricular dilatation and one-year mortality. Moreover, with treatment, improvements in left ventricular dilatation are associated with better long-term outcomes, including survival (Sharpe and Doughty 1998). Patients predicted to be at risk for long-term left ventricular dilatation have an increased risk of mortality and heart failure at 6 months (de Kam et al 2002). Moreover, 15 to 35 % of patients with a disease entity that is diagnosed on the basis of the presence of dilated cardiac chambers, namely idiopathic dilated cardiomyopathy (IDC), die within the first year after diagnosis and only 25 to 40 % survive for 5 years (Fuster et al 1981; Johnson and Palacios 1982; Cohn et al 1984; Unverferth et al 1984; von Olshausen et al 1984). How does cardiac dilatation produce cardiac decompensation?

2.1.2.4 Effect of cardiac dilatation on pump function

As indicated above there is now strong evidence to indicate that ventricular dilatation contributes to progressive systolic (pump) dysfunction. Yet the mechanisms of this effect have largely been inferred from existing physical laws. La Place's law, in which ventricular wall stress or tension is proportional to the product of pressure (developed mainly during systole) and chamber radius and inversely proportional to twice the wall thickness of the chamber, could account for pump dysfunction in a dilated ventricle. Indeed, dilatation is associated with an increased end systolic volume and hence radius, and a reduced wall thickness, effects that will increase wall tension or stress. As wall stress determines myocardial oxygen consumption, the argument is that a dilated ventricle produces an increased myocardial oxygen demand-to-supply ratio. A demand-to-supply mismatch may subsequently decrease cardiac contraction. However, when systolic function is measured using a stress (or load)-independent measure of pump function (end systolic elastance) in an animal model of congestive cardiac failure

and pump dysfunction associated with massive cardiac dilatation (Norton et al 2002), pump dysfunction was noted to be reduced without parallel changes in myocardial contractility. These data would suggest that a mechanism unrelated to stress or load-induced effects contributes to pump dysfunction in cardiac dilatation. One potential explanation is that since remodeling of the chamber occurs in cardiac dilatation, inefficient force transduction during myocyte contraction may lead to pump dysfunction.

2.1.2.5 Cellular and neurohumoral mechanisms responsible for cardiac dilatation

A number of mechanisms have been proposed to explain the development of cardiac dilatation. These may be viewed as either cellular or neurohumoral and the following discussion will underscore the issues related to each of these. The cellular mechanisms have briefly been outlined above (Figure 4) and these essentially include myocyte remodeling mechanisms, interstitial changes and the impact of cell death. However, the following sections deal with each of these issues in detail and Figure 5 summarizes the potential mechanisms involved.

2.1.2.5.1 Cardiomyocyte lengthening versus side-to-side slippage

Gerdes et al (1992) first proposed that cardiac dilatation was the consequence of an inappropriate hypertrophic process occurring in cardiomyocytes, where excessive increases in myocyte length relative to increases in cell width result in a dilated chamber (Figures 4 and 5). These authors were able to demonstrate these changes in patients with ischaemic dilated cardiomyopathy (Gerdes et al 1992) and supported their findings in subsequent studies of ischaemic dilated hearts (Gerdes and Capasso 1995) as well

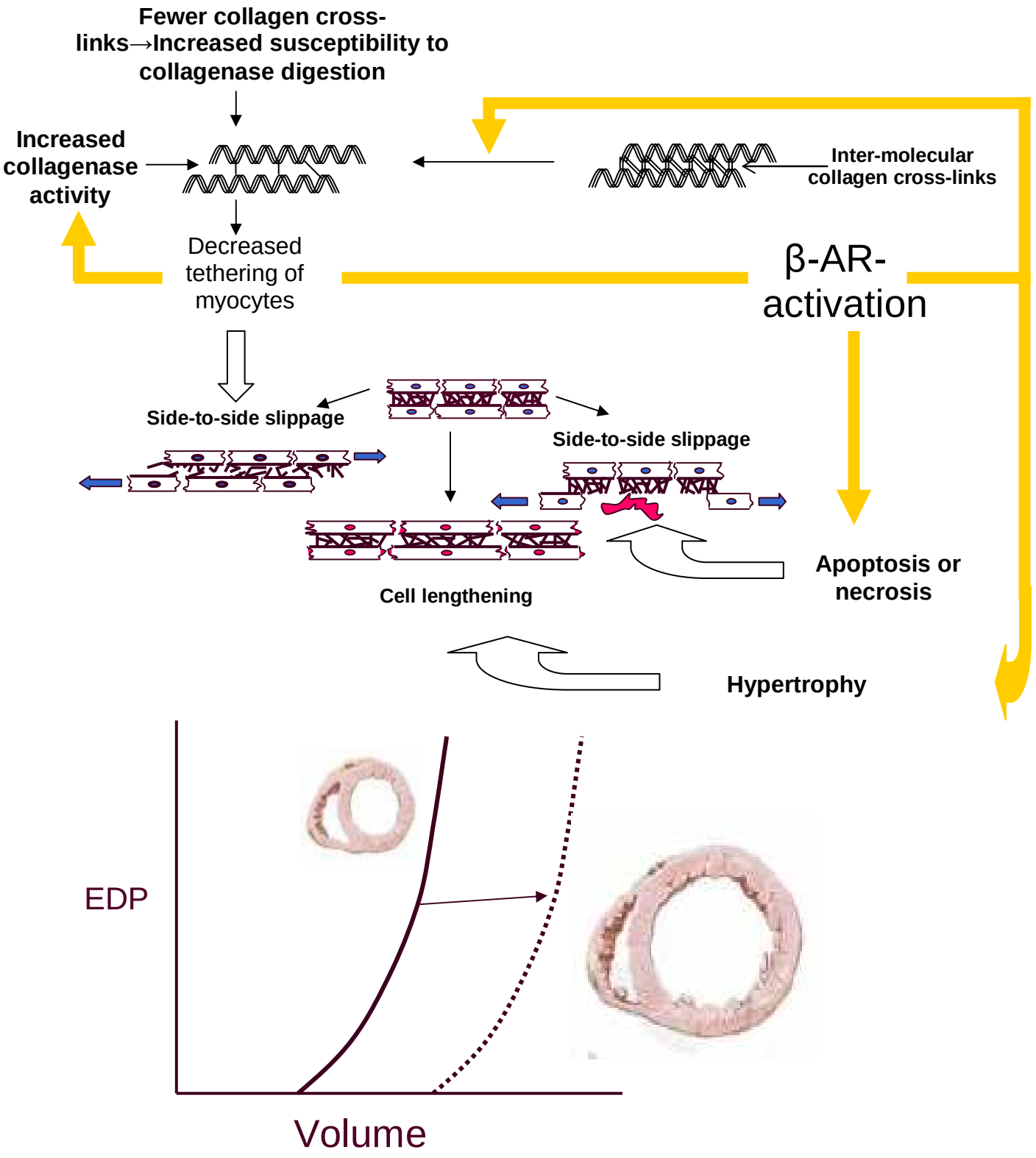


Figure 5. Cellular and molecular changes that may promote cardiac dilatation. See text for description of this figure.

as in hypertensive heart failure (Tamura et al 1998). Moreover, Anand et al (1997) demonstrated that an increase in remodeled myocyte length is associated with left ventricular remodeled volume and inversely associated with left ventricular function. Data from other animal models of heart failure further support the concept that myocyte lengthening may be a hallmark of ventricular dilatation (Zimmer et al 1990; Spinale et al 1991a).

In contrast to the “cell lengthening” hypothesis of cardiac dilatation, a more recently favored view is that cardiac dilatation occurs as a consequence of side-to-side slippage of cardiomyocytes (Figures 4 and 5). Myocyte slippage implies that muscle cells are capable of undergoing side-by-side translocation, leading to wall thinning and chamber dilatation (Olivetti et al 1990) (see Figures 4 and 5). Myocyte slippage in end stage dilated cardiomyopathy has been well documented (Linzbach 1960; Beltrami et al 1995), but the measurement approaches have not been reproduced by Gerdes et al (1992). Whether myocyte side-to-side slippage or cell lengthening or both contribute to cardiac dilatation is controversial.

2.1.2.5.2 Collagen changes in cardiac dilatation

Many forms of cardiac dilatation are accompanied by increases in myocardial collagen concentrations (Fielitz et al 2001). However, the role of increased myocardial collagen concentrations as determinants of cardiac dilatation has been questioned. Indeed, increases in myocardial collagen concentrations are thought to determine left and not right shifts in chamber diastolic P-V relations (Weber 1992). Moreover, reverse remodeling following the use of assist devices is generally accompanied by increases and not decreases in myocardial collagen concentrations (Scheinin et al 1992; Li et al 2001), and pacing-induced cardiac dilatation (Spinale et al 1991b) and β-adrenergic-

induced cardiac dilatation (Woodiwiss et al 2001) are accompanied by decreases rather than increases in myocardial collagen concentrations. A more recent view of how changes in myocardial collagen could contribute toward dilatation is through a loss of collagen support due to an increased degradation of mature collagen. Degradation of collagen may contribute directly to left ventricular dilatation by encouraging side-to-side slippage of cardiomyocytes. These changes are proposed to occur as a consequence of activation of matrix metalloproteinases (MMP) (Jugdutt 2003).

Matrix metalloproteinases are an endogenous family of proteolytic enzymes that degrade all components of the myocardial extracellular matrix (Gunasinghe et al 2001). Increased myocardial expression and activation of MMPs have been demonstrated in patients with congestive heart failure implicating a role in the left ventricular remodeling process (Li et al 2000; Spinale et al 2000; Spinale 2002). Moreover, an increased myocardial MMP activity contributes to left ventricular remodeling in pacing-induced heart failure (Spinale et al 1999). MMP inhibition has been shown to attenuate left ventricular remodeling in animal models of myocardial infarction (Rohde et al 1999; Mukherjee et al 2003) and heart failure in the spontaneously hypertensive rat (Peterson et al 2001). A loss of MMP inhibitory control through the tissue inhibitor of the matrix metalloproteinase-type 1 (TIMP-1) gene deletion has also been shown to cause left ventricular dilatation in mice (Roten et al 2000). With respect to the MMP responsible for mediating chamber dilatation, MMP-2 and perhaps MMP-1 are the only two MMPs that show consistent activity change in models of heart failure. Although Li et al (1998) have suggested that MMP-9 activity is also increased, this has not been reproduced in patients with aortic stenosis (Polyakova et al 2004).

Although non-selective MMP inhibitors consistently inhibit the development of cardiac dilatation, those that do not target MMP-1, but do target MMP-2 and -9 have no effect on cardiac dilatation (King et al 2003). A potential reason for the lack of effect of

selective MMP inhibitors on cardiac dimensions in models of heart failure is that they do not necessarily modify the increased susceptibility of collagen to degradation that often accompanies heart failure. Increases in susceptibility to degradation are probably related to reductions in myocardial collagen cross-linking (Capasso et al 1989; Gunja-Smith et al 1996; Spinale et al 1996; Woodiwiss et al 2001). Indeed, by genetically decreasing the susceptibility of collagen to degradation, a reduced degree of dilatation accompanies pressure-overload states (Lindsey et al 2003).

2.1.2.5.3 Apoptosis and necrosis

As indicated in Figure 4, cardiomyocyte cell death is thought to promote the development of cardiac dilatation by encouraging side-to-side cell slippage. A number of factors may mediate necrosis, but these have been less extensively investigated as compared to the factors that promote apoptosis. Apoptosis involves activation of Nix proteins (Yussman et al 2002). β-adrenoreceptor-induced apoptosis in cardiac myocytes involves activation of a JNK-dependent mitochondrial death pathway and caspase (Remondino et al 2003). Apoptosis in non-cardiomyocytes could also contribute to the remodeling that occurs in the transition from compensatory hypertrophy to decompensated heart failure (Ikeda et al 1999).

2.1.2.6 Role of the sympathetic nervous system in cardiac dilatation

What is the role of the sympathetic nervous system in cardiac dilatation? As indicated in the above discussion, basic science studies suggest that sympathetic activation promotes the development of cardiac dilatation. Indeed, transgenic animal models with decreased adrenergic activation are protected against the development of

dilatation and heart failure when exposed to pressure-overloads (Esposito et al 2002). Moreover, blockade of β-adrenoreceptors prevents the transition from cardiac hypertrophy to a dilated ventricle in hypertension (Chan et al 2004). However, whether cardiac dimension changes in these studies were secondary to reductions in contractile function and subsequent increases in cardiac preloads, or through primary changes mediated by adrenergic activation is unclear. Nevertheless, clinical studies also support the notion that chronic sympathetic activation promotes cardiac dilatation. Indeed, measures of neurohumoral activation are closely associated with cardiac dimensions and hence are thought to play a critical role in cardiac dilatation or right shifts in diastolic P-V relations (Patten et al 1998; Davila et al 2000). Indeed, it has been suggested that reverse left ventricular remodeling (an attenuation of cardiac dilatation) is an important mediator of the clinical benefit of β-adrenergic receptor blockers (Doughty et al 1997; Sharpe and Doughty 1998). However, despite these lines of evidence, the impact of β-adrenergic receptor activation or β-adrenergic receptor blockade on cardiac dimensions is still generally perceived to be secondary to changes in cardiac contractility and subsequent alterations in cardiac preloads. What has not been given due consideration is that a primary target of β-adrenergic receptor activation or blockade is the cellular mechanisms responsible for cardiac dilatation. There are indeed a number of reasons to believe that chronic sympathetic activation in heart failure contributes to cardiac dilatation through direct cellular effects, rather than through indirect effects mediated by changes in cardiac contractility and subsequent alterations in cardiac preloads.

The following arguments support the hypothesis that chronic sympathetic activation in heart failure could contribute to cardiac dilatation through direct cellular effects: Circulating concentrations of norepinephrine may contribute to myocyte hypertrophy, either directly through stimulation of α_1- and β-adrenoreceptors or secondarily by activating the renin-angiotensin-aldosterone system (Schrier and

Abraham 1999). These changes may promote chamber dilatation through cell lengthening. Second, MMPs may be activated through both α- and β-adrenoreceptor pathways (Menon et al 2005; Karkoulias et al 2006), myocardial collagen synthesis by β-adrenoreceptor pathways (Grimm et al 1998), and as indicated above, apoptosis and necrosis through either α- or β-adrenoreceptor pathways (Communal and Colucci 2005). These changes could promote side-to-side slippage and cardiac dilatation.

Despite the evidence to suggest that chronic sympathetic activation in heart failure could contribute to cardiac dilatation through direct cellular effects, there is little clear evidence to support this notion. Thus, as part of the present thesis I sought to explore the impact of chronic adrenergic activation on cardiac chamber P-V relations, dimensions and systolic function. As described in this thesis and published in-part in the journal *Hypertension* (Badenhorst et al 2003b) I have been able to show that chronic adrenergic activation causes cardiac chamber dilatation through direct effects on remodeling mechanisms and not secondary to myocardial systolic dysfunction. The arguments in favor of this conclusion have been underscored in chapter 2.

In the present thesis, I noted that myocardial collagen concentrations were increased rather than decreased following chronic β-adrenoreceptor stimulation (chapter 3). Many forms of cardiac dilatation are accompanied by increases in myocardial collagen concentrations (Fielitz et al 2001). However, as discussed above, the role of increased myocardial collagen concentrations as determinants of cardiac dilatation has been questioned. Indeed, increases in myocardial collagen concentrations are thought to determine left and not right shifts in chamber diastolic P-V relations (Weber 1992; Norton et al 1997). Moreover, reverse remodeling following the use of assist devices is generally accompanied by increases and not decreases in myocardial collagen concentrations (Scheinin et al 1992; Li et al 2001). Further, pacing-induced cardiac dilatation (Spinale et al 1991b) and β-adrenergic-induced cardiac dilatation (Woodiwiss

et al 2001) are accompanied by decreases rather than increases in myocardial collagen concentrations. Nevertheless, I also noted that the cross-linking of myocardial collagen was reduced following chronic β-adrenoreceptor stimulation. As reductions in myocardial collagen cross-linking increase the susceptibility of collagen to MMP degradation, I proposed this to be a mechanism of the deleterious effects of chronic β-adrenoreceptor stimulation on chamber dimensions (chapter 3). Indeed, increases in the susceptibility of collagen to degradation are probably related to reductions in myocardial collagen cross-linking (Capasso et al 1989; Gunja-Smith et al 1996; Spinale et al 1996; Woodiwiss et al 2001). Further, by genetically decreasing the susceptibility of collagen to degradation, a reduced degree of dilatation accompanies pressure-overload states (Lindsey et al 2003). However, there is presently insufficient evidence to support the notion that increases, rather than decreases in myocardial collagen concentrations can lead to cardiac dilatation.

To confirm the hypothesis that increases in myocardial collagen concentrations of the non-cross-linked form could explain cardiac dilatation following chronic β-adrenoreceptor stimulation, I performed a further study. As described in chapter 4, I evaluated whether in various animal models of hypertensive hypertrophy the qualitative characteristics of myocardial collagen that accumulate determine the impact of increases in myocardial collagen concentrations on function. These data provided strong evidence to support this hypothesis. These data were published in *Cardiovascular Research* (Badenhorst et al 2003a) and generated an editorial review on the issue (Koshy et al 2003).

3. Gene variants that modify sympathetic effects as potential determinants of progressive heart failure

As discussed in 1.3 above, although excessive sympathetic activation is a major determinant of disease progression in heart failure it is not entirely clear why some patients respond particularly well to blockers of neurohumoral activation, whilst others progress rapidly to death. Moreover, it is uncertain why some patients manifest heart failure whilst others do not, despite a similar myocardial insult. A recent hypothesis is that genetic variants that influence the proteins involved in mediating neurohumoral effects may modify the natural history of disease progression or disease development (Figure 1). In the present thesis, I therefore also studied the role of gene variants that modify sympathetic effects as potential determinants of heart failure or progressive heart failure. The data that implicate genetic variants that control sympathetic effects as determinants of disease progression or development in heart failure are discussed in the following section. However, first, I will outline the evidence that genetic factors can contribute to cardiac dilatation and heart failure.

3.1.1 Are genetic factors important in dilated cardiomyopathies?

Idiopathic dilated cardiomyopathy (IDC) is one of the leading causes of severe heart failure (Diaz et al 1987; Keren et al 1990; Sugrue et al 1992). Despite improvements in therapy, mortality rates are still very high (Caforio et al 1990; Eichhorn 2001). The disease has a heterogeneous aetiology. Genetic factors have been identified to be potentially important in the manifestation of this disease. About one-third to one-half of patients with IDC have a family history of the disease in one or more relatives (Hershberger 2005) and the frequency of genetic transmission of the disease varies from 20% (Michels et al 1992) to 48% (Baig et al 1998). β-myosin heavy chain and cardiac troponin T genes have been shown to have mutations responsible for familial dilated cardiomyopathy (Kamisago et al 2000). Actin (Olson et al 1998), desmin (Li et al 1999)

and lamin (Fatkin et al 1999) gene mutations have also been implicated in familial dilated cardiomyopathy. However, these mutations are generally infrequent in IDC and hence their identification may not be of clinical use.

What has recently been considered is that common functional mutations in genes that produce changes in physiological responsiveness may modify the phenotypic expression of a disease entity such as IDC. These genetic variants are unlikely to cause disease processes, but rather determine clinical outcomes such as the progression of the disease or how early the disease manifests. It is hoped that by identifying the multiple genetic loci that influence either the development or severity of IDC, a significant proportion of biological variability will be accounted for and gene markers may therefore be used to implement more effective therapeutic strategies in individual patients. In this regard, likely gene candidates are those that modify the impact of the sympathetic nervous system in heart failure.

3.1.2 A potential role in heart failure for genes that influence sympathetic effects

As indicated in the above discussion (2.1.1), in the failing myocardium β-adrenoreceptor density is reduced and inhibitory G-protein concentrations are increased (Feldman et al 1988; Neumann et al 1988; Böhm et al 1990; Eschenhagen et al 1992). Consequently, genetic effects that may contribute toward disease progression or development in heart failure may involve any gene variant that modifies sympathetic activation. In this regard a number of variants of genes for proteins depicted in Figure 2 have been described.

3.1.3 Adrenergic receptor gene variants

Figure 6 illustrates those adrenergic receptor gene variants, shown to be associated with functional changes in receptor function, that were studied in the present thesis. Figure 6 also shows the portion of the adrenergic receptor that is modified by these variants. With regards to the β_2-adrenoreceptor, nine different polymorphisms of the β_2-adrenoreceptor gene have been described, of which four change the amino acid sequence of the receptor (Arg16 to Gly, Gln27 to Glu, Val34 to Met and Thr164 to Ile) (Reihsaus et al 1993). Figure 6 illustrates the two variants studied in the present thesis. The Arg16 to Gly variant leads to enhanced down-regulation of the β_2-adrenoreceptor in response to agonist stimulation, whereas the Gln27 to Glu is characterized by decreased down-regulation (Buscher et al 1999). Thr164 to Ile leads to several functional effects, which include lower binding affinities for agonists and deficient coupling of the receptor to adenylate cyclase (Buscher et al 1999). However, the prevalence of the Thr164 to Ile is too low to perform statistically powered association studies and hence was not studied in the present thesis. The Val34 to Met variant is extremely rare and is found in less than 1% of the population (Leineweber and Brodde 2004), thus also making this variant uninformative in association studies.

With respect to the β_1-adrenoreceptor, a polymorphism in the intracellular cytoplasmic tail near the seventh transmembrane-spanning segment of the human β_1-adrenoreceptor has been identified (Mason et al 1999) (Figure 6). At amino acid position 389, Gly or Arg may be found (Mason et al 1999). This polymorphism is associated with alterations in adenylyl cyclase activities and responses to the β-adrenoreceptor agonist, isoproterenol (Joseph et al 2004). Hence this polymorphism is likely to result in alterations of the β_1-adrenoreceptor-Gs interaction with functional signal transduction consequences (Mason et al 1999).

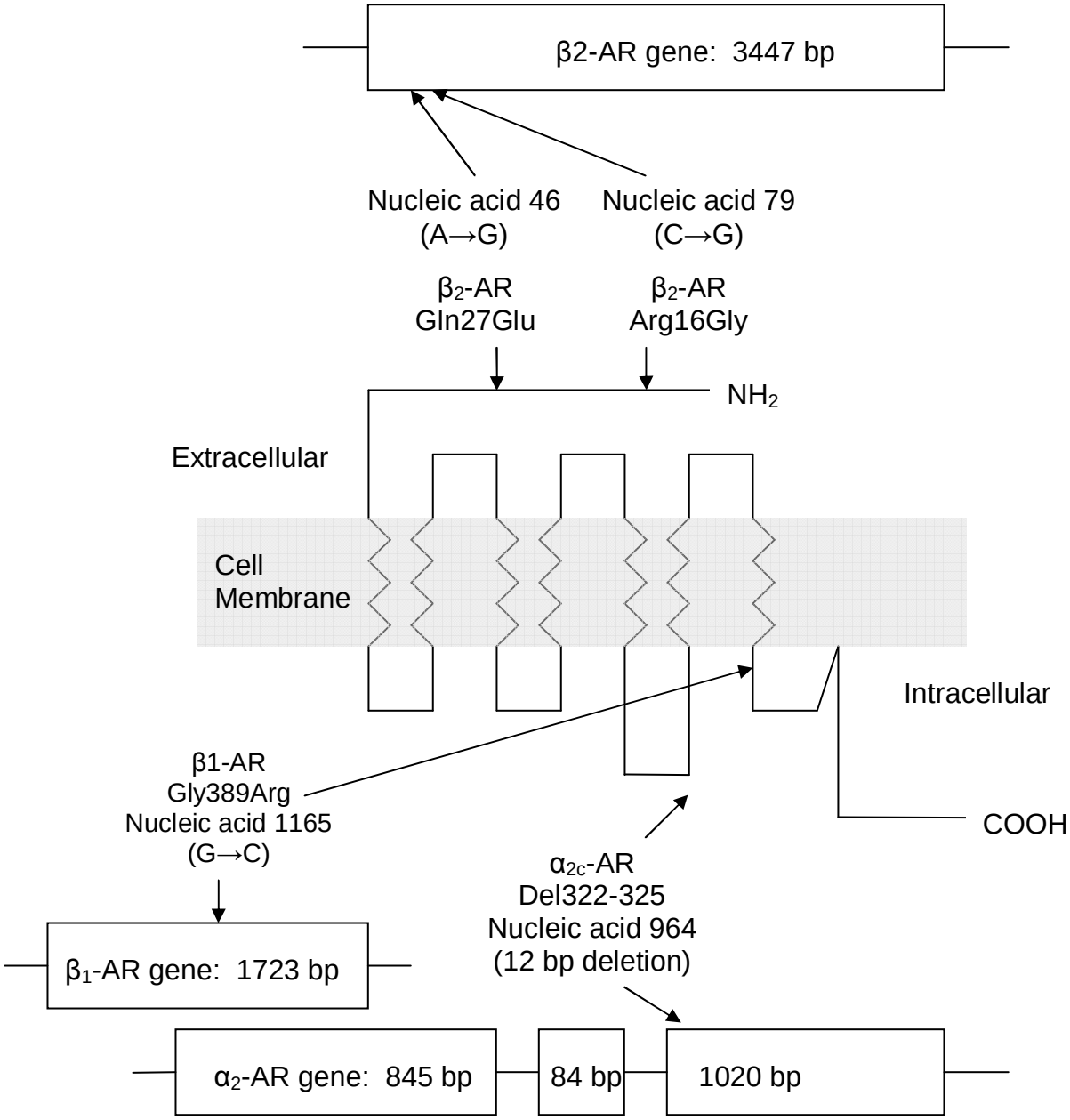


Figure 6. Adrenoreceptor structure and maps of genes depicting the variants that modify the structure of the adrenoreceptor. See text for the functional effects of these variants. Figure modified from Small et al (2003).

3.1.4 Association of adrenergic receptor gene variants with heart failure

Recent data indicate that a variety of variants of the β_2-adrenoreceptor gene are associated with alterations in mortality and morbidity (Liggett et al 1998; Forleo et al 2004), or exercise capacity (Wagoner et al 2000) in heart failure. Although inconsistencies exist in data with regards to the allele that exerts detrimental effects for the Gln27Glu variant (Wagoner et al 2000; Forleo et al 2004) and whether clinical outcomes are modified by the Thr164Ile variant of the β_2-adrenoreceptor gene (Liggett et al 1998; Forleo et al 2004) consistently, the Gly16 allele of the Arg16Gly polymorphism has been demonstrated by two separate groups of investigators to be associated with clinical disadvantages (Wagoner et al 2000; Forleo et al 2004). Although these data indicate an important role for the Arg16Gly polymorphism of the β_2-adrenoreceptor gene in heart failure, the mechanisms involved are unclear. Indeed, these studies were cross-sectional rather than prospective analyses, conducted in patients with diverse cardiac pathologies and performed in patients of whom a proportion were receiving β-adrenoreceptor blocker therapy (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004). The presence of β-adrenoreceptor blocker therapy make the data from these studies (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004) particularly difficult to interpret as some studies have shown β_2-adrenoreceptor genotype-specific effects on LV structure and function after β-adrenoreceptor blocker therapy (Kaye et al 2003).

A potential mechanism that could explain a decreased exercise-induced increase in oxygen consumption, as well as an increased morbidity and mortality in chronic heart failure (Wagoner et al 2000; Forleo et al 2004), is a reduction in pump function due to a reduced β_2-adrenoreceptor-mediated signaling. The reduction in pump function may be

a consequence of either an impaired contractile response to receptor agonists, or a decrease in the protective effects of β_2-adrenoreceptors on apoptotic signaling, thus promoting cardiac dilatation (see sections 2.1.1 and 2.1.2). However, this hypothesis has not formally been tested. To test this hypothesis I conducted the study described in chapter 5 of this thesis. This work is presently *in press* in the journal *Pharmacogenomics* (Badenhorst et al *in press*).

With respect to the β_1-adrenoreceptor gene polymorphism at codon 389 (Gly389Arg), although there is a greater response to β-adrenoreceptor-blocker therapy in patients homozygous for the Arg389 allele (Johnson et al 2003; Liu et al 2003; Mialet Perez et al 2003), there is no clear independent association between this variant of the β_1-adrenoreceptor gene and heart failure (Tesson et al 1999; Small et al 2002; Mialet Perez et al 2003). Nevertheless, the Arg389 allele of the β_1-adrenoreceptor gene, when present with a polymorphic α_{2c}-adrenoreceptor gene variant, is a risk factor for human heart failure in Black African subjects (Small et al 2002). As discussed in section 2.1.1 above, α_2-adrenoreceptors operate as presynaptic inhibitory receptors that control the release of norepinephrine and influence the progression of heart failure (Brede et al 2002). These receptors may thus prevent excess sympathetic activity and thus disease progression in heart failure. However, the relationship between the β_1-+α_2-adrenoreceptor gene variants and IDC in Black African subjects was identified in a small study sample of patients of African descent (Small et al 2002). In the present study I therefore further explored whether this relationship existed in a larger study sample of a group of African descent living in Africa. These data are described in chapter 6.

CHAPTER 2

β-adrenergic activation initiates chamber dilatation and pump dysfunction in concentric hypertrophy

ABSTRACT

It is uncertain whether chronic β-adrenoreceptor (β-AR) activation in hypertension could initiate the progression from compensated left ventricular hypertrophy (LVH) to pump dysfunction, and if this effect is through adverse LV remodeling (chamber dilatation with wall thinning and pump dysfunction) or intrinsic myocardial contractile dysfunction. I evaluated the effect of 5 months of isoproterenol (ISO, 0.02 $mg.kg^{-1}.d^{-1}$) on haemodynamics, LV wall thickness, and cavity size in spontaneously hypertensive rats (SHR) with compensated LVH. In the absence of either changes in volume preload, pressure afterload and heart rate, myocyte necrosis, or decreases in baseline systolic myocardial elastance (load independent measure of intrinsic myocardial contractility), ISO produced a right shift in LV diastolic pressure-volume (P-V) relations (chamber dilatation); a decrease in LV wall thickness despite a further increase in LV weight in SHR; and LV pump dysfunction (right shift in LV systolic P-V relations). The ISO-induced LV geometric and chamber performance changes were similar to alterations noted during decompensation in older SHR. In summary, in the absence of baseline intrinsic myocardial contractile dysfunction, chronic β-AR activation induces adverse chamber remodeling (dilatation) and hence pump dysfunction. These data suggest that chronic sympathetic activation initiates the progression from compensated concentric LVH in hypertension to cardiac dysfunction primarily through deleterious cardiac remodeling rather than intrinsic myocardial contractile dysfunction.

INTRODUCTION

Left ventricular hypertrophy (LVH) is an independent risk factor for the development of heart failure (Levy et al 1990). Although LVH in hypertension can be considered an adaptive response to reduce wall stress (Grossman et al 1975), LVH precedes the development of chronic heart failure (Spann et al 1967; Inoko et al 1994; Bing et al 1995). Indeed, present guidelines have been adapted to underscore the evolution and progression from hypertension via LVH to overt heart failure with dilatation and systolic dysfunction (Hunt et al 2001). Despite the acknowledged importance of LVH as a risk factor for heart failure, the fundamental mechanisms involved in contributing to the progression from compensatory LVH in hypertension to heart failure are largely undefined.

It is now well recognized that adrenergic activation in heart failure (Hasking et al 1986) contributes to progressive cardiac dysfunction (Cohn et al 1984; Bristow 1997). There is also evidence to suggest that excessive adrenergic activation may also promote the progression from LVH to LV decompensation. First, increased myocardial norepinephrine concentrations are measured in the coronary sinus in patients with hypertensive hypertrophy prior to the development of heart failure (Agabiti-Rosei et al 1987; Kelm et al 1996; Schlaich et al 2003). Second, transgenic animal models with decreased adrenergic activation are protected against the development of dilatation and heart failure when exposed to pressure-overloads (Esposito et al 2002). Third, in compensated hypertensive hypertrophy excessive adrenergic activation is associated with downregulation of β-adrenergic systems (Limas and Limas 1978; Castellano et al 1993; Böhm et al 1994a; Böhm et al 1995), changes that could promote the development of contractile dysfunction. Lastly, blockade of β-adrenoreceptors prevents the transition from cardiac hypertrophy to heart failure in hypertension independent of blood pressure effects (Chan et al 2004).

The mechanisms which underlie cardiac dysfunction are a decreased intrinsic myocardial contractility, adverse chamber remodeling or a combination of these. In patients with heart failure it has been postulated that adrenergic activation contributes to progressive cardiac dysfunction through intrinsic myocardial contractile dysfunction and subsequently chamber dilatation (Sabbah 1999). However, recently it has been suggested that deleterious cardiac remodeling rather than decreased intrinsic myocardial contractility may be a precursor of heart failure (Vasan et al 1997; Norton et al 2002).

In the present study I hypothesized that excessive adrenergic activation could initiate the progression from compensated LVH in hypertension to cardiac dysfunction and that this effect is primarily through adverse LV remodeling (dilatation). In order to examine these hypotheses I evaluated whether chronic β-adrenoreceptor (AR) activation, following daily low-dose isoproterenol administration, could induce premature progression to LV pump dysfunction through either intrinsic myocardial contractile dysfunction or LV dilatation in spontaneously hypertensive rats (SHR) with compensated concentric LVH. The response to β-AR antagonists was not assessed as their antihypertensive action would be difficult to distinguish from direct myocardial effects.

METHODS

The present study was approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC approval numbers 99/01/2b, 2002:37:5 and 2002:39:5).

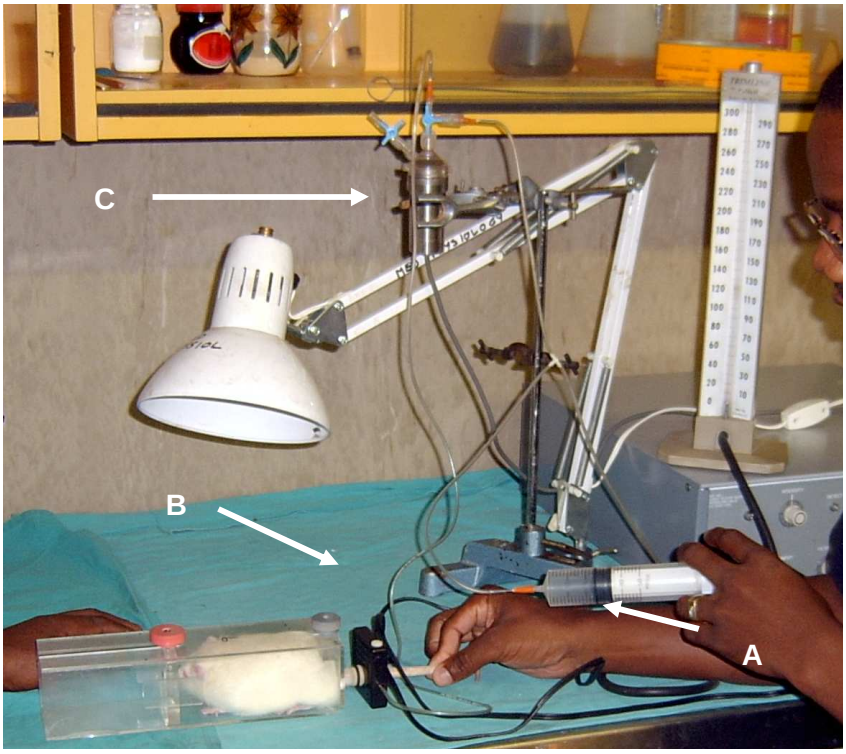
Experimental groups. Seven month old SHR (n=32; OLAC, UK) and Wistar Kyoto controls (WKY, n=34; Kleinterfarm Madorin Ltd, Germany) were assigned to a group of rats (11 SHR and 8 WKY) who received isoproterenol (ISO, Imuprel, Adcock Ingram) at a dose of 0.02 $mg.kg^{-1}.d^{-1}$ subcutaneously (≈0.1 ml) for 5 months (Woodiwiss

et al 2001), to a group of rats (11 SHR and 14 WKY) who received the same volume of the vehicle of ISO (0.1 ml of 0.9% saline) for 5 months, and to a group of rats (10 SHR and 12 WKY) who received no treatment until haemodynamic changes were evaluated at 21-22 months of age. Rats at 21-22 months of age were included in the study in order to assess whether the effect of ISO on LV geometry and haemodynamics in SHR has similarities consistent with the LV changes noted in SHR during the period when decompensation and dilatation occurs (Bing et al 1995; Norton et al 1997; Tsotetsi et al 2001). None of the rats died during ISO or vehicle administration, and 1 untreated SHR died of an indeterminate cause at 10 months of age (not included in the above sample numbers).

In order to evaluate whether similar effects of ISO could be reproduced in older SHR with more advanced LVH, in a separate study, 14 month old SHR (n=18) and WKY (n=9) were assigned to a group of rats (9 SHR) who received ISO for 5 months, and to a group of rats (9 SHR and 9 WKY) who received the same volume of the vehicle of ISO for 5 months. I did not study a group of WKY rats receiving ISO from 14 months of age, as 5 months of ISO administration was unable to produce pump dysfunction in 7 month old WKY rats. No deaths were noted prior to the end of this study.

Chronic ISO administration may produce an enhanced LV filling volume through β_2-AR-mediated vasodilation and/or sympathetic effects on the kidney, and consequently mediate LV remodeling through indirect mechanisms (preload-induced changes). To assess this hypothesis I also evaluated whether a relatively short period of ISO (4 weeks) administered to SHR (9 ISO treated and 8 controls) produced alterations in LV filling dimensions as determined in intact animals.

Systolic blood pressures. Non-invasive systolic blood pressures (SBP) were measured as previously described (Norton et al 1993) at regular periods throughout the studies. The experimental setup for these studies is depicted in Figure 7. Rats were



A = To inflate tail cuff

B = Photoelectric diode and tail cuff

C = Pressure transducer

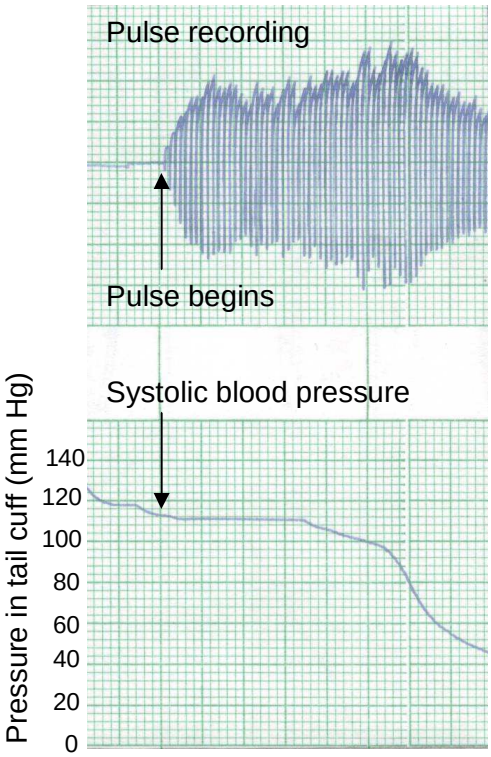


Figure 7. Photograph of the experimental setup used to measure tail artery systolic blood pressures and heart rate in rats (upper panel) and an example of a recording obtained (lower panel).

placed in restrainers for an hour a day on five separate days prior to the first measurement in order to habituate them to the procedure. In order to determine BP and heart rate, rat tails were pre-warmed until the tail artery pulse could be detected with a photoelectric diode (model 29 Pulse Amplifier). A tail-cuff coupled to a pressure transducer was placed on the rat tail proximal to the photoelectric diode and inflated until the tail pulse disappeared. The tail cuff pressures were then slowly released until the tail artery pulse returned. Systolic BP was taken as the cuff BP at which the tail artery pulse returned. Recordings of the tail cuff pressure and the pulse were made on a Beckman model R511A recorder (Figure 7).

LV cavity size and geometry determined in vivo in open-chest rats. LV dimensions and geometry were determined in anaesthetized, ventilated, open-chest rats using piezoelectric ultrasonic transducers placed across the short axis of the LV. The surgery, instrumentation, experimental techniques, and calculations used in the present study have previously been described and validated (Bing et al 1995; Woodiwiss and Norton 1995; Tsotetsi et al 2001). The experimental setup for these studies is depicted in Figure 8.

Rats were anaesthetized using ketamine ($75mg.kg^{-1}$) and xylazine ($15mg.kg^{-1}$) and a polyethylene PP25 catheter inserted into the carotid artery. A tracheostomy was then performed and positive pressure ventilation initiated just prior to performing a midline thoracotomy. Once the chest was opened ventilation volumes and rate were adjusted to achieve a PaO_2 of 90-110 mm Hg measured from a carotid arterial blood sample. LV short axis external diameters were measured throughout the cardiac cycle using piezoelectric ultrasonic transducers placed across the short axis of the heart by means of a cradle designed and validated in our laboratory (Norton et al 1993; Trifunovic et al 1995; Woodiwiss and Norton 1995; Norton et al 1997) (Figure 8a). Left ventricular end diastolic pressures (LVEDP) were measured using a fluid-filled catheter inserted

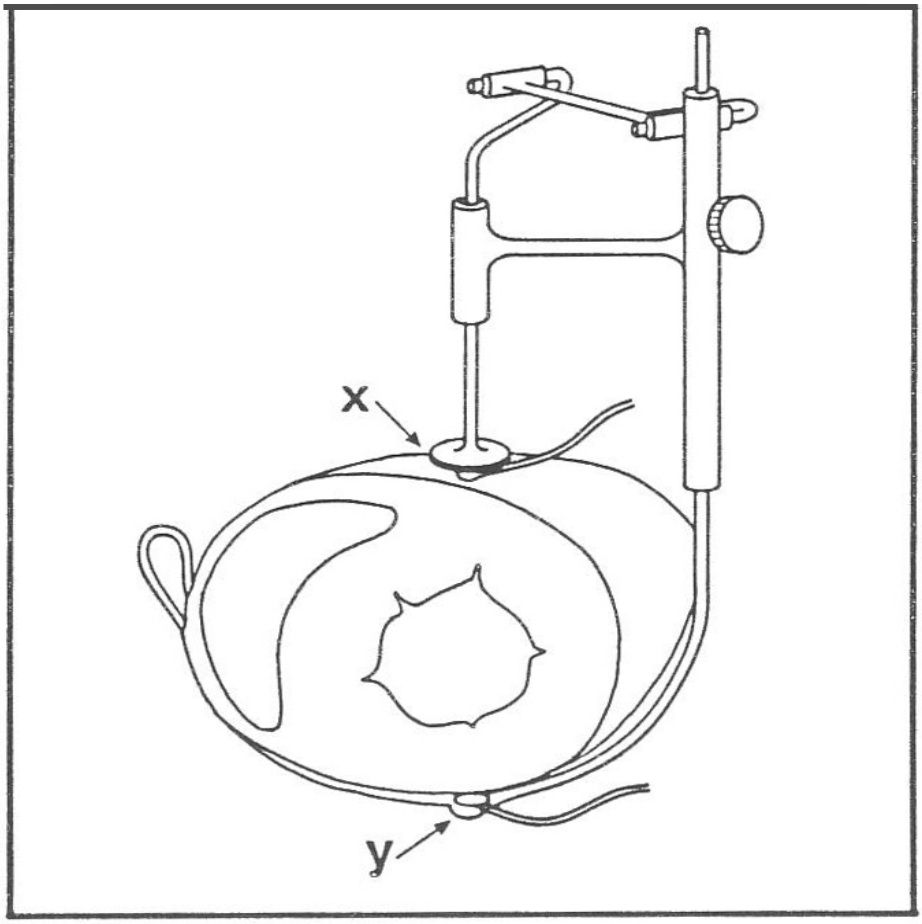


Figure 8a. Schematic to illustrate the experimental setup used to determine short axis dimension measurements over a range of preloads in rats (upper panel). Representative data obtained are shown in Figure 8b. ‘x’ and ‘y’ indicate the transmitting and receiving piezoelectric transducers respectively.

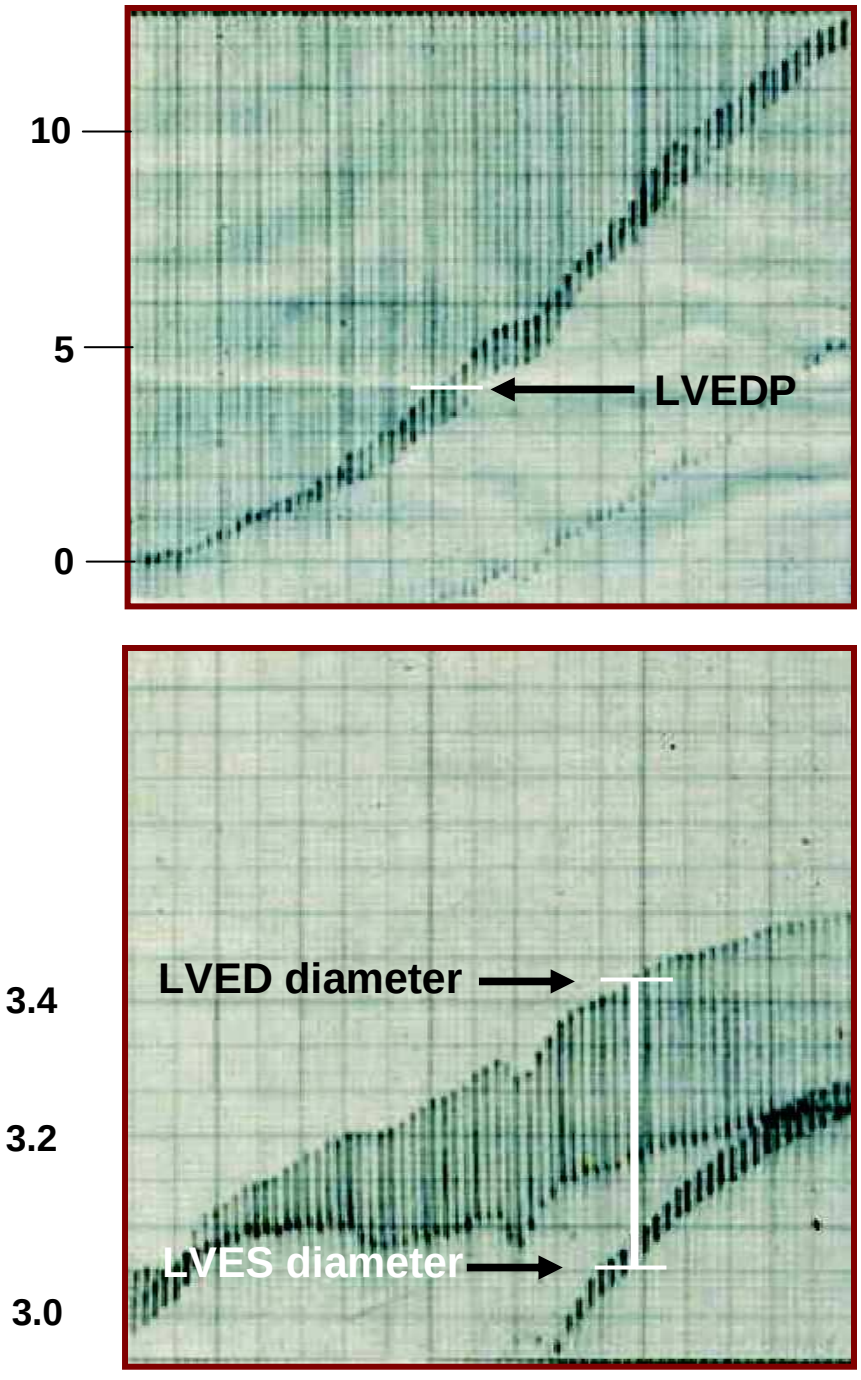


Figure 8b. Representative data obtained from the experimental setup shown in Figure 8a. LVED, left ventricular end diastolic, LVEDP, LVED pressure.

through the apex of the LV (Figures 8a and 8b). The amplitude-frequency response of the catheter, dome and pressure transducer combination was uniform to 10 Hz (Norton et al 1997). Measurements of LVED external diameters and LVEDP were obtained over a range of LVEDP values by manipulating blood volume using an iso-oncotic solution (Dextran) as well as through inferior vena cava occlusion. The iso-oncotic solution was first 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 (Figure 8b). Inferior vena cava occlusion was employed to obtain data over a range of LVEDP values less than 5 mm Hg in some rats. LVED radius (r) and wall thickness (h) was determined from previously described formulae (Tsotetsi et al 2001) as follows:

$$\text{LVED r} = \sqrt[3]{[\text{LVED external diameter}/2]^3 - \text{LV wall volume}[3/4\pi]}$$

where LV wall volume = 0.943xLV wet weight and LVED h = (LVED external diameter-2r)/2. Relative wall thickness was determined from LVED h/r. LV remodeling was also assessed from the LVEDr intercept of the LVEDP-LVEDr relation ($LVEDr_0$). An appropriate range of LVEDP and LVED external diameter measurements were successfully obtained in all rats surviving the duration of the study.

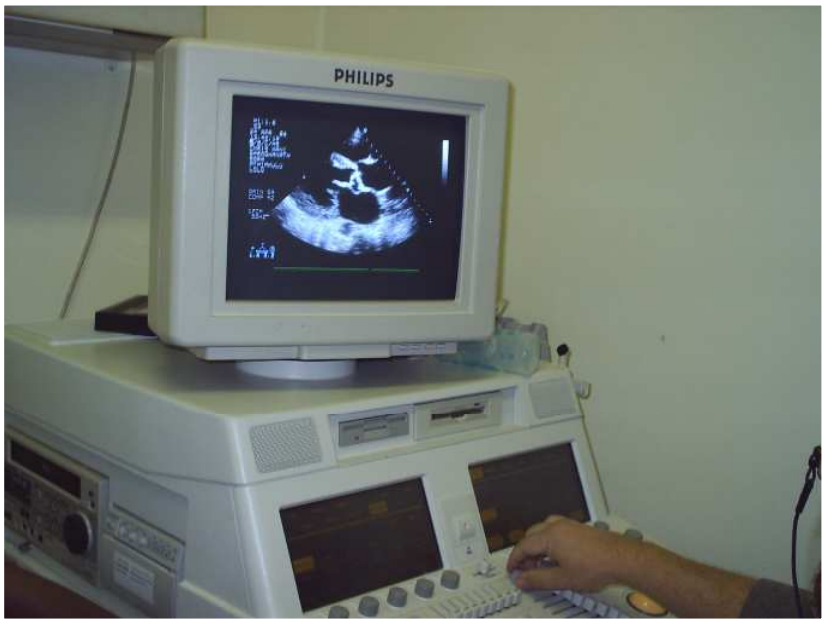
LV cavity size and geometry determined in vivo in closed chest rats. In the group of rats studied from 14 to 19 months of age, echocardiography was performed 24 hours after the last dose of ISO and a day prior to assessing LV geometry and dimensions using piezoelectric transducers. In SHR used to assess the effects of ISO on preload, a day prior to assessing LV geometry and dimensions using piezoelectric transducers, echocardiography was performed approximately 1 hour after the last dose of ISO and following 4 days of daily injections of ISO. Echocardiography was not performed in younger SHR as the degree of concentric LV remodeling prohibited the accurate

measurement of end systolic dimensions. The anaesthetic and the methodological approach employed to perform echocardiography have previously been described (Chung et al 1998). The measurement setup is illustrated in Figure 9.

Briefly, rats were anaesthetized with ketamine ($75mg.kg^{-1}$) and xylazine ($15mg.kg^{-1}$) their chests shaved and placed in a prone position in a box with windows cut out both beneath the nose and mouth of rats to prevent asphyxiation and beneath the anterior chest wall of rats to allow for transducer placement. A 7.5 MHz transducer was then used to acquire M-mode recordings in the short axis of the LV at the level of the papillary muscles using two-dimensional guidance. All data were acquired using a model 2000 Hewlett Packard echocardiograph (Figure 9) and recordings were made by an echocardiographer without knowledge of the rat's identity. Analysis of at least three consecutive beats was performed according to the leading edge method described by the American Society of Echocardiography (Sahn et al 1978). Analysis was not performed on-line, but rather print-outs of recordings were obtained, scanned into a digital computer, coded by an independent individual and read by the investigator without knowledge of the rat's identity by code. Left ventricular end diastolic (LVEDD) and end systolic (LVESD) internal diameters and LV end diastolic ($PWT_{diastole}$) and end systolic ($PWT_{systole}$) posterior wall thickness values were determined (Figure 9). Left ventricular endocardial fractional shortening (LV FS_{end}) and midwall fractional shortening (LV FS_{mid}) were determined from the following equations and used as an assessment of LV chamber and myocardial systolic function respectively (Norton et al 2002). These equations assume a similar anterior as compared to posterior wall thickness. Anterior wall thickness values were not used as the correlation coefficient between observers was poor.

LV FS_{end} = LVEDD-LVESD/LVEDD x 100

LV FS_{mid} = (LVEDD+$PWT_{diastole}$)-(LVESD+$PWT_{systole}$)/(LVEDD+$PWT_{diastole}$) x 100



A = Left ventricular end diastolic diameter
B = Left ventricular end systolic diameter
C = Left ventricular end diastolic posterior wall thickness
D = Left ventricular end systolic posterior wall thickness

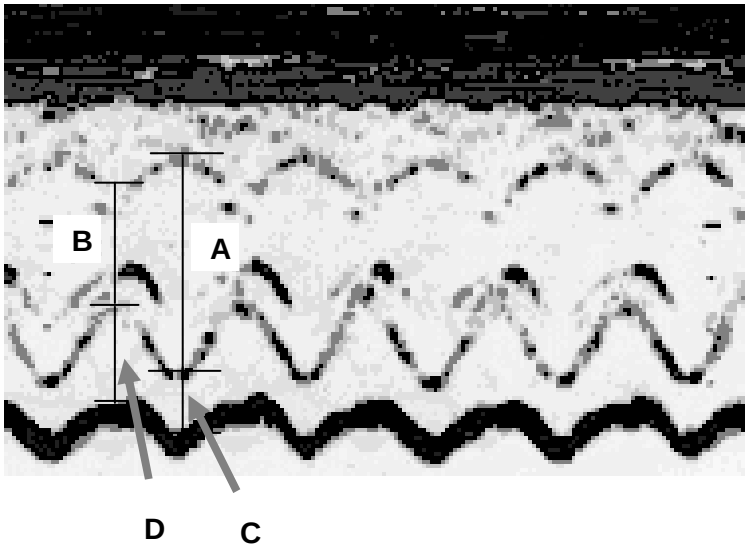


Figure 9. Echocardiograph used to assess cardiac structure and function in rats (upper panel) and representative recording showing the measurements made (lower panel).

Isolated perfused heart preparations. Following the collection of LVEDP and LVED external diameter data *in vivo*, LV systolic function and LV remodeling was assessed *in vitro* in isolated perfused heart preparations as previously described (Woodiwiss et al 2001). Whilst rats were still anaesthetized, hearts were removed from the chest cavity and placed in an ice-cold saline solution until they were mounted on a perfusion apparatus illustrated in Figure 10. Hearts were perfused retrogradely (via the aorta) at a constant flow (12 $ml.min^{-1}$.g wet heart weight) with 37℃ physiological saline solution consisting of (in mM) 118.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$ and 10.0 glucose with a pH of 7.4. The solution was saturated with 95% O_2 and 5% CO_2 gas and carefully filtered through a size 0.45μm Millipore Durapore membrane filter. Hearts were paced at 360 $beats.min^{-1}$ with platinum electrodes attached to the left atrium and the apex of the heart. An empty latex balloon with a known wall volume (determined with a water displacement technique), coupled to a Statham P23 pressure transducer and a micromanipulator (see Figure 10) via a polyethylene catheter, was inserted through the mitral valve into the LV lumen. LV systolic and diastolic pressures were determined over a range of as many multiple small increments in volume as were practically possible to improve on the accuracy of curve fitting during later analysis (Figure 10). Balloon volume was increased by means of adjusting the micromanipulator.

LV systolic chamber performance (a measure of systolic pump function) was determined *in vitro* from the slope (E) of the linear portion of the LV peak systolic P-V relation. Intrinsic myocardial systolic performance (a load-independent measure of intrinsic myocardial contractility) was assessed *in vitro* from the slope (E_n) of the systolic stress (σ)-strain relation (Norton et al 2002). Systolic σ and strain were calculated using previously described formulae (Weber et al 1988) assuming a thick-

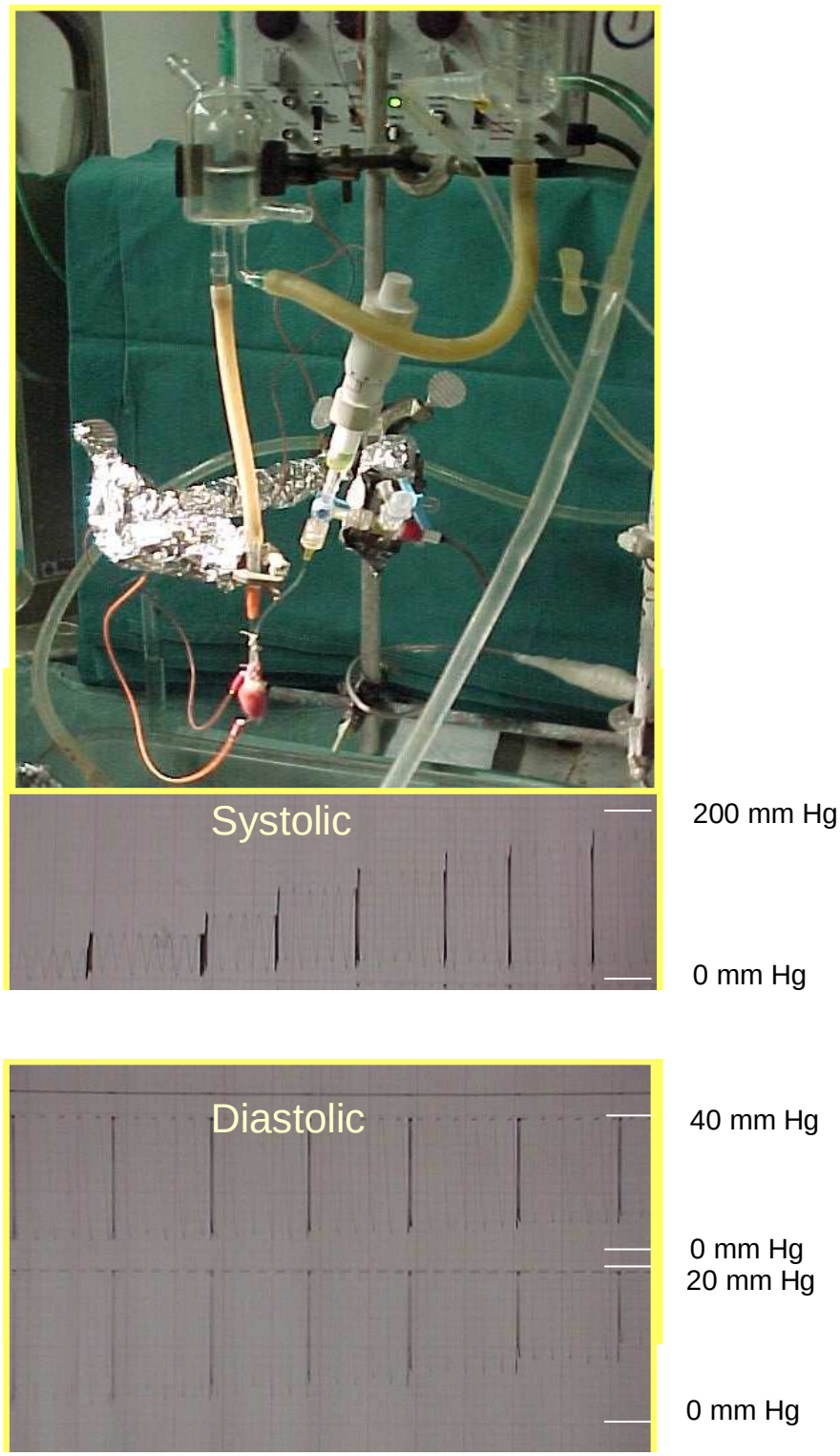


Figure 10. Experimental setup for the isolated, perfused heart apparatus (upper panel) and typical recordings obtained (lower panel).

walled, spherical model of LV geometry as follows:

$\sigma = [1.36\ P\ V^{2/3}]/[(V+V_m)^{2/3}-V^{2/3}]$

$\text{strain} = \{[V^{1/3}+(V+V_m)^{1/3}]/[V_0^{1/3}+(V_0+V_m)^{1/3}]\} - 1$

where P=pressure, V=volume, V_m=left ventricular muscle volume, V_0=unstressed left ventricular volume

LV remodeling was assessed in isolated, perfused heart preparations from the volume intercept (V_0) of the LV diastolic P-V relation (Woodiwiss et al 2001).

Myocardial norepinephrine. In order to ensure that LVH in SHR is indeed associated with excessive myocardial catecholamine release, norepinephrine (NE) concentrations were determined in the coronary effluent of isolated, perfused hearts in 7 and 14 month old SHR and WKY rats. For this purpose a sample of coronary effluent was collected for one minute in pre-chilled containers at a constant coronary flow (12 $ml.min^{-1}$.g wet heart weight). A pilot study conducted in SHR established that NE release decreased when measured at incremental LV filling volumes from 0.20 mls (0.34±0.03 $nmol.ml^{-1}$) to 0.23 mls (0.26±0.03 $nmol.ml^{-1}$, $p<0.01$ versus 0.20 mls) to 0.27 mls (0.22±0.02 $nmol.ml^{-1}$, $p<0.001$ versus 0.20 mls)(the filling volumes at which most hearts exhibited a wide range of filling volumes). Consequently, all measurements of NE release were performed at 0.23 ml filling volumes. Coronary effluent was stabilized with Na_2EDTA and $HClO_4$ (0.01$mol.l^{-1}$ and 0.025% respectively). Norepinephrine was immediately extracted from 1 ml of coronary effluent using alumina (Sigma) adsorption with a Tris buffer at pH 8.6 eluted with 0.1M $HClO_4$ (Ganhao et al 1991), stored at -70℃ and concentrations determined using reversed phase, ion-exchange high performance liquid chromatography with electrochemical detection (Ganhao et al 1991). The equipment set-up and a typical print-out obtained are illustrated in Figure 11. As all

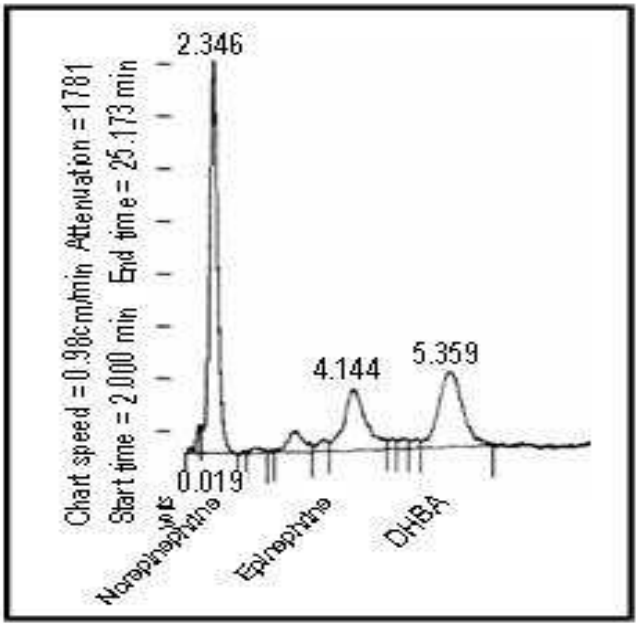


Figure 11. High performance liquid chromatograph used to determine coronary effluent norepinephrine concentrations (upper panel) and typical example of data obtained when determining monoamine concentrations in coronary effluent samples (lower panel).

hearts were perfused at the same flow rates per gram of tissue, myocardial NE release was expressed as the concentration of NE in the effluent.

3,4-dihydroxybenzylamine (DHBA, Sigma) was used as an internal standard for catecholamine detection. Standard curves for DHBA and NE were generated on each day that measurements were obtained. The inter-assay variance was very low for both DHBA (1.9%) and NE (3.2%).

Myocyte necrosis. A longitudinal slice of the LV from the apex to the base through the LV free wall was obtained from all rats for histology. LV tissue was stored in formalin for subsequent histology. LV tissue was processed routinely for light microscopy and 50 μm-thick sections of the long axis circumference were cut through the full thickness of the LV wall. Ten slices were obtained at 1-mm intervals and stained with Masson's trichrome from which haematoxylin was omitted (study conducted in young SHR) or van Gieson's stain (older SHR). After staining a pathological grade was assigned, where 0 indicates no damage; 1 and 2, patchy fibrosis in less than or more than 20% of the field respectively; 3 and 4, diffuse contiguous subendocardial fibrosis in less than or more than 50% of the field respectively and 5 and 6, full thickness fibrosis in less than or more than 50% of the field respectively (Teerlink et al 1994; Woodiwiss et al 2001). Representative slides showing fibrotic areas identified using either of the staining techniques is illustrated in Figure 12.

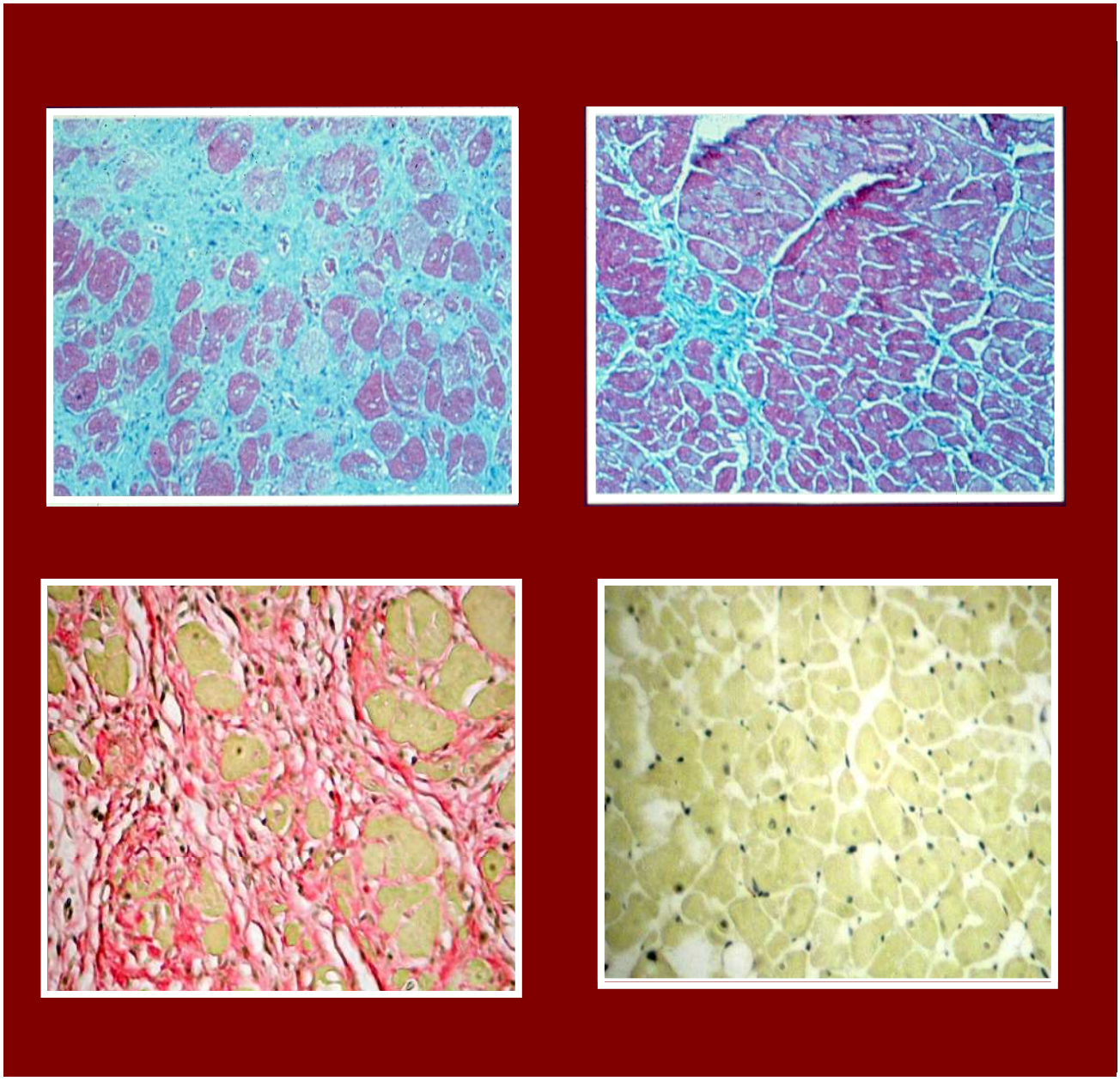


Figure 12. Histological images obtained using light microscopy from cross-sections of myocardial tissue stained with either Masson's trichrome from which haematoxylin was omitted (upper panels) or van Gieson's stain (lower panel). The slides show evidence of tissue necrosis and diffuse fibrosis following cell death (left panels) as compared to normal hearts (right panels). The slides on the left were from a heart of an SHR with advanced heart failure.

Data analysis. Regression analysis was used to determine the lines of best fit for the cardiac function relations. LV systolic P-V and LV systolic σ-strain relations were found to best fit a linear function. Differences in LV geometry, LV chamber and myocardial performance, haemodynamics and myocyte necrosis between groups were identified by a one-factor ANOVA followed by a Tukey *post hoc* test. All values in the text are represented as mean ± SEM.

RESULTS

Blood pressure and heart rate. As determined in unanaesthetized, restrained rats SHR had an increased SBP, but no differences in heart rate, as compared to WKY controls throughout the study (SBP a week prior to assessing LV geometry and function: SHR=185±8 mm Hg, WKY=122±6 mm Hg, $p<0.001$). Similarly, throughout the duration of the study, ISO administration failed to influence either SBP (SHR receiving ISO=180±9 mm Hg determined 2 weeks after initiating ISO injections and 182±8 mm Hg determined a week prior to LV geometry and function measurements) or heart rate (SHR not receiving ISO=462b.min^{-1}, SHR receiving ISO for 2 weeks=472 b.min^{-1} and SHR receiving ISO=470 b.min^{-1}as determined a week prior to LV geometry and function measurements and 45 minutes after ISO injection) in either SHR or WKY rats. The lack of effect of ISO on SBP and heart rate in unanaesthetized, restrained rats was noted throughout the study irrespective of whether measurements were taken within 1 hour, 2 hours or 12 hours of ISO injection (data not shown). Moreover, ISO failed to influence carotid SBP, diastolic BP or pulse pressure (in mm Hg: SHR=44 mm Hg, ISO-treated SHR=48 mm Hg) as determined in anaesthetized rats.

LV and body weight. SHR at all ages had an increase in LV weight as compared to age-matched WKY controls (see Tables 2 and 3 for data in rats studied from 7 to 12 months of age and in older SHR). The increased LV weight in SHR was augmented with

Table 2. Effect of chronic isoproterenol administration from 7-12 months of age on left ventricular and body weight in spontaneously hypertensive and Wistar Kyoto control rats. Data from 21-22 month old SHR are included as a comparator.

	SHR	SHR	SHR	WKY	WKY	WKY
Age (months)	12	12	21-22	12	12	21-22
Treatment	-	ISO	-	-	ISO	-
LV weight (g)	1.08±0.03*	1.30±0.03**††	1.34±0.04**††	0.95±0.03	1.02±0.05	0.98±0.02
Body weight (g)	355±8**	369±7	351±12*	432±14	383±19	405±9
LV/BW x 10^{-3}	3.05±0.05**	3.53±0.09**†	3.85±0.20**††	2.47±0.05	2 .66±0.08	2.41±0.03

SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto control; ISO, isoproterenol; LV, left ventricle; BW, body weight.

* p<0.05, ** p<0.001 versus age-matched WKY; † p<0.01, †† p<0.001 versus untreated 12 month old SHR group.

Table 3. Effect of chronic administration of isoproterenol (ISO) from 14-19 months of age on left ventricular (LV) and body weight, and posterior wall thickness in spontaneously hypertensive rats (SHR). WKY rats are age-matched.

	WKY	SHR	SHR+ISO
N=	9	9	9
LV weight (g)	1.08±0.04	1.32±0.06**	1.56±0.09**†
Body weight (BW) (g)	438±12	359±6**	353±10**
LV/BW x 10^4	2.45±0.14	3.66±0.14**	4.41±0.34**†
LV posterior wall thickness# (cm)	0.19±0.01	0.28±0.01**	0.21±0.02†

at end diastole; WKY, Wistar Kyoto control. * $p<0.05$, ** $p<0.01$ versus WKY group, † $p<0.01$ versus SHR group.

age (Tables 2 and 3). ISO administration further increased LV weight in SHR to values not significantly different from SHR at 21-22 months of age (Tables 2 and 3). Although ISO administration to WKY rats tended to reduce body weight and to increase LV weight, these effects were not statistically significant (Table 2). Body weights were consistently lower in the SHR as compared to age-matched WKY (Table 2).

LV cavity dimensions.

Ultrasonic transducers. Consistent with the development of LV remodeling (LV dilatation), at 21-22 months of age SHR developed an increased $LVEDr_0$ (Figure 13). Chronic ISO administration to SHR at both 7 and 14 months of age produced a marked increase in LVED internal radius and $LVEDr_0$ to values comparable with those noted in SHR at 21-22 months of age (Figures 13 and 14). Although there was a trend for ISO administration to increase LV cavity size in WKY rats, this effect was statistically insignificant (Figure 13).

Isolated, perfused hearts. Data obtained in isolated, perfused hearts essentially reproduced that obtained with ultrasonic transducers. Consistent with the development of LV remodeling (LV dilatation), at 21-22 months of age SHR developed an increased LVV_0 (Figure 15). Chronic ISO administration to SHR at both 7 and 14 months of age produced a marked increase in LVV_0 to values comparable with those noted in SHR at 21-22 months of age (Figures 15 and 16). Although there was a trend for ISO administration to increase LV cavity size in WKY rats, this effect was statistically insignificant (Figure 15).

Echocardiography. At 19 months of age, SHR had similar LV internal dimensions (LVEDD and LVESD) as compared to age-matched WKY (for LVEDD see Figure 17). ISO administration increased both LVEDD and LVESD in SHR (for LVEDD see Figure 17). However, ISO administered for 1 month failed to increase LVED internal diameters (LVEDD in cm; ISO=0.799±0.018, CONTROL=0.807±0.014).

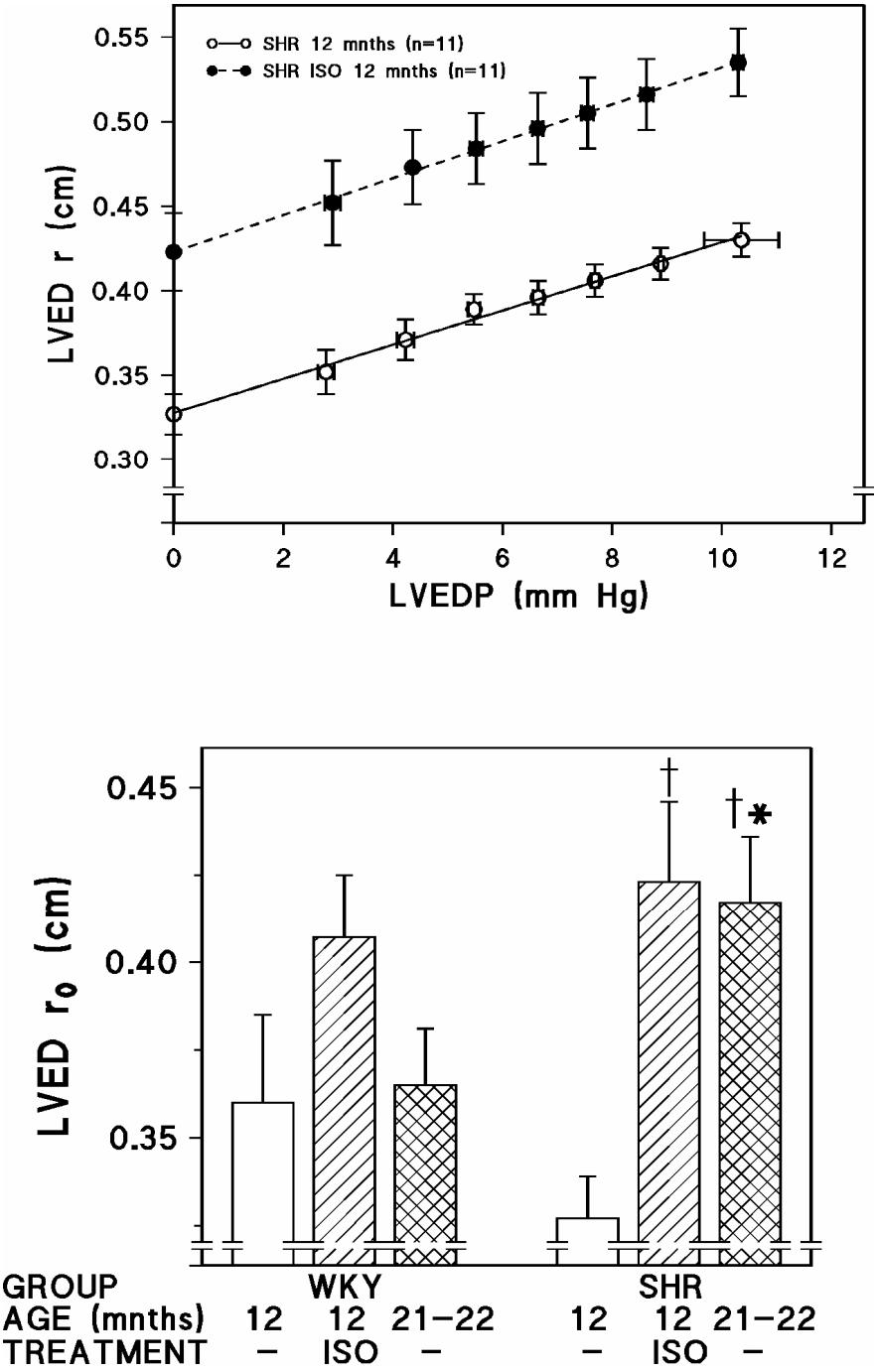
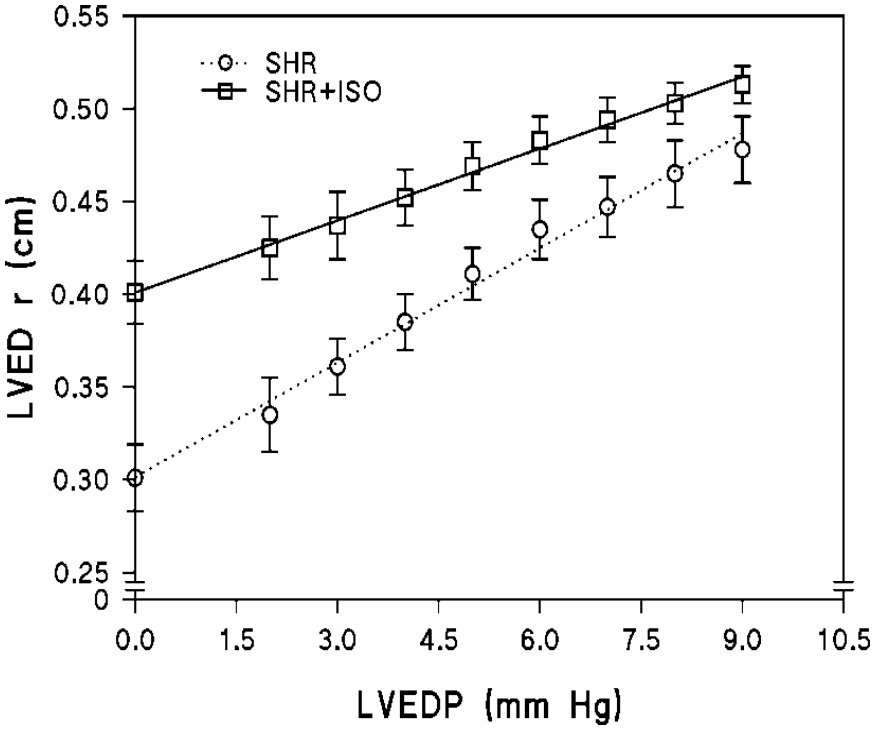


Figure 13. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular end diastolic pressure-internal radius (LVEDP-LVEDr) relations (upper panel) and LVEDr and intercepts at 0 mm Hg ($LVEDr_0$) (lower panel) in 7 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 12 month old untreated SHR group.



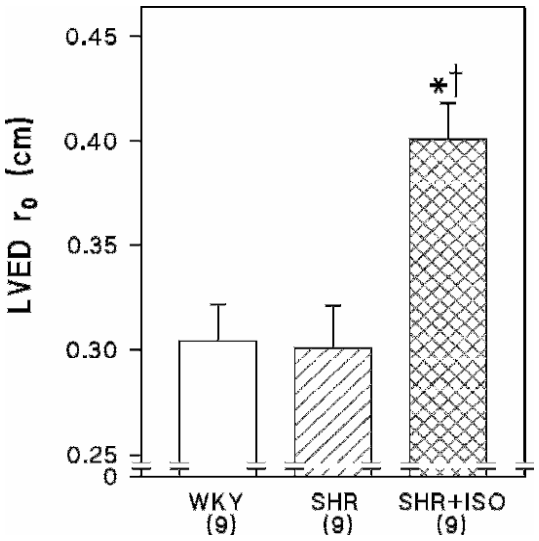


Figure 14. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular end diastolic pressure-internal radius (LVEDP-LVEDr) relations (upper panel) and LVEDr and intercepts at 0 mm Hg ($LVEDr_0$) (lower panel) in 14 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 19 month old untreated SHR group.

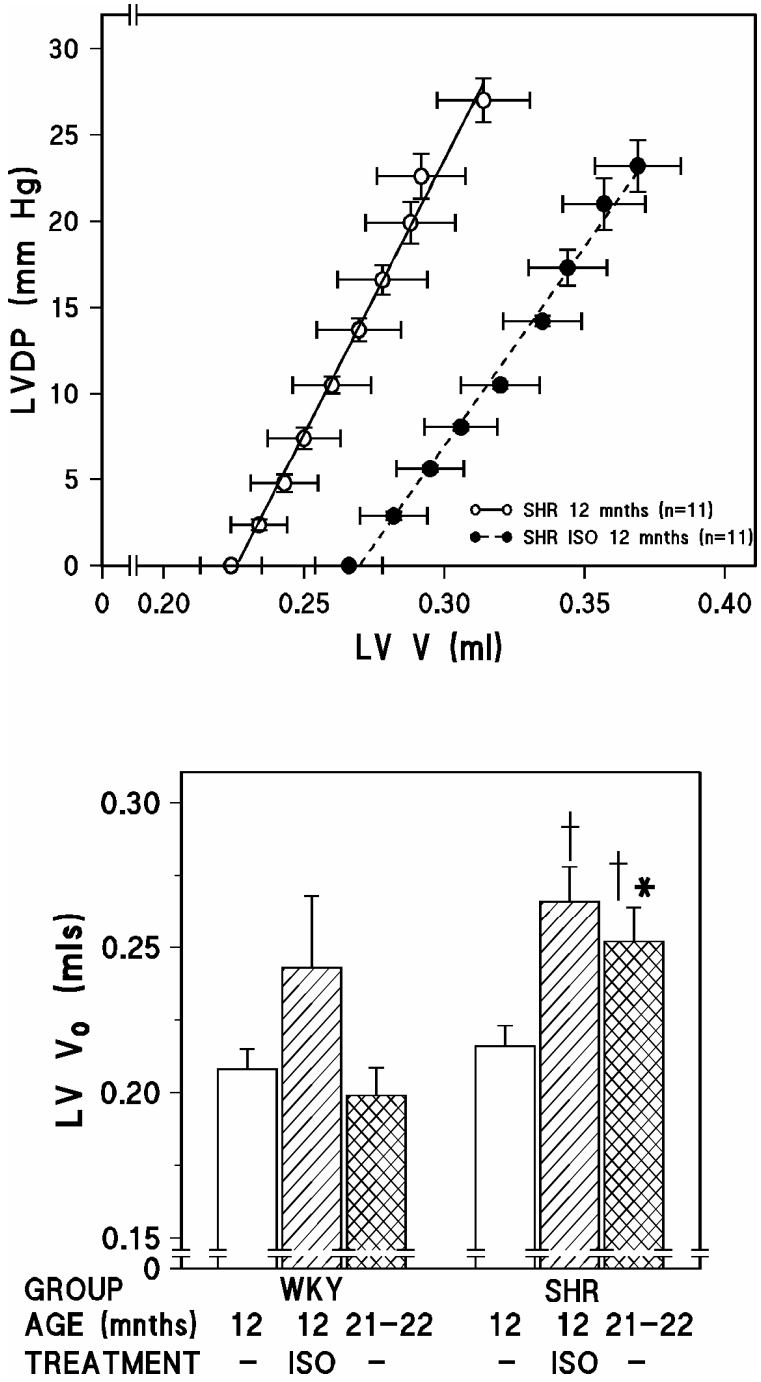


Figure 15. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular (LV) diastolic pressure-volume (LVDP-LVV) relations (upper panel), and LVV intercepts at 0 mm Hg (LVV_0) (lower panel) in 7 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * p<0.01 versus age-matched WKYgroup; † p<0.01 versus 12 month old untreated SHR group.

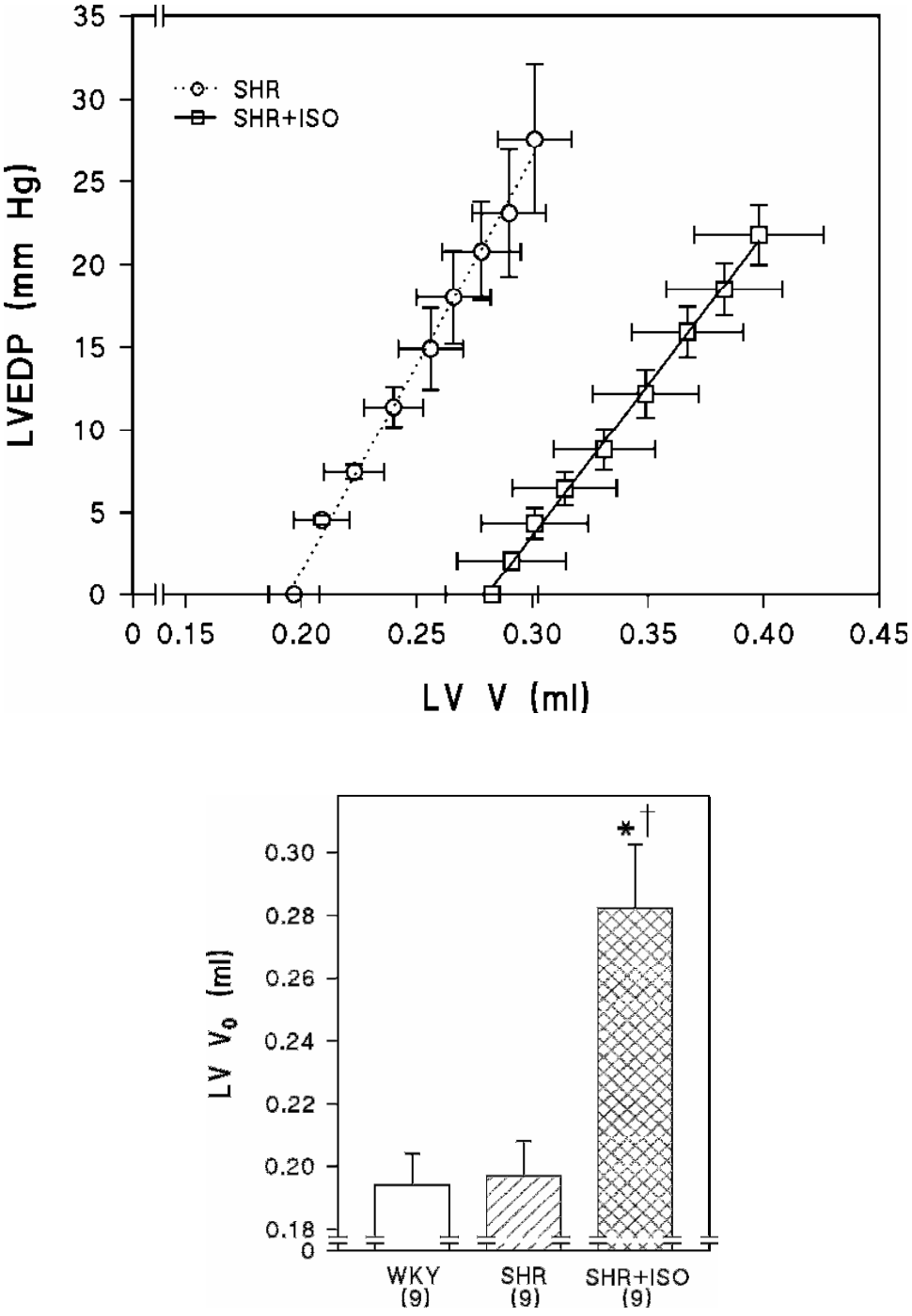


Figure 16. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular (LV) diastolic pressure-volume (LVDP-LVV) relations (upper panel), and LVV intercepts at 0 mm Hg (LVV_0) (insets) in 14 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 19 month old untreated SHR group.

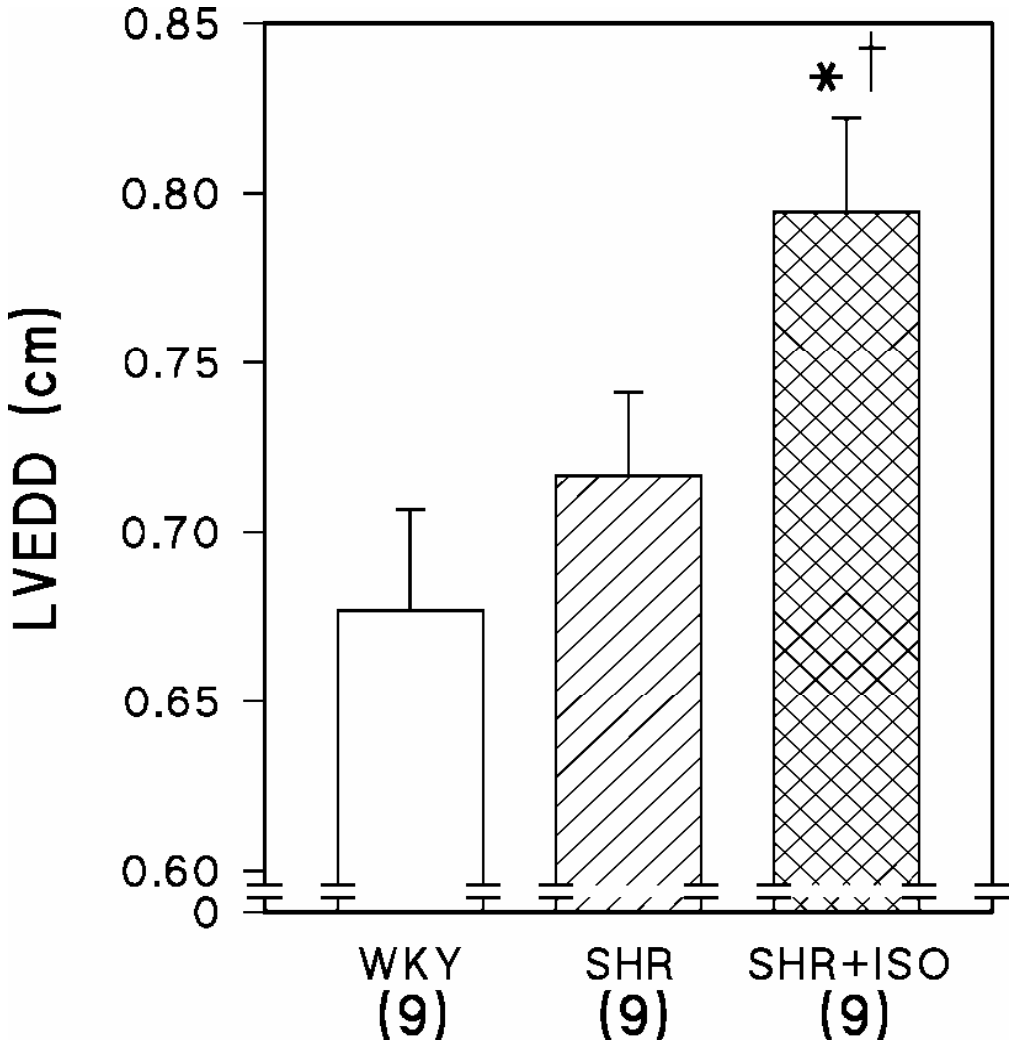


Figure 17. Impact of chronic isoproterenol (ISO) administration from 14-19 months of age on left ventricular end diastolic diameters (LVEDD) in spontaneously hypertensive rats (SHR). * $p<0.01$ versus age-matched Wistar Kyoto (WKY) group; † $p<0.01$ versus 19 month old untreated SHR group.

LV relative wall thickness.

Ultrasonic transducers. Consistent with concentric LVH, 12 month old and 19 month old untreated SHR had increases in LVED relative wall thickness (h/r) as determined at controlled filling pressures (Figures 18 and 19). In contrast, consonant with deleterious LV remodeling, at 21-22 months of age LVED h/r was diminished in SHR (Figure 18) despite the marked increase in LV weight noted at this time (Table 2). Similarly, although ISO administration to SHR from 7-to-12 months of age and from 14-to-19 months of age produced a further increase in LV weight to values comparable with those obtained in 21-22 month old SHR (Table 2), ISO treated SHR developed a reduced LVED h/r in comparison to untreated age-matched SHR (Figures 18 and 19). Despite the extent of the increment in LV weight noted in ISO treated SHR and in 21-22 month old SHR in comparison to the WKY control groups (Table 1), LVED h/r values in ISO treated SHR and 21-22 month old SHR were only comparable with those of WKY controls (Figures 18 and 19). ISO administration to WKY rats failed to influence LVED h/r (Figure 18).

Echocardiography. SHR at 19 months of age had a marked increase in LV posterior wall thickness as compared to WKY (Table 3). Despite ISO administration augmenting LV hypertrophy in SHR (Tables 2 and 3), ISO reduced LV posterior wall thickness to values similar to WKY control values (Table 3).

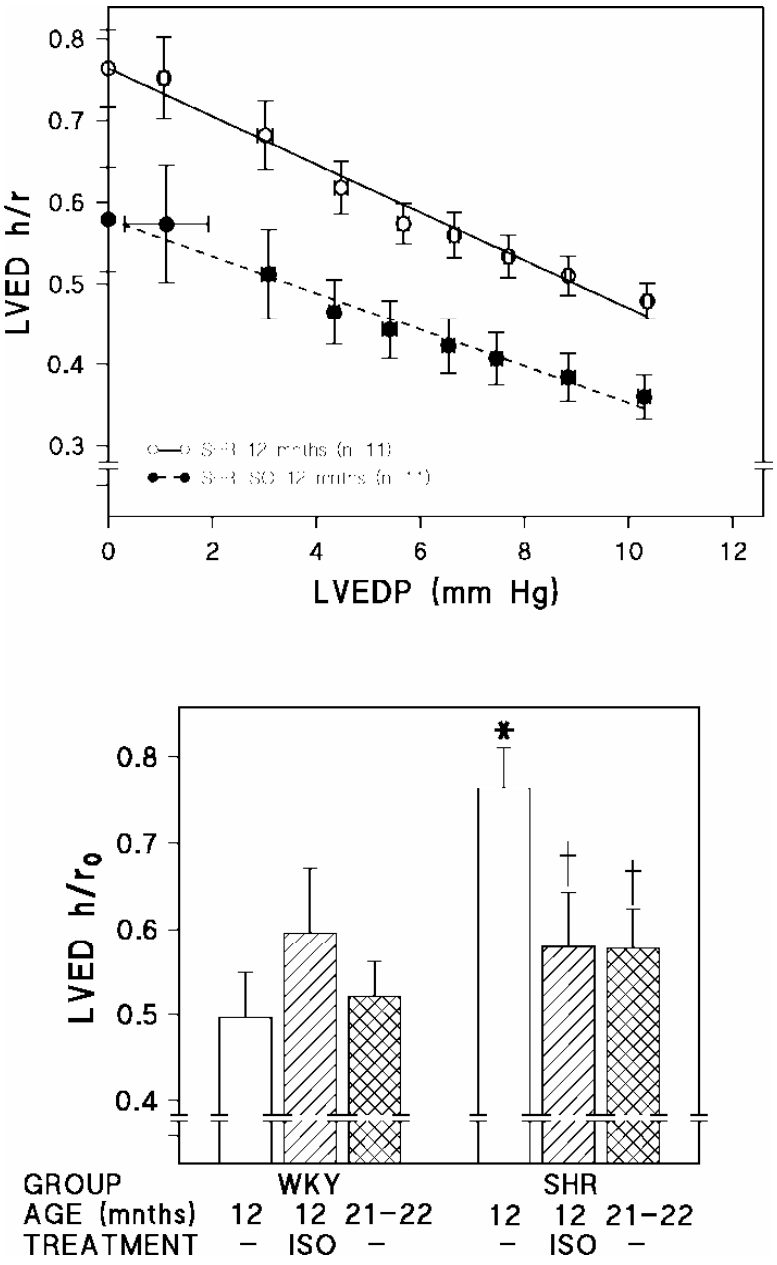


Figure 18. Effect of 5 months of isoproterenol (ISO) administration on left ventricular end diastolic (LVED) relative wall thickness (wall thickness [h]-to-radius[r] values determined over a range of LVED pressures (LVEDP)) and the LVED h/r intercept at 0 mm Hg ($LVEDh/r_0$) in 7 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 12 month old untreated SHR group.

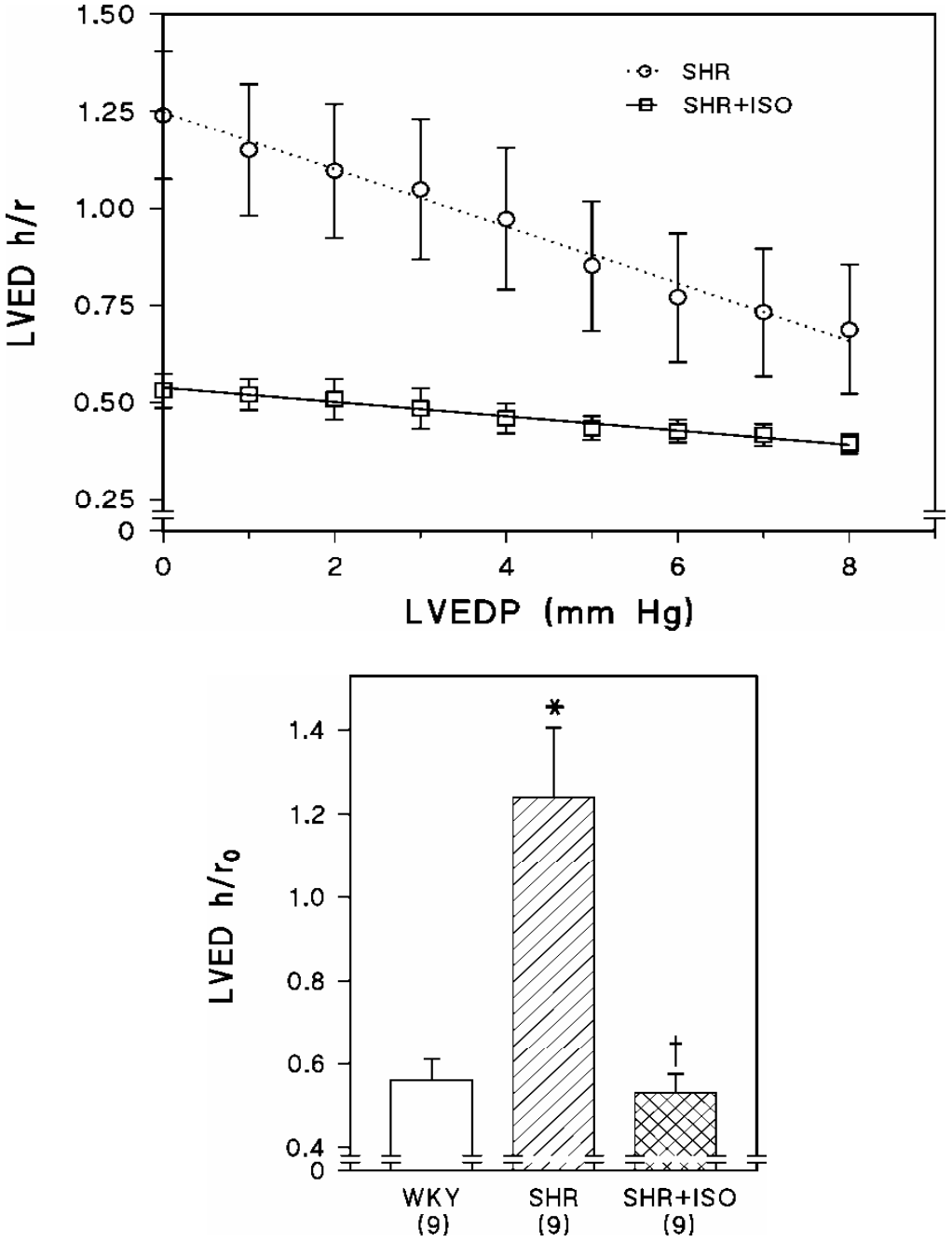


Figure 19. Effect of 5 months of isoproterenol (ISO) administration on left ventricular end diastolic (LVED) relative wall thickness (wall thickness [h]-to-radius[r]) values determined over a range of LVED pressures (LVEDP) and the LVED h/r intercept at 0 mm Hg ($LVEDh/r_0$) in 14 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 19 month old untreated SHR group.

LV systolic chamber performance and intrinsic myocardial contractility.

Isolated, perfused heart studies. Untreated SHR at 12 months of age had an increased LV systolic chamber performance (E) but a similar intrinsic myocardial contractility (E_n) as compared to untreated WKY controls (Figures 20 and 21). In contrast, 21-22 month old SHR and SHR treated with ISO from 7-to-12 months of age developed a reduced LV systolic chamber function (E) as compared to untreated SHR, but no change in intrinsic myocardial contractility (E_n) (Figures 20 and 21). ISO administration to WKY rats also tended to reduce systolic chamber performance, but not intrinsic myocardial contractile function, however this effect failed to reach statistical significance (Figures 20 and 21).

In contrast to 12 month old SHR who had an enhanced LV systolic chamber performance (E) as compared to WKY (Figure 20), SHR at 19 months of age had an LV E value that was comparable with age-matched WKY (Figure 22). Again however, intrinsic myocardial contractility (E_n) was not different in untreated SHR as compared to untreated WKY controls (Figure 23). ISO administration to SHR from 14-to-19 months of age reduced LV systolic chamber function (E) as compared to untreated SHR, but did not change in intrinsic myocardial contractility (E_n) (Figures 22 and 23).

Echocardiography. SHR at 19 months of age had similar systolic chamber (LV FS_{end}) and myocardial (LV FS_{mid}) function as compared to age-matched WKY controls (Figure 24). However, ISO administered to SHR decreased systolic chamber function as assessed *in vivo* (Figure 24, LV FS_{end}). In contrast, ISO failed to modify intrinsic myocardial systolic function as assessed *in vivo* (Figure 24, LV FS_{mid}).

Pathological score. Neither untreated, nor ISO-treated SHR at 12 months of age had evidence of significant myocyte necrosis (Table 4). However, 21-22 month old SHR had evidence of myocyte necrosis (Table 4). A trend for an increase in pathological

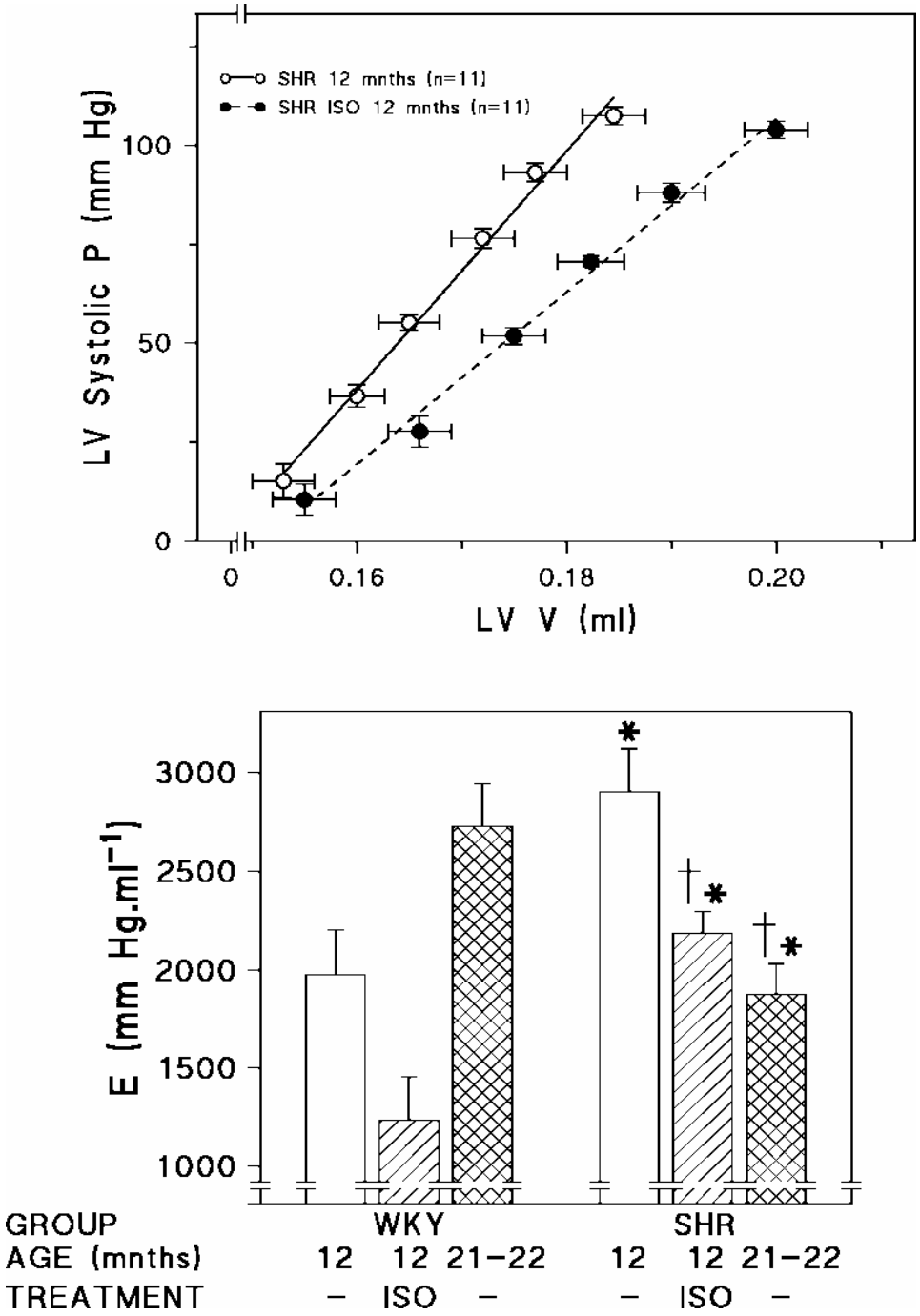


Figure 20. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular systolic pressure-volume (LV systolic P-LVV) relations (upper panel), and the slopes of these relations (systolic chamber function [E]) (lower panel) in 7 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 12 month old untreated SHR group.

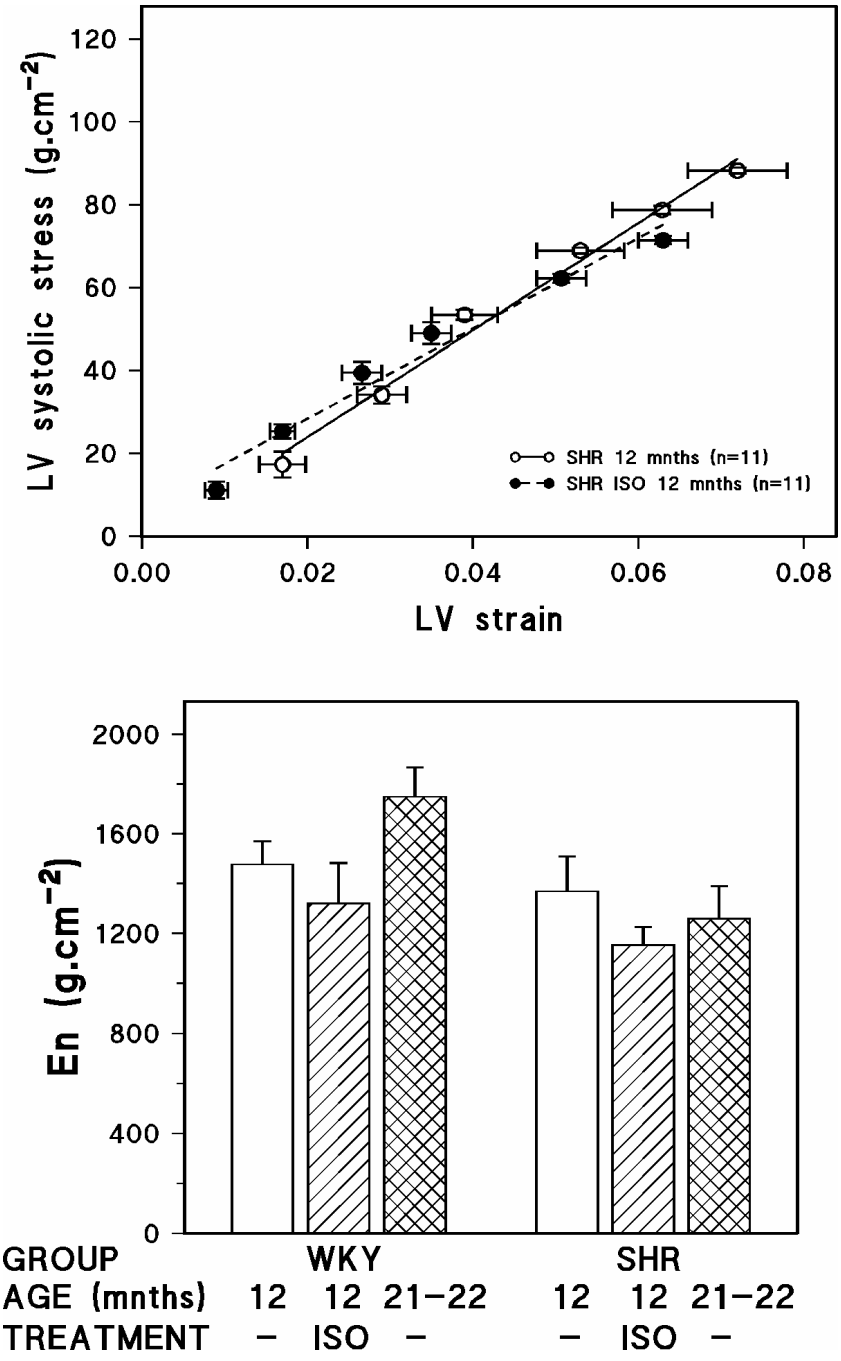


Figure 21. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular systolic stress (σ)-strain relations (upper panel), and the slopes of these relations (systolic myocardial [E_n] elastance) (lower panel) in 7 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 12 month old untreated SHR group.

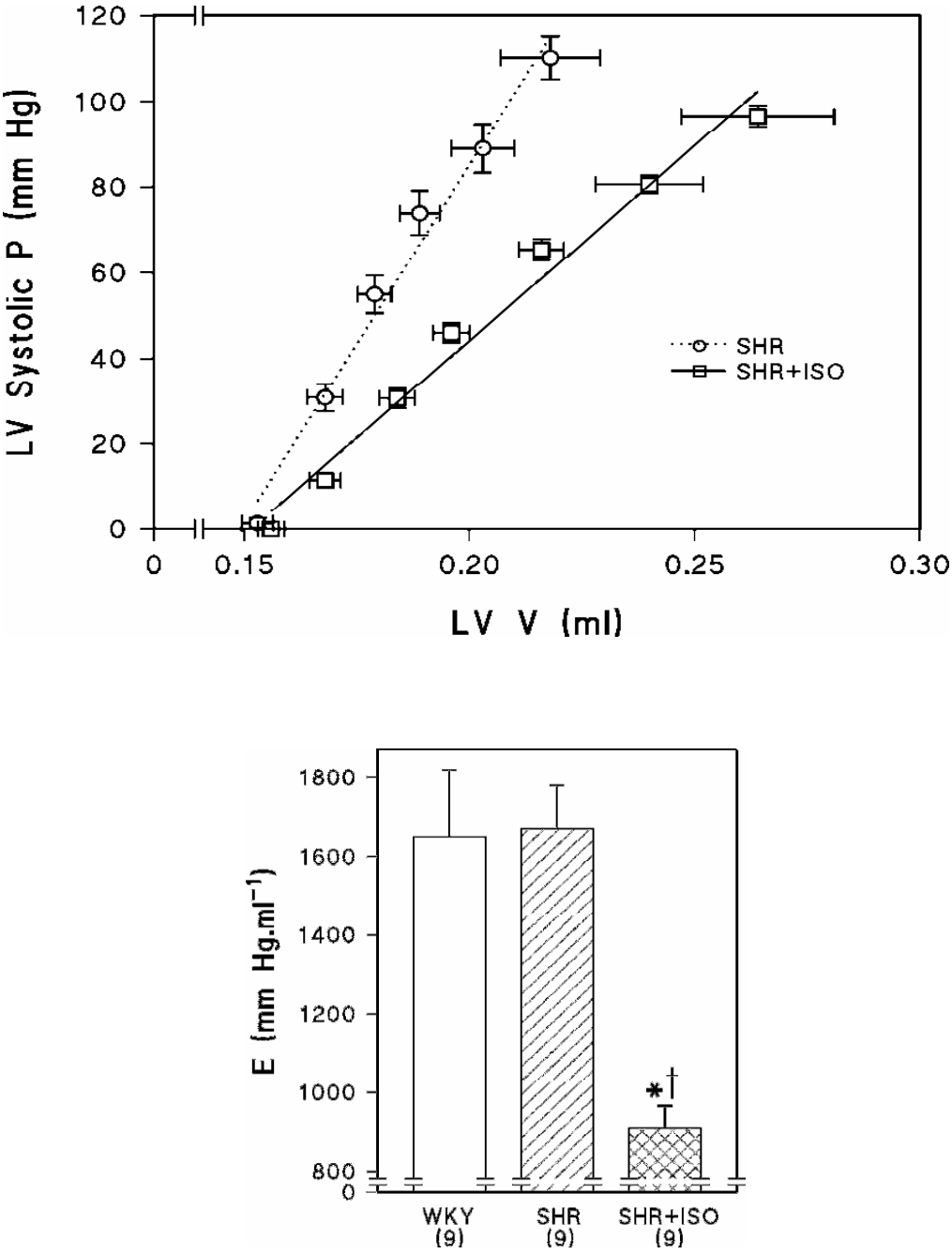


Figure 22. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular systolic pressure-volume (LV systolic P-LVV) relations (upper panel), and the slopes of these relations (systolic chamber function [E]) (lower panel) in 14 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 19 month old untreated SHR group.

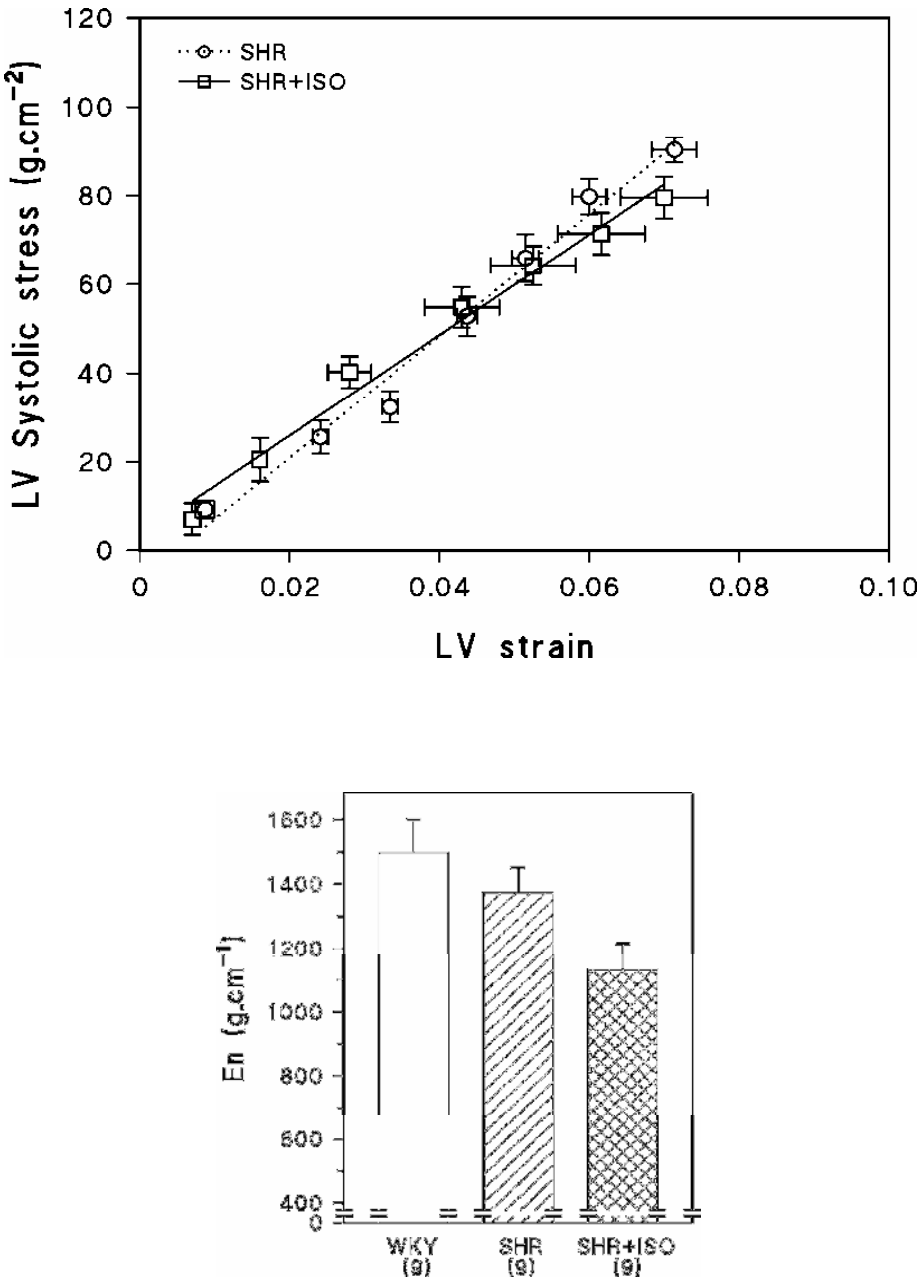


Figure 23. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular systolic stress (σ)-strain relations (upper panel), and the slopes of these relations (systolic myocardial [E_n] elastance) (lower panel) in 14 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. No differences were noted between the groups.

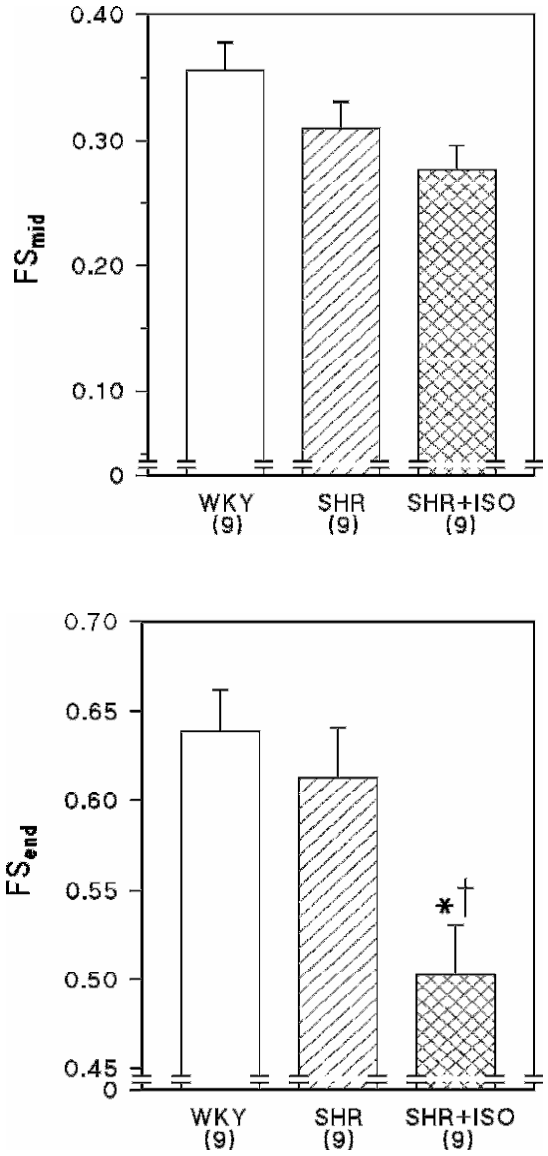


Figure 24. Effect of 5 months of daily isoproterenol (ISO) administration on left ventricular systolic chamber (LV endocardial fractional shortening- FS_{end}) and myocardial (LV midwall fractional shortening- FS_{mid}) function in 14 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. * $p<0.01$ versus age-matched WKY group; † $p<0.01$ versus 19 month old untreated SHR group.

Table 4. Effect of chronic isoproterenol (ISO) administration on myocardial necrosis in spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats.

Rat strain	Age	ISO	Pathological score
SHR	12 months	-	0.4±0.1
SHR	12 months	+	0.5±0.2
SHR	19 months	-	1.9±0.3
SHR	19 months	+	3.1±0.1*
SHR	21-22 months	-	3.7±0.7*
WKY	12 months	-	0.3±0.1
WKY	12 months	+	0.4±0.1
WKY	19 months	-	0.5±0.2
WKY	21-22 months	-	0.3±0.1

* $p<0.01$ versus age-matched WKY control.

score occurred in untreated SHR at 19 months of age as compared to WKY controls, an effect that achieved significance in SHR receiving ISO (Table 4).

Myocardial norepinephrine. SHR at 19 months of age had a marked increase in myocardial NE release (Figure 25). ISO administration failed to influence myocardial NE release in either younger (ISO administration from 7-12 months old) or older (ISO administration from 14-19 month old) SHR (Figure 25).

DISCUSSION

The principal finding of the present study is that chronic administration of ISO to SHR, without mediating intrinsic myocardial contractile dysfunction or altering BP or LV filling volumes, promotes the progression from compensated concentric LVH to LV dilatation and pump dysfunction in hypertension. Chronic β-AR activation in SHR produced a further increment in LV weight which was accompanied by an increased chamber volume, wall thinning, and decrements in chamber performance but not intrinsic myocardial contractility. This deleterious LV geometric remodeling effect mediated by chronic β-AR activation, was compatible with changes noted during LV decompensation in old SHR (SHR 22 months). The impact of chronic β-AR activation was noted in both modest LVH (12 month old SHR) as well as advanced LVH (19 month old SHR). Although the doses of the β-AR agonist used in the present study tended to promote myocyte necrosis, this was only noted in older SHR.

Importantly, in the present study LV remodeling and function were assessed using three approaches each with its own strengths and limitations. Cardiac dilatation and pump dysfunction following chronic β-AR activation in SHR was detected using echocardiography in anaesthetized rats, piezoelectric ultrasonic transducers in anaesthetized, ventilated, open-chest rats, and from LV volume measurements in isolated, perfused heart preparations. The value of echocardiography is that *in vivo*

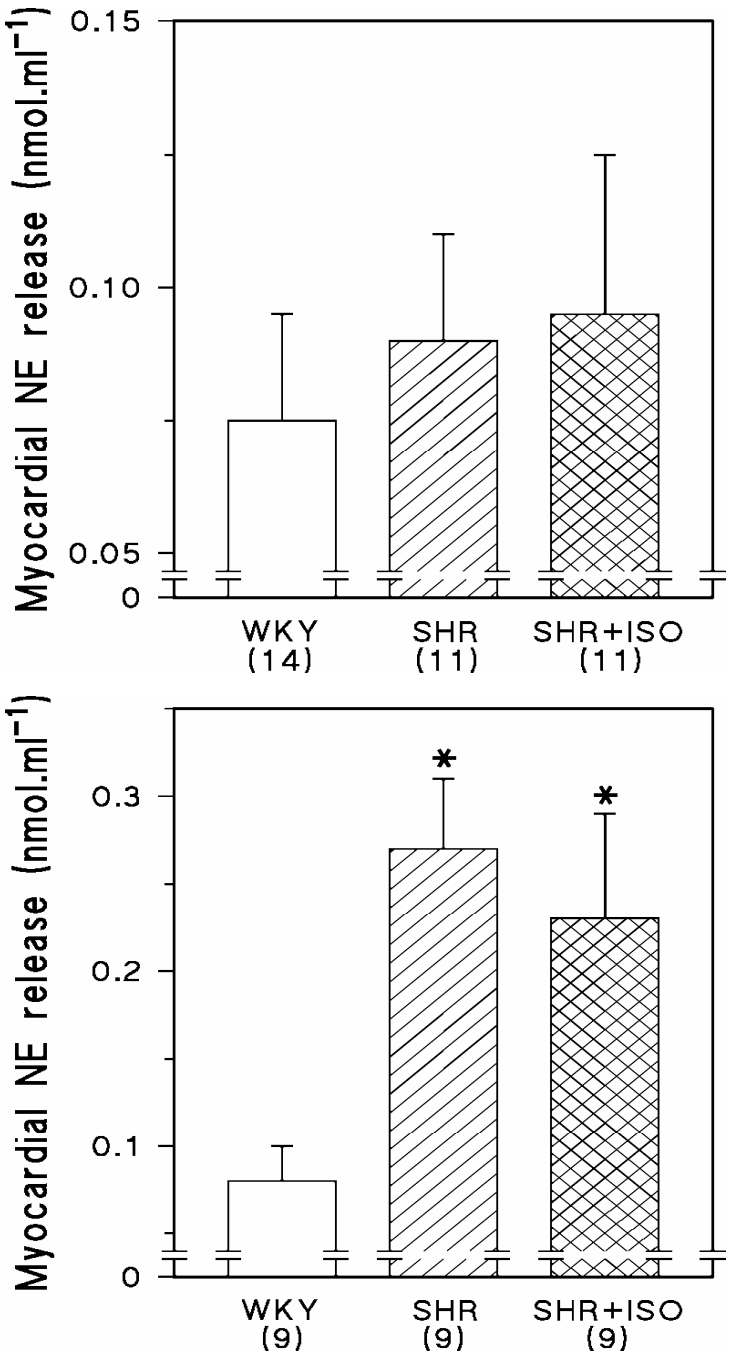


Figure 25. Myocardial norepinephrine (NE) release in spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) after 5 months of isoproterenol (ISO) administration. Data obtained in rats studied from 7-12 (upper panel) and from 14-19 (lower panel) months of age are shown. * $p<0.01$ versus age-matched WKY group.

assessments are performed in closed-chest rats and that dimensions can be measured in an emptying and filling ventricle. The limitations of the use of echocardiography are however numerous and include the fact that loading conditions and coronary flow are not controlled, and that measurements are made in a single axis of the heart and performed under anaesthesia. The value of piezoelectric ultrasonic transducer assessments is that preloading conditions are controlled and that dimensions can be measured in an emptying and filling ventricle. The limitations of the use of piezoelectric tranducers are however numerous and include the fact that neither coronary flow nor afterload are controlled; measurements are made in a single axis of the heart; internal dimensions are calculated; and that the measurements are performed under anaesthesia under unphysiological conditions (open-chest and ventilated rats). The values of the isolated, perfused heart assessments are that loading conditions and coronary flow are controlled, LV volume measurements are made using direct techniques, and the effect of anaesthesia is eliminated. The limitations of the use of isolated, perfused heart preparations are however numerous and include the fact that measurements are made under unphysiological conditions in a preparation that is not filling or emptying. Despite the varying strengths and weaknesses of each approach, the fact that the same outcomes were reproduced using all three techniques and in two separate experiments (both in younger and older SHR) provides substantial support for the notion that the data are indeed reliable.

LV dilatation is perceived to be secondary to a number of sympathetic-mediated intrinsic myocardial functional changes (alterations in cell signaling and calcium handling, and effects mediated through necrosis and apoptosis), all of which are thought to contribute to initiating pump dysfunction and subsequently producing chamber remodeling (Sabbah 1999). In contrast, in the present study, although baseline intrinsic myocardial systolic function as determined both *in vivo* (LV FS_{mid})(load-dependent) and

ex vivo (LV E_n)(load-independent) was maintained, pump dysfunction was noted both *in vivo* (LV FS_{end})(load-dependent) and *ex vivo* (LV E)(load-independent). The reduction in systolic pump function was attributed to adverse chamber remodeling. The present study therefore suggests that chronic β-AR activation in hypertensive LVH can mediate pump dysfunction through primary rather than secondary effects on chamber remodeling. Hence our data support the concept originally proposed by Cohn (1995), and subsequently substantiated by data obtained in human (Vasan et al 1997) and animal (Norton et al 2002) studies, that cardiac dilatation is a precursor of LV dysfunction. Although previous data have indicated that non-necrotic doses of ISO in normotensive rats are able to produce LV dilatation and pump dysfunction (Woodiwiss et al 2001), no distinction was made in this study between myocardial and geometric effects on pump abnormalities.

The present study is in apparent contrast to the notion that chronic sympathetic activation, through β-AR downregulation and effects mediated through apoptosis, mediates intrinsic myocardial dysfunction (Sabbah 1999). This apparent conundrum has nevertheless been recently clarified by our laboratory (Osadchii et al 2007). In this study (Osadchii et al 2007) we were able to show that three months administration of ISO to rats resulted in left ventricular (LV) pump failure as evidenced by reduced LV endocardial fractional shortening and a decrease in the slope of the LV systolic pressure-volume relation. Similar to the present study, changes in intrinsic myocardial contractility could not explain a reduced pump function. Indeed, LV midwall fractional shortening and the slope of the LV systolic stress-strain relation were unchanged. Nevertheless, chronic β-AR activation promoted apoptosis (TUNEL score) and induced β_1- and β_2-AR-mediated inotropic down-regulation as evidenced by attenuated contractile responses to dobutamine and salbutamol. The sustained intrinsic myocardial function despite the presence of apoptosis and downregulation of β-AR contractile

responsiveness was attributed to up-regulation of α-AR mediated contractile responsiveness, as determined by phehylephrine infusions. Indeed, norepinephrine-induced contractile responses were preserved in ISO-treated heart preparations. LV pump failure in this study was also attributed to LV dilatation as evidenced by increased LV internal dimensions and volume intercept of the LV diastolic pressure-volume relation.

Increases in LV cavity size with a proportionately greater increase in LV weight and hence an enhanced relative wall thickness, as previously shown to follow chronic administration of non-necrotic doses of ISO to rats (Woodiwiss et al 2001), may not represent LV remodeling consistent with advanced heart failure. In advanced heart failure, LV cavity size increases out of proportion to the growth of the LV wall, and a resultant decrease in absolute or relative wall thickness is thought to contribute to pump dysfunction (Cohn 1995; Mann 1999; Cohn et al 2000). In the present study, the β-agonist-mediated reduction in wall thickness, despite mediating further increases in LV weight in SHR, represents the first evidence to indicate that chronic β-AR activation in LVH produces LV dilatation together with wall thinning without necessarily inducing myocyte necrosis.

If, as the data from the present study indicate, LV remodeling is not secondary to intrinsic myocardial contractile dysfunction, what are the potential mechanisms responsible for geometric changes? Chronic β-AR activation may mediate LV remodeling indirectly through alterations in haemodynamic loads. Chronic β_2-AR activation may induce sustained increments in LV volume preload through vasodilatation. However, LV filling dimensions remained unchanged in intact rats after a relatively short period (1 month) of ISO administration. Moreover, chronic vasodilator therapy with the potent non-specific vasodilator, hydralazine, has previously been shown to prevent rather than encourage the development of LV dilatation and wall thinning in

SHR (Tsotetsi et al 2001). Chronic β_1-AR activation could also lead to LV remodeling through an enhanced afterload secondary to increases in contractility and heart rate. However, we have been unable to detect alterations in SBP, diastolic BP, pulse pressure, or heart rate when measured an hour following ISO administration to SHR (Gibbs et al 2004, Osadchii et al 2007).

Despite marked ISO-induced effects on LV remodeling in SHR, the same dose of ISO produced only a trend for LV remodeling in WKY control rats. Although this finding does not affect the interpretation of our data (evaluations of the actions of ISO on WKY rats was not a primary goal of the present study), these data suggest that animals with LVH are more sensitive to the detrimental effects of chronic β-AR activation, a finding that requires confirmation with larger sample sizes in the WKY group. As left ventricular tissue NE and angiotensin II concentrations are reported to be increased in SHR compared to WKY (Dang et al 1999) it is likely that SHR are predisposed to cardiac remodeling. Indeed, in the present study, SHR had a considerably greater coronary effluent NE concentration at 19 months of age. These findings are consistent with the increased NE released from the myocardium in hypertensive LVH in humans (Kelm et al 1996). The mechanisms responsible for an increased NE release in hypertensive LVH are likely to include a reduced NE re-uptake and an increased sympathetic activity (Rumantir et al 2000; Schlaich et al 2003). In the present study an increased NE released into the coronary effluent cannot be attributed to an enhanced sympathetic activity as hearts were isolated from sympathetic effects. Further studies are required to assess NE re-uptake in isolated perfused hearts from SHR. The study sample size that would be required to show significant differences in LVED internal dimensions between WKY and WKY receiving ISO with 80% power was calculated to be 45 rats in each group. Consequently, the clinical value of a potential ISO effect on LV dimensions in WKY would have to be questioned.

In conclusion, I have been able to show that chronic administration of a β-AR-agonist to rats with compensated concentric LVH promotes the development of pump dysfunction through a mechanism which is independent of intrinsic myocardial contractility changes and necrosis, but associated with chamber remodeling. These data suggest that adrenergic activation contributes to the progression from compensated concentric LVH to pump dysfunction in hypertension through a novel mechanism (primary effects on cardiac remodeling).

CHAPTER 3

Mechanisms of β-adrenergic-induced chamber dilatation and pump dysfunction in concentric hypertrophy

ABSTRACT

As indicated in chapter 2, excessive β-adrenergic activation initiates the development of pump dysfunction in hypertensive left ventricular hypertrophy (LVH) through direct effects on cardiac chamber remodeling (dilatation). Although β-adrenergic activation has been shown to induce apoptosis and interstitial changes, whether these alterations account for β-adrenergic-induced LV dilatation in hypertensive LVH is uncertain. I evaluated the effect of isoproterenol (ISO, 0.02 $mg.kg^{-1}.d^{-1}$) on myocardial collagen characteristics, matrix metalloproteinase (MMP) activity and expression, tissue inhibitor of matrix metalloproteinase (TIMP) expression and apoptosis (TUNEL) in spontaneously hypertensive rats (SHR) with compensated LVH. The ability of 5 months of ISO administration to promote LV dilatation in SHR has been reported on in chapter 2. SHR not receiving ISO had a marked increase in apoptosis and myocardial total, cross-linked (resistant to digestion), and type I and type III collagen concentrations. However, neither myocardial collagen susceptible to digestion (non-cross-linked collagen), nor MMP activity (zymography) was increased in SHR at 19 months of age. However, ISO administered to SHR from 7-to-12 months and from 14-to-19 months of age resulted in an increase in myocardial collagen susceptible to digestion (non-cross-linked collagen). Moreover, ISO administered over short-term periods (4-5 days) promoted increases in MMP-2 activity and this was sustained after 5 months of administration. However, ISO failed to modify collagen type I-to-III ratios or to induce further myocardial apoptosis in SHR despite promoting marked apoptosis in WKY controls. Neither MMP-2 nor TIMP-2 expression was altered by ISO administration to either SHR or WKY controls. In conclusion, these data suggest that excessive β-adrenergic activation promotes the transition from compensated left ventricular hypertrophy (LVH) to LV dilatation and hence pump dysfunction through interstitial changes including activation of MMPs and the accumulation of myocardial collagen susceptible to MMP digestion.

INTRODUCTION

Excessive β-adrenoreceptor (AR) activation is now well recognized as contributing toward progressive cardiac dilatation in heart failure (Sabbah 1999; Gibbs et al 2004). A close relationship exists between sympathetic activation and left ventricular (LV) cavity dimensions (Iwase et al 1997). Moreover, β-AR-blockers mediate beneficial effects in heart failure to a large extent through a reduction in cardiac cavity dimensions (Lekven 1975; Waagstein et al 2003). There is also now substantial evidence to indicate that in hypertension, the transition from compensated left ventricular hypertrophy (LVH) to LV dilatation also involves chronic β-AR activation. Indeed, in LVH, increased circulating norepinephrine (NE) concentrations (Agabiti-Rosei et al 1987; Kelm et al 1996) as well as myocardial NE release (chapter 2) (Schlaich et al 2003) precede heart failure. In addition, in hypertensive LVH, prolonged β-AR agonist administration increases the susceptibility to LV dilatation and decompensation without blood pressure (BP) changes (chapter2) (Badenhorst et al 2003b). Moreover, genetically-induced down-regulation of sympathetic effects (Esposito et al 2002) or β-AR-blockade (Chan et al 2004) prevent the development of LV dilatation and pump dysfunction in pressure overload states without mediating BP changes.

Despite well documented evidence to indicate that chronic β-AR activation promotes LV dilatation, the mechanisms responsible for this effect are nevertheless uncertain. In heart failure, LV dilatation is thought to occur through a number of mechanisms including haemodynamic and cellular changes. With respect to haemodynamic changes, cardiac dilatation is thought to follow increases in LV preloads through decreases in myocardial contractility and fluid accumulation. However, as discussed in chapter 2 I have provided data to indicate that neither of these two haemodynamic effects plays a major role in the ability of excessive β-AR activation to promote the transition from compensated LVH to LV dilatation.

With regards to cellular changes responsible for LV dilatation, two potential alterations are of importance, namely cell lengthening and cell slippage (see section 2.1.2.5.1 of chapter 1). The modern view of LV dilatation has favored the cell slippage hypothesis. Cell slippage is thought to occur through two major changes, namely cell death through necrosis/apoptosis (Cheng et al 1996) and/or through degradation of myocardial collagen by matrix metalloproteinases (MMPs) (D'Armiento 2002). Although β-AR stimulation activates MMPs in cultured cardiomyocytes (Menon et al, 2005) whether MMP activation occurs in a model of β-AR-induced cardiac dilatation has not been determined. Myocardial collagen synthesis is stimulated by β-AR pathways (Grimm et al 1998), but as discussed in section 2.1.2.5.2, decreases and not increases in myocardial collagen synthesis are thought to promote cardiac dilatation. Lastly, apoptosis and necrosis may be induced through β-AR pathways (Communal and Colucci 2005). As indicated in chapter 2, myocyte necrosis does not appear to play a major role in mediating the impact of excessive β-AR activation in the transition from compensated LVH to LV dilatation. However, whether apoptosis occurs in a model of LV dilatation mediated by β-AR activation has not been determined. The uncertainty regarding the mechanisms of β-AR-induced cardiac dilatation and pump dysfunction prompted me to attempt to further explore this question.

The aim of the present study was therefore to evaluate whether chronic β-AR activation in spontaneously hypertensive rats (SHR) with compensated LVH could promote LV dilatation in association with either apoptosis or alterations in the cardiac interstitium.

METHODS

Experimental groups and haemodynamic assessments. The majority of the experimental groups and haemodynamic assessments have already been described in

chapter 2. Importantly, however, an additional study (AESC approval number: 2006/37/04) was performed to determine the impact of a 4-5 day period of daily ISO administration (short-term) on myocardial apoptosis, MMP activity and expression and TIMP expression. This approach was adopted to establish a direct 'cause-effect' relationship between excessive β-AR activation and either apoptosis, MMP activity and expression and TIMP expression, as these changes determined later in the study could be secondary to the long-term effects of β-AR activation (i.e receptor and cell-signaling changes, cardiac hypertrophy or cardiac dysfunction), rather than to the direct effects of β-AR activation. For the short-term study 12 month old SHR (n=16) and Wistar Kyoto controls (WKY, n=18) were assigned to a group of rats (8 SHR and 10 WKY) who received ISO at the dose described in chapter 2, intraperitoneally (≈0.1 ml) for 4-5 days, and a group of rats (8 SHR and 8 WKY) who received the same volume of the vehicle of ISO for the same duration. For the expression studies an additional 2 WKY rats not receiving ISO, 8 SHR not receiving ISO, and 4 SHR receiving ISO were studied in order to achieve statistical power. Rats were killed and their hearts removed and processed within 90 minutes of receiving the last dose of ISO.

Myocardial collagen. For the studies described in chapter 2, samples of LV tissue were weighed and stored at -70℃ prior to tissue analysis. Myocardial hydroxyproline concentration ([HPRO]) was determined after acid (HCl) hydrolysis (Norton et al 1997; Tsotetsi et al 2001; Woodiwiss et al 2001; Norton et al 2002; Badenhorst et al 2003b). Myocardial collagen was also extracted and digested with cyanogen bromide (CNBr) (Norton et al 1997; Tsotetsi et al 2001; Woodiwiss et al 2001; Badenhorst et al 2003b). A portion of the CNBr digested collagen sample was subjected to acid hydrolysis and [HPRO] determination. The amounts of non-cross-linked (soluble) and cross-linked (insoluble) collagen in the myocardium were ascertained based on the solubility of myocardial collagen to CNBr digestion (Norton et al 1997; Tsotetsi et al 2001;

Woodiwiss et al 2001; Badenhorst et al 2003b). Using the remaining portion of the CNBr digested sample, polyacrylamide gel electrophoresis was subsequently performed on vertical gels by stacking and separation gel concentrations of 3% and 12.5% respectively, and the type I-to-III collagen ratio was determined following gel scanning (Norton et al 1997; Tsotetsi et al 2001; Woodiwiss et al 2001). Myocardial type I and III collagen concentrations 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). Representative examples of polyacrylamide electrophoretic gels and densitometry patterns used to determine collagen type I and III ratios and concentrations are illustrated in Figure 26. Electrophoretic patterns of myocardial collagen extracts were obtained and the bands corresponding to collagens type I and III identified from collagen type I (Sigma) and III (Calbiochem) standards. The relative amounts of type I:III collagen were determined from the relationship between the quantity of collagen applied to the gel and the relative area under the densitometry curve corresponding to bands G [α_1(I)-CB-8] and H [α_2(I)-CB-3] (type I) (Laurent et al 1981) and band M [α_1(III)-CB-5 plus α_1(III)-CB-9] (type III) (Laurent et al 1981; Mukherjee and Sen 1990). Bands G, H and M were chosen because they contain very little interference from comigrating peptides of other collagen types.

Myocyte apoptosis. The degree of apoptosis was quantified on myocardial tissue sections obtained from the same tissue blocks used to assess the pathological score as described in chapter 2. In addition, tissue samples were collected from the 12 month old animals receiving ISO/vehicle for 4-5 days. For each tissue block, 5 μm thick sections were stained and evaluated. Nuclear deoxyribonucleic acid (DNA) fragments in the tissue sections were detected using a non-radioactive *in situ* apoptotic cell death detection kit (DeadEnd™ Colorimetric TUNEL system, Promega, Madison, WI, USA), where terminal deoxynucleotidyl transferase (TdT) was used to incorporate biotinylated

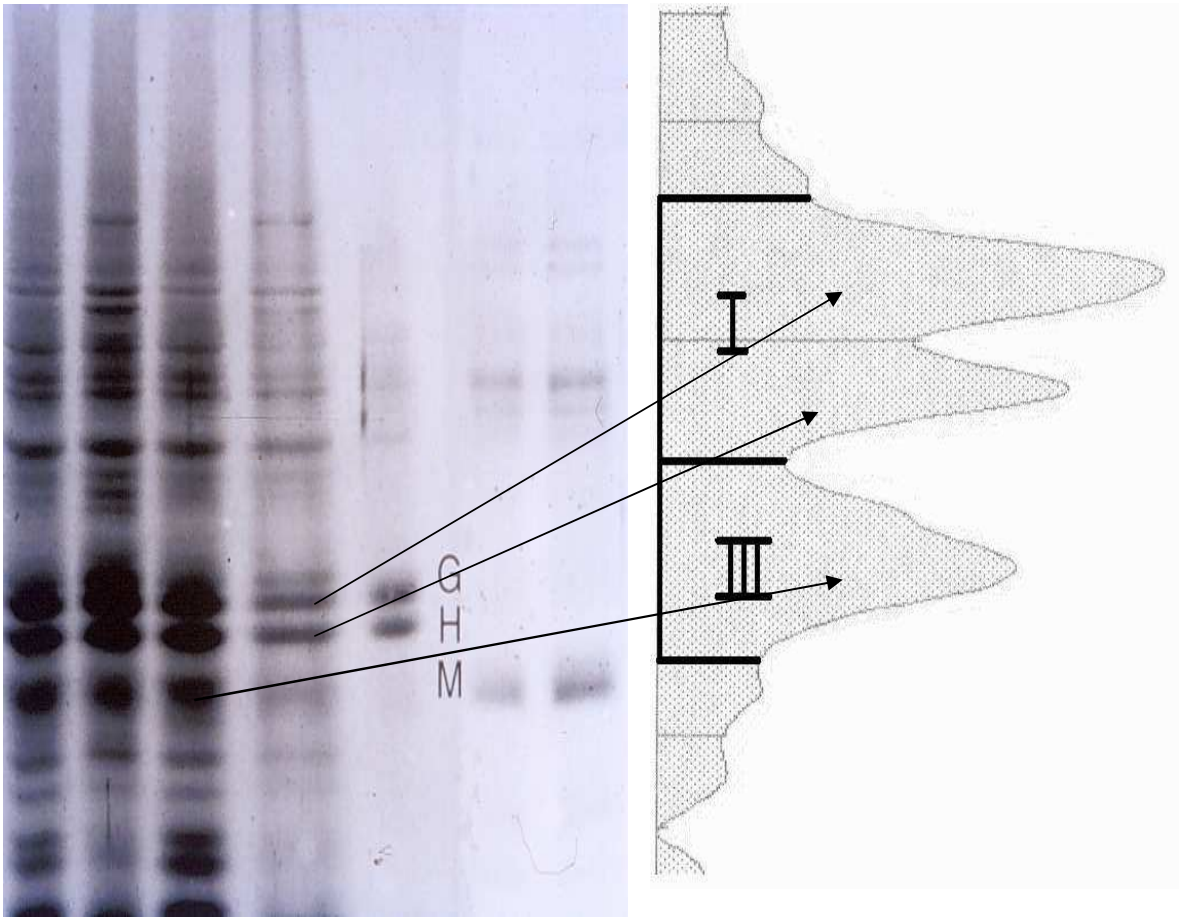


Figure 26. Polyacrylamide electrophoretic gel showing typical banding patterns for myocardial collagen (left panel) and densitometry patterns determined from the gel (right panel). See text for explanation.

nucleotide at the 3'-OH DNA ends. Horseradish-peroxidase-labeled streptavidin binds to biotinylated nucleotides, which subsequently stain dark brown in response to hydrogen peroxide and diaminobenzidine (Agarwala and Kalil 1998). Both positive (DNase treated) and negative (no addition of TdT) control tissue sections were incorporated into each assay. The number of apoptotic cardiomyocyte nuclei and the total number of cardiomyocyte nuclei (hematoxylin and eosin stain) in each slide were counted on ten evenly spaced fields from the apex to the base using a computer-based image acquisition and analysis system at 400 times magnification (Axiovision 3, Carl Zeiss, Gottingen, Germany). Apoptotic cardiomyocyte nuclei were expressed as a percentage of the total number of cardiomyocyte nuclei. Representative examples of stained sections for the samples assessed and from positive and negative controls and from an SHR are illustrated in Figure 27.

Matrix metalloproteinase activity. Gelatin zymography was performed as previously described (Tyagi et al 1993) in order to determine the activity of the gelatinase MMP-2. For this purpose, tissue from the lateral wall of the LV was analyzed. Tissue samples used were frozen in liquid nitrogen within 5 minutes of removing hearts from the thoracic cavity and then stored at -70℃ until analysis. Tissue samples were collected from the 19 month old animals (5 months ISO/vehicle administration to 14 month old animals) as well as the 12 month old animals receiving ISO/vehicle for 4-5 days. Cardiac tissue protein was extracted for zymography as follows: tissue was ground to a powder under liquid nitrogen using a mortar and pestle. The ground tissue was placed in an eppendorf, weighed and 100 µl extraction buffer (50 mM Tris, 0.1% SDS) added per 100 mg tissue powder. The samples were vortexed and incubated for 18 h at 4℃, thereafter the samples were centrifuged at 12 000 rpm for 10 minutes and the supernatant containing the soluble extracted protein was decanted and stored at -70℃.

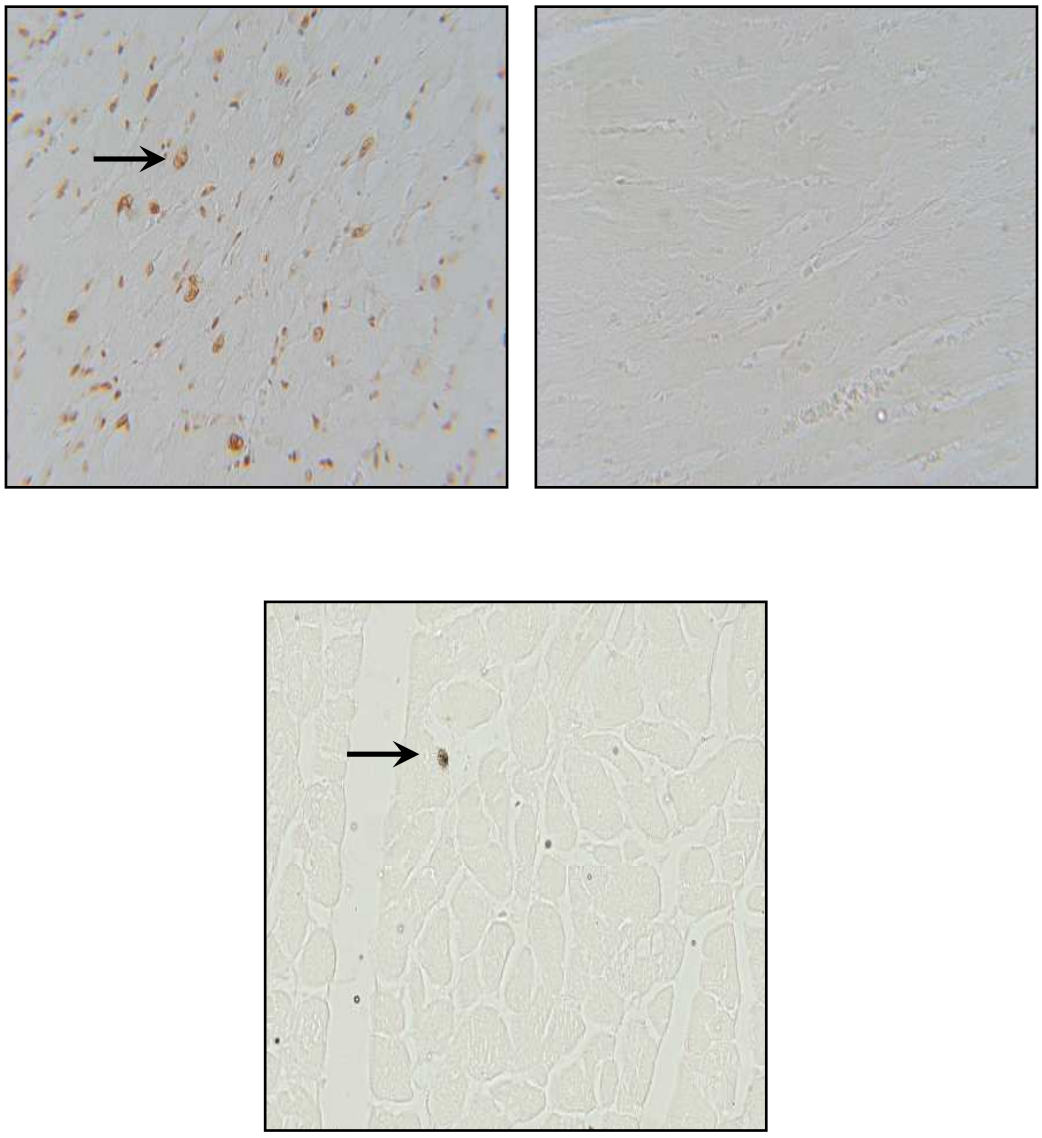


Figure 27. Histological sections of the myocardium stained for apoptotic nuclei. The upper panels illustrate sections obtained from a positive (left) and a negative (right) control and the lower panel a section from the myocardium of a heart from a spontaneously hypertensive rat. Arrows indicate one apoptotic cardiomyocyte nucleus in the positive control and one in the myocardium of a heart from a spontaneously hypertensive rat.

The protein concentrations of the supernatants were estimated using a modified Lowry/Folin technique (Lowry et al 1951). In order to determine the relative activity of MMP-2 in each sample, 20 µg of protein was loaded into each well of a 10% polyacrylamide gel containing $1mg.ml^{-1}$ of type A gelatin. The proteins were separated electrophoretically over 1.5 hours at 30 mA. A single standard of rat MMP-2 (Sigma, purity >95% by SDS-PAGE visualized by silver staining) was included on each gel to locate the MMP-2 bands. The gels were then incubated overnight in substrate buffer (Tris 50 mM pH 8, $CaCl_2$ 5 mM) to allow degradation of gelatin. The gels were then stained for protein with Coomassie blue dye resulting in a gel with a dark background and light bands (Figure 28A), the intensity of which indicate the activity of MMP. The gels were scanned using a flat bed transmission scanner (Cano Scan 4200 F, Cannon Solutions, China). Images were inverted (Figure 28B) and the density of the MMP band analyzed compared to a standard extract MMP sample using digital densitometry with LabWorks Software Version 4.5 (UVP, Upland, USA).

Matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase expression. mRNA was directly extracted from rat hearts obtained from 12 month old animals receiving ISO/vehicle for 4-5 days using the *Oligotex Direct mRNA Mini Kit* (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Heart tissue was homogenized using QIAshredder homogenizers (Qiagen, Hilden, Germany) and tissue debris and protein were removed to create optimal conditions for hybridization of poly A+ mRNA to Oligotex. cDNA was synthesized from the mRNA complex using the *Transcriptor First Strand cDNA Synthesis Kit* (Roche, Mannheim, Germany) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was carried out in a lightcycler 1.2 instrument (Roche, Germany) using a *LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit* (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cDNA labeled with SYBR Green I, a

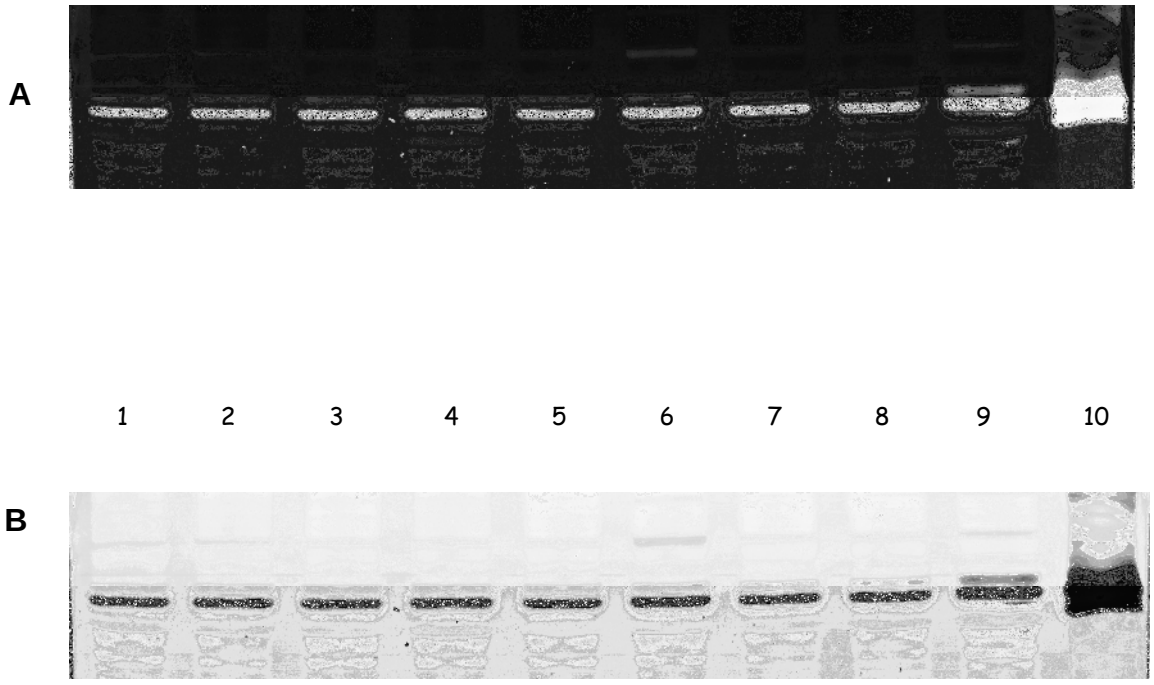


Figure 28. A representative example of a normal (A) and inverted image (B) of a zymogram illustrating banding patterns obtained for matrix metalloproteinase (MMP) 2 activity. Lanes 1-9 represent samples, lane 10 represents MMP-2 standard.

fluorescence label which intercalates into the DNA double helix, was amplified by PCR using appropriate primers and conditions. The following primers were employed for amplification of specific mRNA targets:

GAPDH forward: 5'-CTCCCTCAAGATTGTCAGCAA-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 GAPDH (housekeeping gene), MMP-2 and TIMP-2 were 56℃, 51℃ and 53℃, respectively. The PCR conditions in each case were 95℃ for 10 minutes followed by 40 cycles of 95℃ for 5 seconds, annealing at the appropriate temperature for 15 seconds and extension at 72℃ for 10 seconds. Analysis was carried out using the Lightcycler Version 4.0 software (Roche, Germany). To quantitate the expression of each mRNA target, the target gene was expressed relative to the housekeeping gene (GAPDH) using a calibrator normalized method. Figure 29 illustrates a representative example of typical amplification curves obtained from real time RT-PCR cardiac cDNA samples and RT-PCR products for GAPDH, MMP-2 and TIMP-2.

Analysis. Differences between groups were identified by a one-factor ANOVA followed by a Student-Newman-Keuls *post hoc* test. All values in the text are represented as mean ± SEM.

RESULTS

Myocardial collagen.

Study 1: 7-to-12 months of age. Untreated 12 month old SHR had an increase in myocardial [HPRO], an effect that was considerably enhanced in 21-22 month old SHR (Figure 30). The increase in myocardial [HPRO] noted in 12 month old SHR was

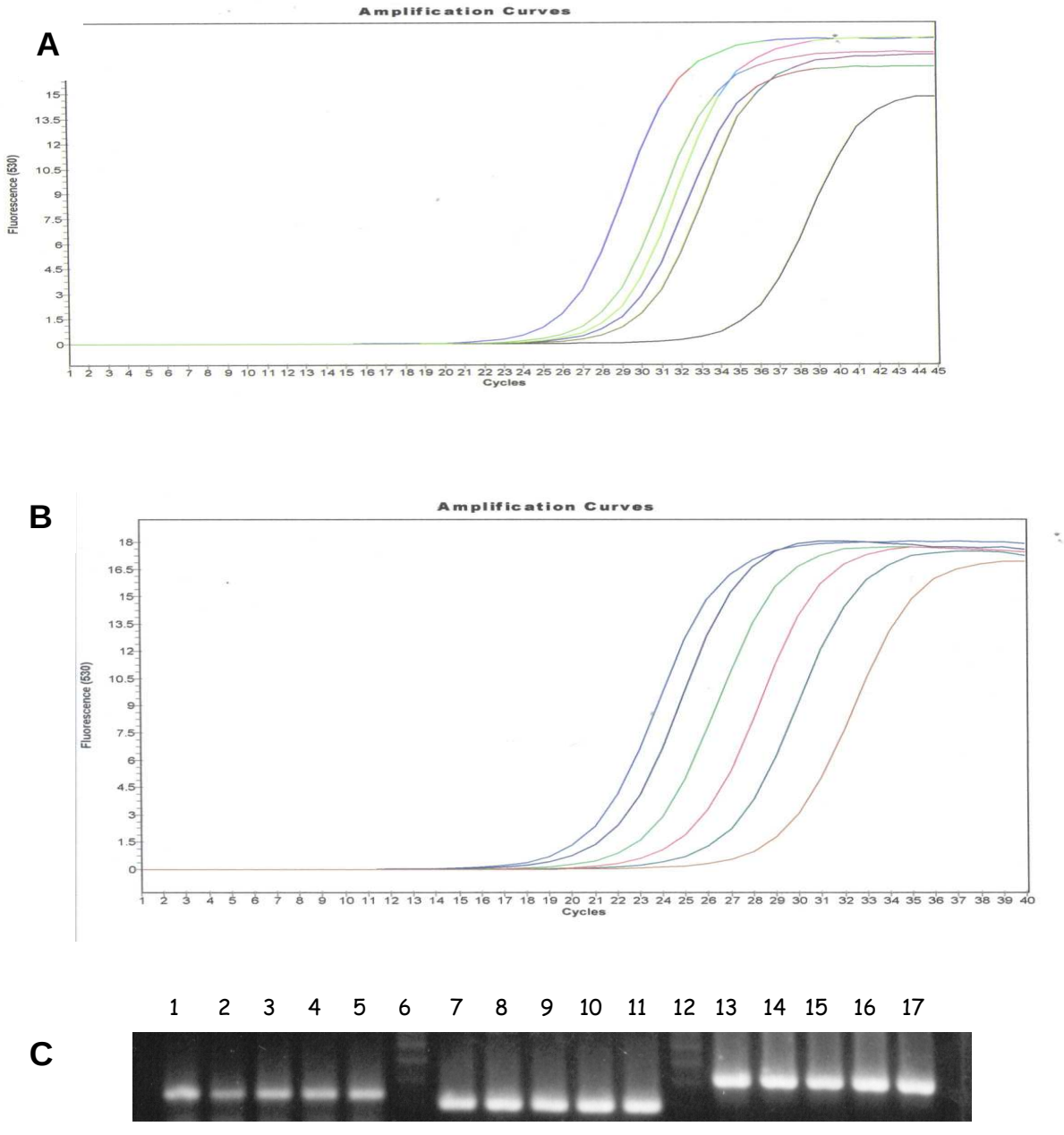


Figure 29. Typical amplification curves obtained from real time RT-PCR cardiac cDNA samples. **A** represents TIMP-2, **B** represents GAPDH. **C** is an agarose gel showing RT-PCR products for MMP-2 (218bp, lanes 1-5, lane 6=molecular weight marker), TIMP-2 (186bp, lanes 7-11, lane 12=molecular weight marker) and GAPDH (289bp, lanes 13-17).

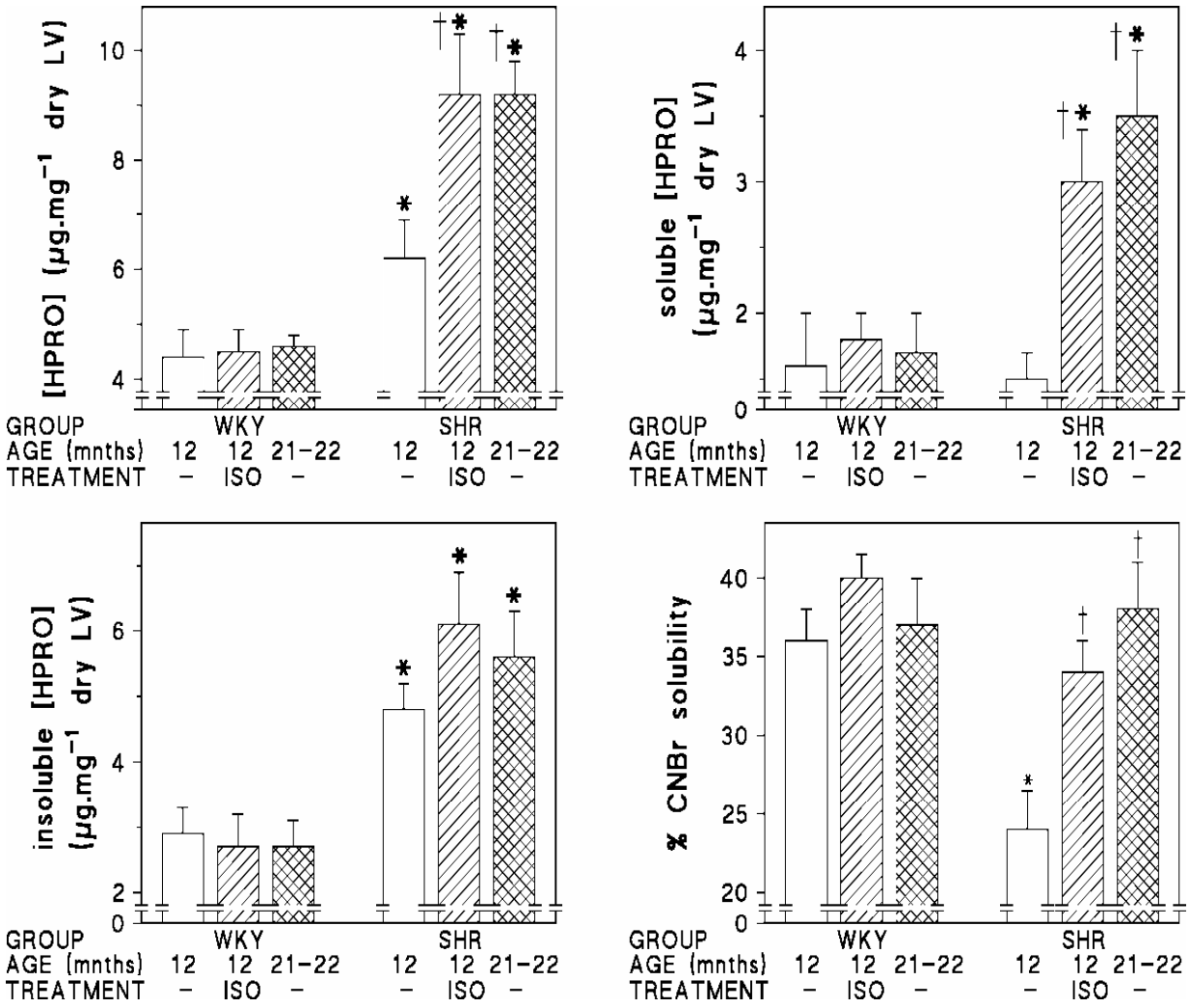


Figure 30. Impact of 5 months of daily isoproterenol (ISO) administration on myocardial collagen characteristics in 7 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats. [HPRO], hydroxyproline concentrations; CNBr, cyanogen bromide. * $p<0.01$ versus WKY group; † $p<0.01$ versus untreated SHR group.

further augmented by the administration of ISO to SHR (Figure 30). In contrast, ISO administration to WKY rats produced no significant change in [HPRO] (Figure 30).

As a consequence of a decrease in the % myocardial collagen soluble to CNBr digestion (Figure 30), untreated SHR at 12 months of age had an increase in insoluble (cross-linked), but not soluble (non-cross-linked) collagen concentrations (Figure 30). In contrast, at 21-22 months of age, SHR had % myocardial collagen solubility which was not different from that of WKY rats. Hence, SHR at 21-22 months of age had marked increases in both insoluble and soluble collagen concentrations relative to WKY rats (Figure 30). ISO administration to 12 month old SHR increased the % myocardial collagen solubility to levels not different from 21-22 month old SHR or WKY rats. Therefore, following ISO-induced increases in myocardial collagen concentrations, both the insoluble and the soluble myocardial collagen concentrations were increased in 12 month old SHR relative to WKY rats (Figure 30). Moreover, ISO administration to SHR increased the concentration of soluble collagen relative to that of untreated SHR. ISO administration to WKY rats produced no significant effect on % myocardial collagen solubility (Figure 30).

Study 2: 14-to-19 months of age. Similar to the data noted in 12 month old SHR, as compared to age-matched WKY, myocardial [HPRO] was greater in 19 month old untreated SHR (Figure 31). Also comparable with the data noted in 12 month old SHR, because of a reduction in collagen solubility (increased cross-linking) in SHR, only insoluble (cross-linked) collagen concentrations were increased (Figure 31). ISO administered to 19 month old SHR potentiated the increment in myocardial [HPRO] but increased the solubility of collagen (decreased cross-linking) to values no different from those of WKY rats (Figure 31). Consequently, the increase in myocardial [HPRO] was associated with an increase in soluble, but not insoluble collagen concentrations relative to untreated SHR (Figure 31).

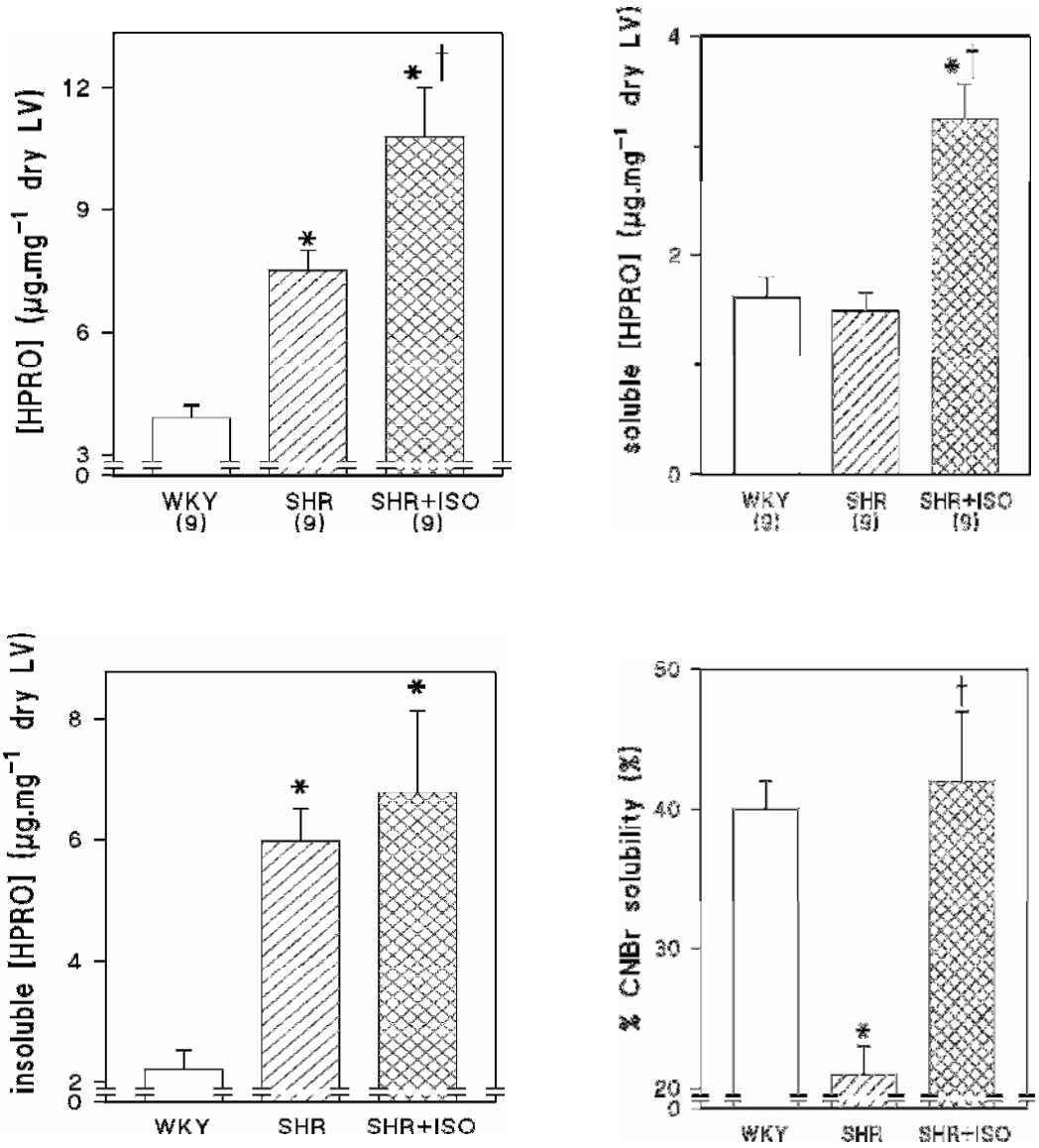


Figure 31. Impact of 5 months of daily isoproterenol (ISO) administration on myocardial collagen characteristics in 14 month old spontaneously hypertensive rats (SHR). WKY, Wistar Kyoto control rats; [HPRO], hydroxyproline concentrations; CNBr, cyanogen bromide. * $p<0.01$ versus WKY group; † $p<0.01$ versus untreated SHR group.

In 19 month old untreated SHR, the ratio of myocardial type I-to-III collagen was similar to that of age-matched WKY, although both type I and III collagen concentrations were increased (Table 5). ISO failed to modify the ratio of myocardial type I-to-III collagen in SHR, but augmented the increments in both type I and III collagen concentrations (Table 5).

Myocyte apoptosis. In the long-term study, an increase in percentage apoptotic cells was noted in untreated SHR as compared to WKY controls, but ISO failed to modify the degree of apoptosis when assessed 24 hours after the last dose of ISO administered (Table 5). In the short-term study, although in WKY control rats ISO produced a marked increase in cardiomyocyte apoptosis, this effect was not reproduced in SHR (Table 6).

Myocardial matrix metalloproteinases and their tissue inhibitors. Although not assessed at 19 months of age, at 12 months of age the activity of MMPs was similar between WKY and SHR groups (Table 6). However, an increase in MMP-2 activity was noted in SHR receiving ISO either after 5 months (Table 5) or for 4-5 days (Table 6). In contrast, ISO administered to WKY rats failed to increase MMP-2 activity (Table 6).

Although not assessed at 19 months of age, neither the expression of MMP-2 nor TIMP-2 was altered in untreated SHR as compared to WKY rats at 12 months of age (Table 6). Although ISO administration failed to significantly alter MMP-2 or TIMP-2 expression in either SHR or WKY rats, a trend effect for a greater MMP-2 expression was noted in SHR receiving ISO (Table 6).

DISCUSSION

The main finding of the present study is that the transition from compensated LVH to cardiac dilatation following chronic β-adrenergic activation in SHR is associated with an increase in myocardial collagen concentrations, enhanced MMP activity and an accumulation of myocardial collagen susceptible to MMP digestion (non-cross-linked

Table 5. Effect of chronic (5 months) administration of isoproterenol (ISO) on myocardial collagen characteristics, apoptosis and matrix metalloproteinase-2 (MMP-2) activity in 14 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats.

	WKY (n=9)	SHR (n=9)	SHR+ISO (n=9)
Type I-to-III collagen ratio	2.89±0.23	3.06±0.13	3.02±0.19
Collagen type I ($\mu g.mg^{-1}$ dry LV)	23±3	46±3*	65±10**†
Collagen type III ($\mu g.mg^{-1}$ dry LV)	8±1	15±1*	21±2**†
% apoptotic/normal nuclei#	1.99±0.37	5.12±0.84*	5.10±0.89*
MMP-2 (relative densitometry units)	-	1.22±0.59	3.30±0.55‡

# indicates average number of apoptotic nuclei expressed as a % of normal nuclei.

* $p<0.05$, ** $p<0.01$ versus WKY group, ‡ $p<0.05$, † $p<0.01$ versus SHR group.

Table 6. Impact of 4-5 days of daily isoproterenol (ISO) administration on myocardial % apoptosis, matrix metalloproteinase (MMP) activity (zymography) and expression (quantitative polymerase chain reaction [PCR] technique) and tissue inhibitor of MMP (TIMP) expression (quantitative PCR) in 12 month old spontaneously hypertensive (SHR) and Wistar Kyoto control (WKY) rats.

	WKY	WKY ISO	SHR	SHR ISO
% apoptotic/normal nuclei#	0.03±0.01 (n=8)	0.23±0.04* (n=10)	0.10±0.02 (n=8)	0.12±0.03‡ (n=8)
MMP-2 (relative densitometry units)	0.89±0.03 (n=8)	0.99±0.06 (n=10)	1.08±0.04 (n=8)	1.29±0.11‡† (n=8)
MMP-2 expression (relative mRNA abundance)	0.49±0.27 (n=10)	1.26±0.67 (n=9)	1.21±0.34 (n=16)	2.19±0.75 (n=12)
TIMP-2 expression (relative mRNA abundance)	0.92±0.26 (n=10)	0.75±0.19 (n=9)	1.10±0.23 (n=16)	0.84±0.30 (n=12)

# indicates average number of apoptotic nuclei expressed as a % of normal nuclei.

* $p<0.001$ versus WKY group, ‡ $p<0.05$ versus WKY ISO group, † $p<0.05$ versus SHR group. One rat in the WKY ISO group was excluded from the expression studies as inappropriate quantities of mRNA were obtained.

collagen). Neither chronic β-adrenergic-induced LV dilatation nor short-term administration of the β-adrenoreceptor agonist was associated with an augmented apoptosis in SHR. Although short-term administration of the β-AR agonist did not alter TIMP-2 expression in SHR, a trend for a greater MMP-2 expression was noted.

Despite clear evidence to indicate that chronic β-AR activation promotes LV dilatation and hence pump dysfunction in cardiac disease (Sabbah 1999; Gibbs et al 2004), the exact mechanism by which this effect occurs is not clear. β-AR activation promotes apoptosis (Colucci et al 2000), and MMP activity and expression (Briest et al 2001; Coker et al 2001) both of which could induce LV dilatation. However, these studies were either conducted *ex vivo* (Colucci et al 2000) or with the use of norepinephrine (Briest et al 2001), which stimulates both α-AR and β-ARs. In contrast, the present study was conducted *in vivo* and with an adrenoreceptor agonist with a high specificity for β-ARs, suggesting that β-ARs and not α-ARs are responsible for the changes noted. Importantly, the present study was conducted in a model shown to result in LV dilatation following prolonged β-AR activation (chapter 2). The present study thus provides strong evidence to support the notion that chronic β-AR activation *in vivo* mediates LV dilatation in association with interstitial changes.

As discussed in chapter 1, adverse chamber remodeling (dilatation) may follow either cardiomyocyte lengthening (mainly through the addition of sarcomeres), cell death (necrosis or apoptosis), or changes in the interstitium (alterations in the quantity and/or quality of myocardial collagen as well as activation of MMPs). With respect to myocardial collagen concentrations, it is well recognised that β-AR activation promotes an increase in myocardial collagen content (Grimm et al 1998). However, whether increases in myocardial collagen concentrations mediate LV dilatation is controversial. Indeed, myocardial collagen concentrations may decrease rather than increase in some models of LV dilatation (Spinale et al 1996; Woodiwiss et al 2001). In the present study SHR at

both 12 and 19 months of age had an increase in myocardial collagen concentrations, an effect that was augmented by ISO administration in both younger and older SHR. Importantly, the consistent myocardial collagen change noted in response to ISO in the present study was an increase in soluble (non-cross-linked) rather than insoluble (cross-linked) myocardial collagen concentrations. This is in contrast to the myocardial collagen change noted in age-matched untreated SHR, where although myocardial collagen concentrations were increased, the enhanced cross-linked properties of collagen (decreased solubility) resulted in increments in the cross-linked, but not in the non-cross-linked portion of myocardial collagen. The ISO-mediated increase in non-cross-linked myocardial collagen was synonymous with interstitial changes noted in older SHR and may have contributed to progressive LV dilatation associated with reductions in wall thickness. Indeed, non-cross-linked myocardial collagen is susceptible to degradation by collagenases (MMPs), thus contributing to side-to-side slippage of cardiomyocytes (Mann and Spinale 1998; Li et al 2001; Woodiwiss et al 2001).

Previous studies have suggested that alterations in the relative abundance of myocardial collagen subtypes (type I and III subtypes) may contribute to adverse cardiac function (Mukherjee and Sen 1990; Lombardi et al 2003). However, this notion is controversial and has not been supported by some studies (Spinale et al 1996; Woodiwiss et al 2001). In the present study the ratio of type I-to-III myocardial collagen ratios was unchanged in 19 month old SHR as compared to WKY controls irrespective of whether or not SHR were receiving ISO. Consequently, the present study does not support the concept that type I and III changes in myocardial collagen contribute to LV dilatation or pump dysfunction.

In the present study MMP activity (MMP-2) was enhanced in SHR following β-adrenoreceptor activation. This is consistent with changes noted in many forms of cardiac dilatation in both human (Reddy et al 2004) and animal models (Peterson et al

2001). Indeed, adrenergic activation has previously been shown to mediate an increase in MMP expression and activity (Menon et al 2005). As non-selective MMP inhibition prevents cardiac dilatation in pacing-induced heart failure (Spinale et al 1999), it is likely that activation of MMPs contributes to increases in LV cavity volumes. The mechanism that may be responsible for the effect of MMPs on LV cavity size is through excessive degradation of myocardial collagen with subsequent cardiomyocyte side-to-side slippage occurring (see chapter 1). However, it is questionable whether it is MMP-1, MMP-2 or MMP-9 activation that contributes to increases in LV cavity size, as selective MMP-2 and -9 inhibition (no direct effect on MMP-1 activity) does not modify cavity volumes in pacing-induced heart failure (King et al 2003). Thus, in the present study it is possible that MMP-2 activation may not be involved in the pathophysiology of adverse cardiac remodeling. As there are no currently accepted methods available for the assessment of MMP-1 activity, alterations in MMP-1 activity still remains a possible mechanism of β-AR-induced cardiac dilatation.

The increase in MMP-2 activity induced by β-AR activation in the present study is neither attributed to a significant increase in MMP-2 expression (although a trend-effect was noted), nor to a decrease in 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 (Goldberg et al 1989; Olson et al 1997) and hence is the most likely TIMP to modify MMP-2 activity. One possible mechanism for the increase in MMP-2 activity noted to occur in the present study is through a direct action of soluble collagen, which, as indicated by the present and a previous (Woodiwiss et al 2001) study, is induced by chronic β-AR activation. Indeed, the presence or addition of soluble collagen *in vitro* may stimulate MMP activity (Tomasek et al 1997; Ruangpanit et al 2001).

In the present study SHR not receiving ISO had similar MMP-2 activities and expression levels as compared to normotensive controls, data that is inconsistent with

an increased MMP-2 expression and/or activity noted in other forms of pressure overload states. Indeed, MMP-2 expression and or activity is increased in Dahl salt-sensitive rats prior to the development of heart failure (Sakata et al 2004) and in humans with aortic stenosis with or without pump dysfunction (Polyakova et al 2004). However, the data obtained in the present study are consistent with a normal MMP-2 activity in SHR prior to the development of heart failure as previously described (Mujumdar and Tyagi 1999).

A number of studies have demonstrated that β-adrenergic activation promotes cardiomyocyte apoptosis and in doing so contributes to LV dilatation (Communal et al 1998; Singh et al 2001) through a mechanism described in chapter 1. However, in the present study, although cardiomyocyte apoptosis was markedly enhanced in SHR, ISO failed to further enhance cardiomyocyte apoptotic scores in SHR, as assessed 24 hours after the last dose of ISO. Moreover, despite producing a striking pro-apoptotic effect on cardiomyocytes in WKY when assessed within 90 minutes after ISO administration, ISO failed to induce apoptosis in SHR. These data would suggest that β-AR activation does not mediate LV dilatation through cardiomyocyte apoptotic effects.

A potential limitation of the present study is that I failed to assess cardiomyocyte dimensions to determine whether LV dilatation could be explained by excessive myocyte lengthening (Gerdes 2002). However, research conducted by other laboratory members has established, using both image analysis and flow cytometry, that the model of ISO-induced cardiac dilatation is indeed not associated with increases in cell length (Woodiwiss et al, personal communications).

In conclusion, the present study is the first to characterize the potential mechanisms responsible for LV dilatation in a model of β-AR-induced LV dilatation. This study supports the notion that β-AR activation promotes the transition from compensated LVH to cardiac dilatation and pump dysfunction mainly through interstitial modifications.

The interstitial changes of importance appear to be an accumulation of myocardial collagen susceptible to MMP digestion (non-cross-linked collagen) and activation of MMPs. The present study does not support the view that cardiomyocyte apoptosis has direct effects on cardiac chamber dilatation.

CHAPTER 4

Cross-linking influences the impact of quantitative changes in myocardial collagen on cardiac stiffness and remodeling in hypertension in rats

ABSTRACT

The impact of quantitative changes in myocardial collagen on cardiac function in left ventricular hypertrophy (LVH) in hypertension is controversial. The aim of the present study was to assess whether the variable impact of quantitative changes in myocardial collagen on LV diastolic myocardial stiffness (myocardial k) and remodeling (increased volume intercept of diastolic pressure-volume relations) in LVH is associated with alterations in myocardial collagen cross-linking. I evaluated myocardial collagen content (hydroxyproline concentrations [HPRO]) and the degree of myocardial collagen cross-linking (solubility to cyanogen bromide digestion) in 14-15 and 21-22 month old spontaneously hypertensive rats (SHR), and in aortic-banded rats with pressure-overload hypertrophy (POH). In rats with POH and in SHR irrespective of age, increases in myocardial [HPRO] were noted. However, hypertensive rats differed in the material and geometric properties of the myocardium, and in qualitative aspects of fibrosis. In 14-15 month old SHR myocardial k (determined from diastolic stress-strain relations) and insoluble (cross-linked) [HPRO] were increased, but no LV remodeling or increases in myocardial soluble (non-cross-linked) [HPRO] were noted. In rats with POH, LV remodeling and increases in soluble myocardial [HPRO] occurred, but no increase in k or insoluble myocardial [HPRO] were observed. In 21-22 month old SHR, increases in k, soluble and insoluble myocardial [HPRO], as well as LV remodeling occurred. In conclusion, collagen cross-linking may determine the diverse relation that exists between increases in myocardial collagen concentrations and either myocardial stiffness or chamber remodeling in hypertension. These findings support the notion that fibrosis contributes to myocardial stiffness as well as LV dilatation in LVH, albeit an effect that is modulated by collagen quality.

INTRODUCTION

Myocardial fibrosis is a potentially important determinant of left ventricular (LV) diastolic properties. Increases in myocardial collagen concentrations are associated with increments in stiffness and hence LV diastolic dysfunction in pressure-overload hypertrophy (POH) (Weber et al 1990). Furthermore, the relationship between reparative tissue fibrosis and adverse chamber remodeling (right shifts in diastolic pressure-volume relations with subsequent dilatation and pump dysfunction) in hypertension (Brooks et al 1997) and other forms of cardiac disease (Gunja-Smith et al 1996), also implicates cardiac fibrosis in the pathogenesis of chamber remodeling.

Despite data suggesting that increases in myocardial collagen concentrations contribute to diastolic heart disease and chamber remodeling, a number of discrepant results remain unexplained. Normal myocardial stiffness (Thiedermann et al 1983) and modest increases in myocardial stiffness (Norton et al 2002) are both associated with increments in collagen concentrations; pharmacological-induced improvements in stiffness may occur without decreases in collagen concentrations (Norton et al 1997); and decrements in collagen concentrations do not necessarily reduce stiffness (Motz and Strauer 1989). Moreover, increments in myocardial collagen concentrations may occur without chamber remodeling (Mann and Spinale 1998); tissue fibrosis is not a necessary prerequisite for chamber remodeling (Spinale et al 1991b; Mann and Spinale 1998; Woodiwiss et al 2001); and reverse remodeling is associated with increases (Scheinin et al 1992; McCarthy et al 1995; Madigan et al 2001) in collagen concentrations. Therefore, the contribution of enhanced myocardial collagen concentrations toward diastolic dysfunction and chamber remodeling is not established.

Recent data suggest that increments in myocardial collagen cross-linking contribute to an enhanced myocardial stiffness (Norton et al 1997), and decreases in cross-linking to chamber remodeling (Woodiwiss et al 2001). An accumulation of

myocardial collagen in the cross-linked form is well recognized as promoting an increase in myocardial stiffness (Iimoto et al 1988; Spinale et al 1991b). Moreover, studies described in chapters 2 and 3 support the notion that the impact of increments in myocardial collagen concentrations on LV cavity volume could be determined by the cross-linked properties of collagen. In these studies I demonstrated that chronic β-AR activation promotes LV dilatation in association with an accumulation of myocardial collagen in the non-cross-linked form. Non-cross-linked collagen is susceptible to matrix metalloproteinase (MMP) digestion and hence could promote the development of breaks or tears in collagen. Tearing of myocardial collagen could in turn encourage side-to-side cardiomyocyte slippage and LV dilatation.

I therefore hypothesized that the diverse relationship that exists between the degree of myocardial fibrosis and both stiffness and chamber remodeling, could in-part be explained through a modulating influence of alterations in myocardial collagen cross-linking on the impact of fibrosis on cardiac diastolic characteristics. In order to examine this hypothesis I evaluated myocardial interstitial changes in rat models of hypertensive heart disease with varying functional abnormalities, including increased stiffness without remodeling, remodeling without increased stiffness, and both remodeling and increased stiffness.

METHODS

The present study was approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC approval numbers; 2000/40/5, 98/28/4, and 97/44/5).

Animal models. To produce a model of POH in rats associated with LV dilatation, but no increase in myocardial stiffness, a suprarenal abdominal aortic stenosis was created in 130-170 g male Sprague Dawley rats as previously described (Chung et al

1998). In the present study a 21 gauge needle was used as a guide to determine the internal diameter of the stenotic lesion. In this model of POH our group have previously shown little effect on myocardial stiffness (Norton et al 2002) and in a pilot study designed to assess the effects of a wider diameter aortic band (21 as opposed to a 22 gauge needle used to determine the internal diameter of the stenotic lesion), although LV dilatation was noted on echocardiography, no changes in myocardial stiffness were noted. Sham operations were performed in a separate group of rats (n=11). Sham-operated rats and rats with POH were assessed for haemodynamic changes and myocardial collagen characteristics 7.5-to-8 months after surgery. Of the rats operated on to produce POH, 37 survived the immediate post-operative period, and 19 died prior to the collections of haemodynamic data. To assess myocardial collagen changes in a model of increased myocardial stiffness without LV dilatation, 14-to-15 month old spontaneously hypertensive rats (SHR, n=14) and age-matched Wistar Kyoto (WKY) controls (n=11) were employed (Norton et al 1997). Moreover, to assess myocardial collagen changes in a model of increased myocardial stiffness with concomitant LV dilatation, 21-to-22 month old SHR (n=10) and age-matched WKY controls (n=12) were employed (Conrad et al 1995; Tsotetsi et al 2001). In the 21-22 month old SHR, 6 animals had clinical evidence of heart failure (either pleuro-pericardial effusions, and/or calcified left atrial thrombi).

LV cavity size and geometry determined in vivo. In rats with POH and in sham-operated controls, LV internal dimensions were determined at regular intervals throughout the study in anaeshetized animals using echocardiography as described in chapter 2 (Chung et al 1998). In this study all data were acquired using a model 2500 Hewlett Packard echocardiograph with a 7.5 MHz transducer.

In SHR with concentric LV geometry (14-15 month old rats), the leading edge of the posterior wall endocardial surface was difficult to identify with accuracy using

echocardiographic techniques, as the papillary muscle obscured the surface. Hence, LV geometry and dimensions were determined at controlled filling pressures in anaesthetized, ventilated, open-chest SHR and WKY rats using alternative techniques developed in our laboratory that have previously been described and validated elsewhere (Woodiwiss and Norton 1995; Norton et al 1997; Tsotetsi et al 2001). These techniques have been described in chapter 2 in detail (pages 41-44) and involve the measurement of LV end diastolic (LVED) short axis external diameters using piezoelectric ultrasonic transducers, and LVED pressures (LVEDP) from a fluid-filled catheter inserted through the apex of the LV. LVED radius (r) and wall thickness (h) were determined from previously described formulae (Tsotetsi et al 2001) (see chapter 2, page 44). LVED relative wall thickness was determined from LVED h-to-r ratios determined over a range of LVEDP values. Statistical comparisons of LVEDr and LVED relative wall thickness values were made on LVEDr and LVEDh/r intercepts of the LVEDP-LVEDr and LVEDP-LVEDh/r relations respectively ($LVEDr_0$ and $LVEDh/r_0$).

Isolated, perfused heart preparations. Following the collection of haemodynamic data *in vivo*, hearts were excised and LV diastolic pressures determined over a range of filling volumes in isolated, perfused heart preparations as described in chapter 2 (Woodiwiss et al 2001). LV remodeling was assessed from the volume intercept (V_0) of the LV diastolic pressure-volume (P-V) relation (Woodiwiss et al 2001). Myocardial stiffness (myocardial k) was determined from the slope of diastolic stress-strain relations using previously described formulae (Weber et al 1988) as described in chapter 2 (page 49).

Myocardial collagen. Samples of LV tissue were weighed and stored at -70℃ for tissue analysis. Myocardial hydroxyproline concentration ([HPRO]) was assessed using the method of Stegemann and Stalder after acid (HCl) hydrolysis (Stegemann and Stalder 1967). Myocardial collagen was also extracted and digested with cyanogen

bromide (CNBr) (Norton et al 2002). The CNBr digested collagen sample was subjected to acid hydrolysis and [HPRO] determination. The amounts of non-cross-linked (soluble) and cross-linked (insoluble) collagen in the myocardium were determined as previously described based on the solubility of myocardial collagen to CNBr digestion (Norton et al 1997; Woodiwiss et al 2001). The methodology has been described in chapter 3 (page 83).

Analysis. Regression analysis was used to determine the lines of best fit for the cardiac function relations. The stress-strain relations were linearized for statistical comparisons. Linear regression analysis and Pearson's correlation coefficient were used to assess the relationship between myocardial stiffness and total collagen concentration as well as each of the fractions. Differences in LV weight, internal dimensions, geometry, performance, and myocardial collagen biochemical analysis between SHR and WKY groups were assessed by a one-factor ANOVA followed by a Tukey *post hoc* test and between POH and SHAM groups by an unpaired Welch t-test. As data obtained in 14-15 and 21-22 month old WKY were shown to be similar, data for all WKY rats were pooled for graphic representations. All values in the text are represented as mean ± SEM.

RESULTS

LV weight. Aortic-banded rats and SHR at both 14-15 and at 21-22 months of age had increases in LV weight as compared to their respective age-matched controls (Table 7).

LV cavity size, remodeling, and geometry. Consistent with LV dilatation, rats with POH developed an increase in LV internal diameters as compared to sham-operated controls throughout the treatment period of the study (data for the final echocardiographic assessment are given in Figure 32, upper left panel). In keeping with

Table 7. Effect of aortic-banding and spontaneous hypertension on left ventricular (LV) and body weight (BW).

	POH	SHAM	SHR		WKY	
Age (months)	-	-	14-15	21-22	14-15	21-22
Sample number	18	11	14	10	11	12
LV weight (g)	1.72±0.04**	1.31±0.05	1.11±0.04*	1.34±0.04**†	0.95±0.04	0.98±0.02
Body weight (g)	681±15	682±23	363±9	351±12*	358±9	405±9
LV/BW x 10^{-3}	2.53±0.07*	2.07±0.07	3.12±0.10*	3.85±0.18**†	2.65±0.07	2.41±0.03

POH, pressure-overload hypertrophy; SHAM, sham-operated controls; SHR, spontaneously hypertensive rats; WKY, normotensive Wistar Kyoto control; LV, left ventricle; BW, body weight. * $p<0.01$, ** $p<0.001$ versus SHAM or age-matched WKY; † $p<0.001$ versus 14-15 month old SHR.

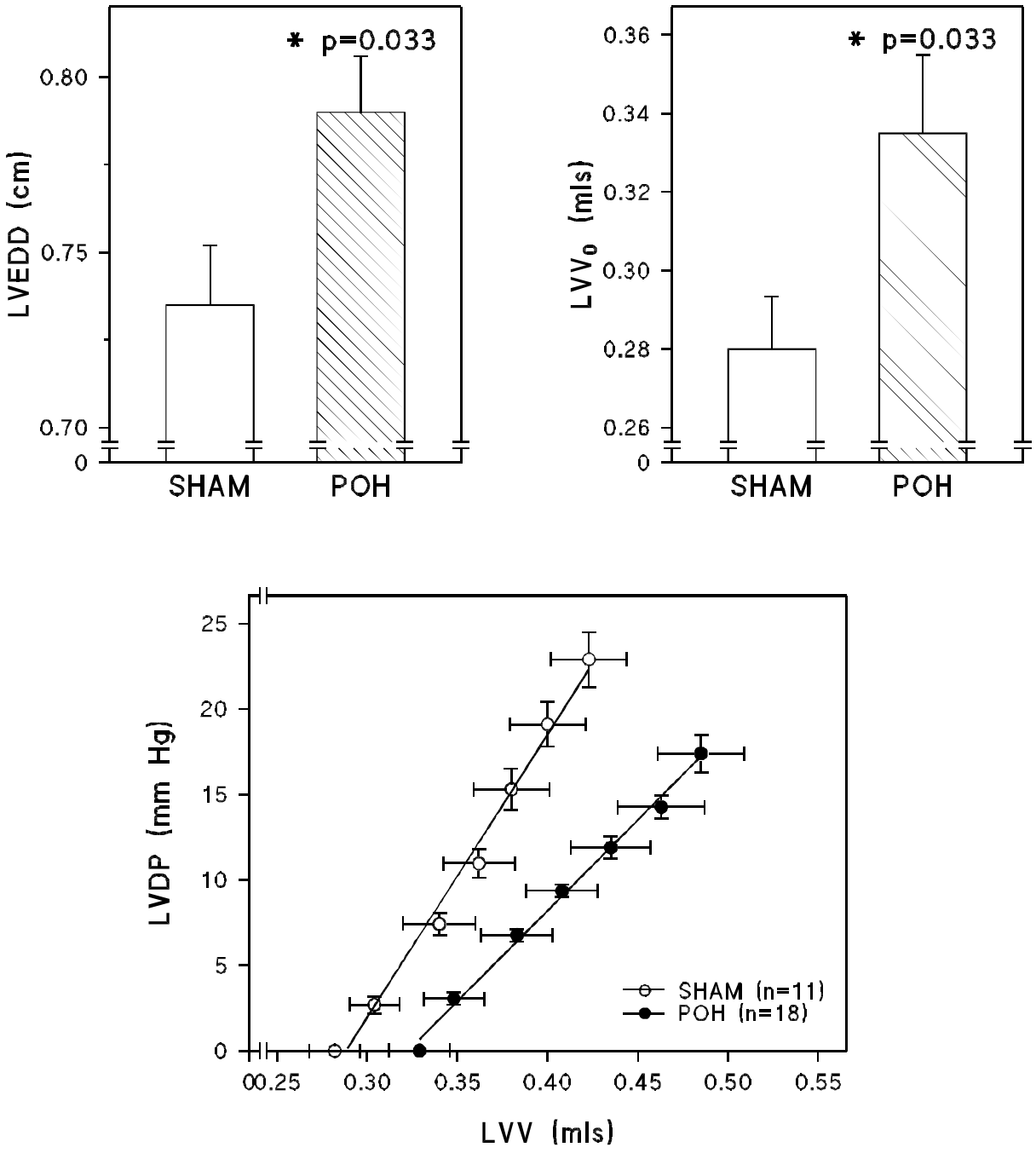


Figure 32. Effect of aortic-banding with pressure overload hypertrophy (POH) on left ventricular end diastolic diameters (LVEDD) (upper left panel), LV diastolic pressure-volume (LVDP-LVV) relations (lower panel), and the volume intercept (LVV_0) of the LVDP-LVV relation (upper right panel). SHAM, sham-operated control. * $p<0.05$ versus SHAM.

LV remodeling, rats with POH had a right shift in the LV diastolic P-V relation and an increase in the LV volume intercept of this relation (Figure 32, lower and upper right panels).

SHR at 14-15 months of age had normal LVEDP-LVED internal radius values ($LVEDr_0$ is illustrated in the upper left panel of Figure 33) and a normal volume intercept of the LV diastolic P-V relation (inset of Figure 34) as compared to age-matched WKY controls. Consequently, the increase in LV weight noted in 14-15 month old SHR (Table 7) translated into an increase in relative wall thickness as determined at controlled filling pressures (Figure 33, upper right and lower panels). However, in keeping with the development of LV remodeling, 21-22 month old SHR developed an increase in LVED internal dimensions as determined at controlled filling pressures (upper left panel of Figure 33), a right shift in the LV diastolic P-V relation (Figure 34) and an increased volume intercept of the LV diastolic P-V relation (inset of Figure 34). Thus, even though 21-22 month old SHR had marked increases in LV weight (Table 7), LVED relative wall thickness was similar to WKY controls and reduced in comparison to 14-15 month old SHR (lower panel and upper right panel of Figure 33), an effect that can only be attributed to LV remodeling.

Myocardial stiffness. Myocardial stiffness was unaltered in rats with POH, but was enhanced in both 14-15 and 21-22 month old SHR (Figure 35).

Myocardial collagen. Rats with POH, as well as SHR at 14-15 and 21-22 months of age developed an increase in myocardial [HPRO] with the greatest increase being in 21-22 month old SHR (Figure 36). However, because of a decrease in % myocardial collagen soluble to CNBr digestion (SHR=33±2, WKY=41±2, $p<0.05$), an increase in insoluble (cross-linked collagen) myocardial collagen concentrations, without a significant increment in the concentration of the soluble portion (non-cross-linked

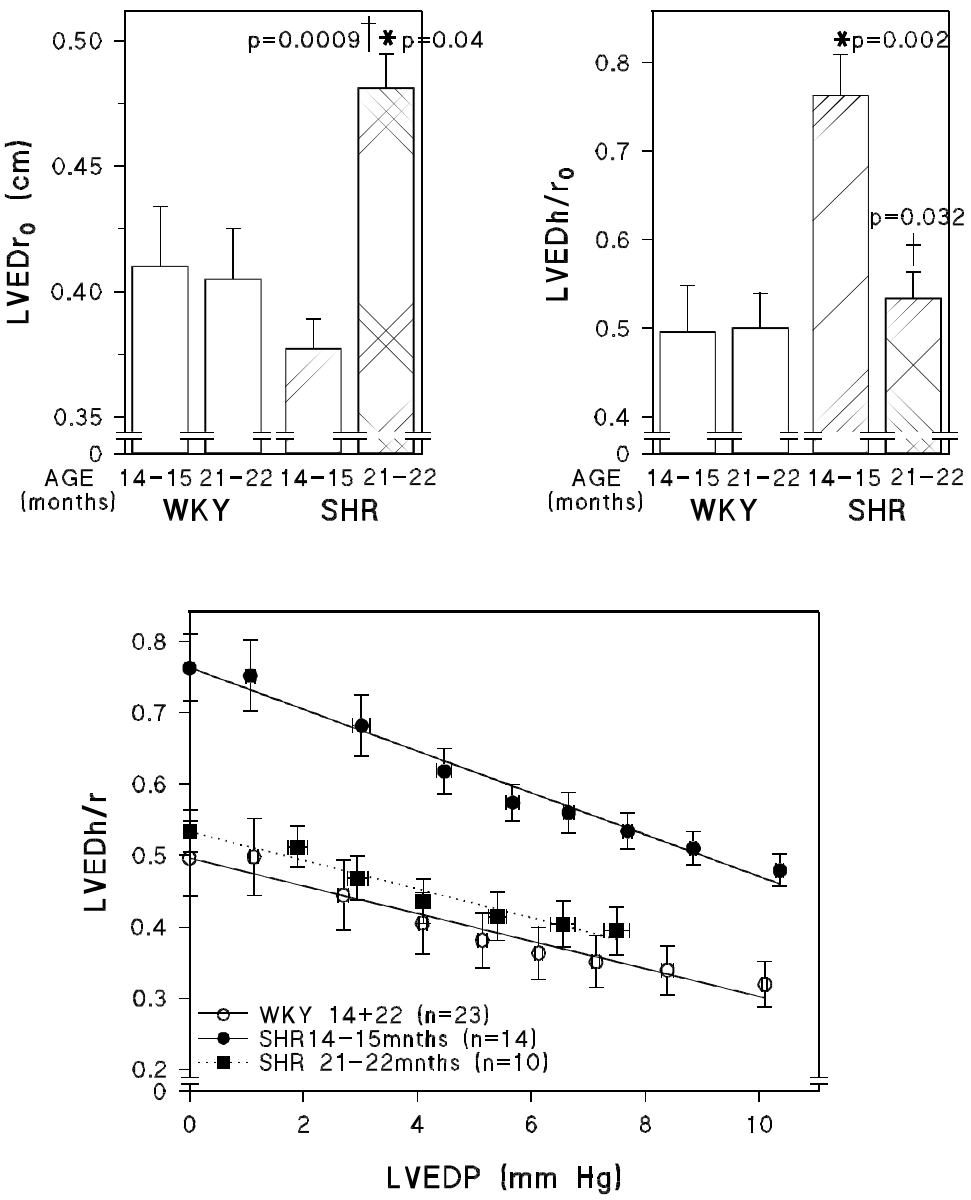


Figure 33. Left ventricular end diastolic pressure (LVEDP)-LVED relative wall thickness (wall thickness-to-radius ratio) (LVEDh/r) relations (lower panel), LVEDh/r intercept ($LVEDh/r_0$, upper right panel) of the LVEDP-LVEDh/r relation, and LVED internal radius (r) intercept ($LVEDr_0$, upper left panel) of the LVEDP-LVEDr relation (not shown) in spontaneously hypertensive rats (SHR) and their normotensive Wistar Kyoto controls (WKY). * $p<0.05$ versus WKY, † $p<0.05$ versus SHR at 14-15 months of age.

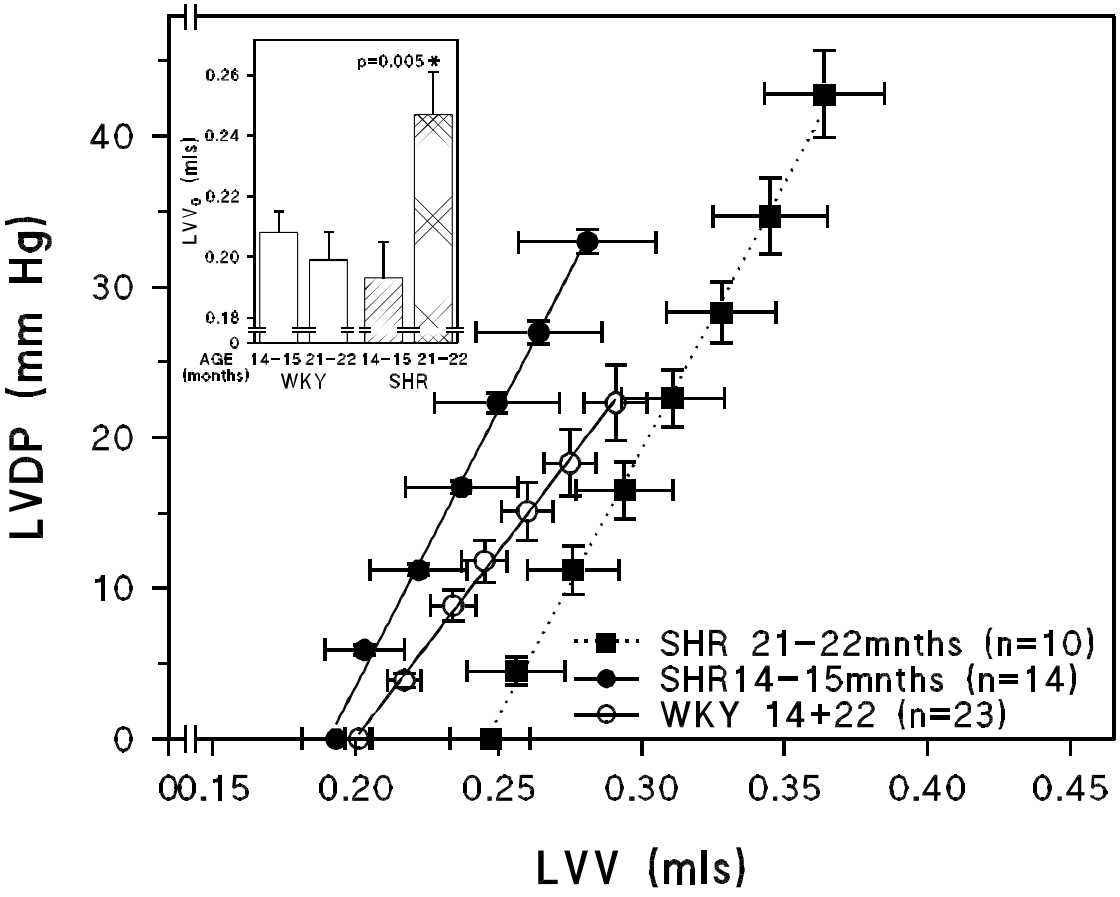


Figure 34. Left ventricular diastolic pressure-volume (LVDP-LVV) relations and the volume intercepts (LVV_0) of the LVDP-LVV relations (inset) in spontaneously hypertensive rats (SHR) and their normotensive Wistar Kyoto controls (WKY). * $p<0.01$ versus the other 3 groups.

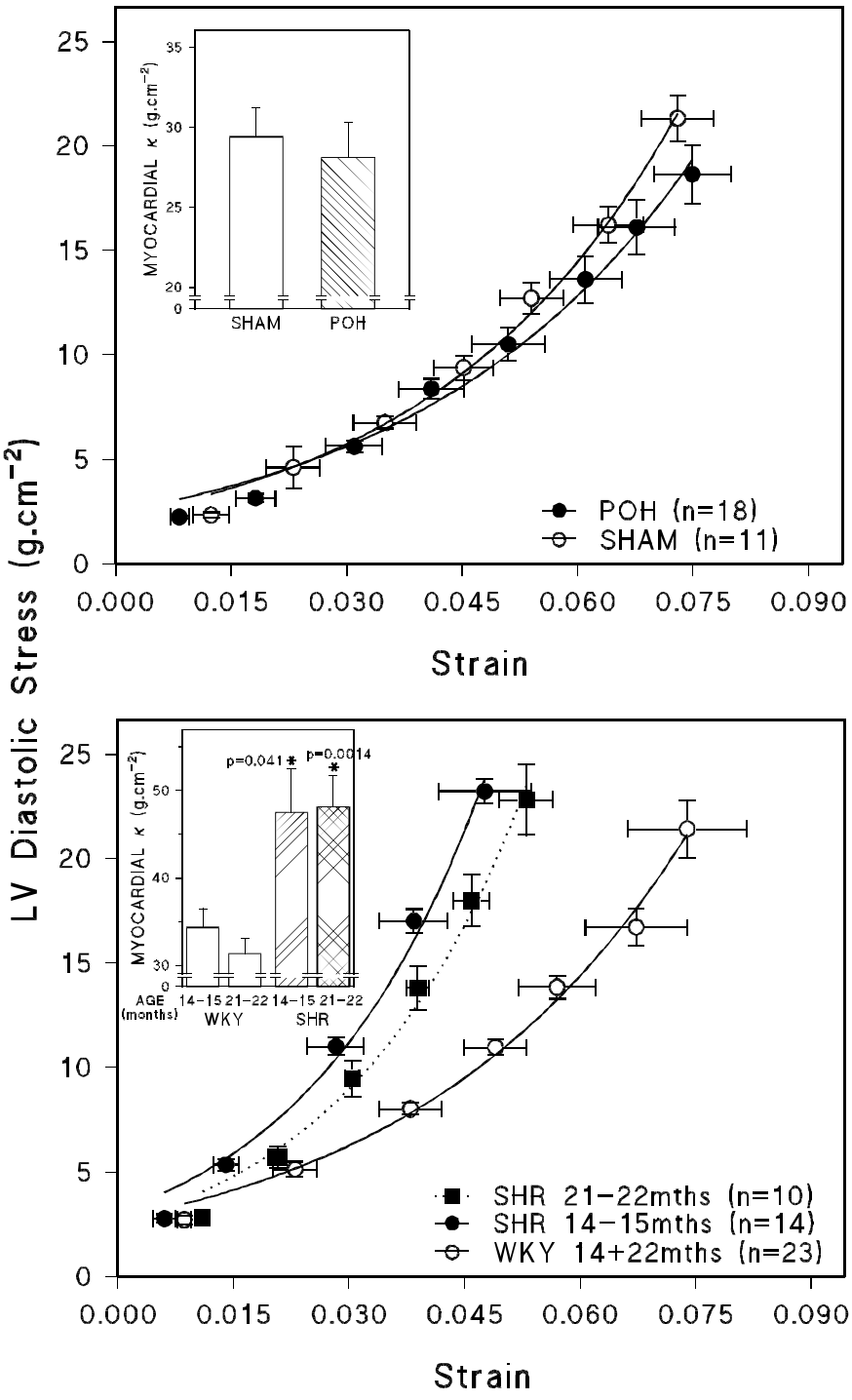


Figure 35. Left ventricular diastolic stress-strain relations and myocardial diastolic stiffness constants (k) in aortic-banded rats with pressure overload hypertrophy (POH) and their sham-operated controls (SHAM) (upper panel) and in spontaneously hypertensive rats (SHR) and their normotensive Wistar Kyoto controls (WKY) (lower panel). * $p<0.05$ versus age-matched WKY controls.

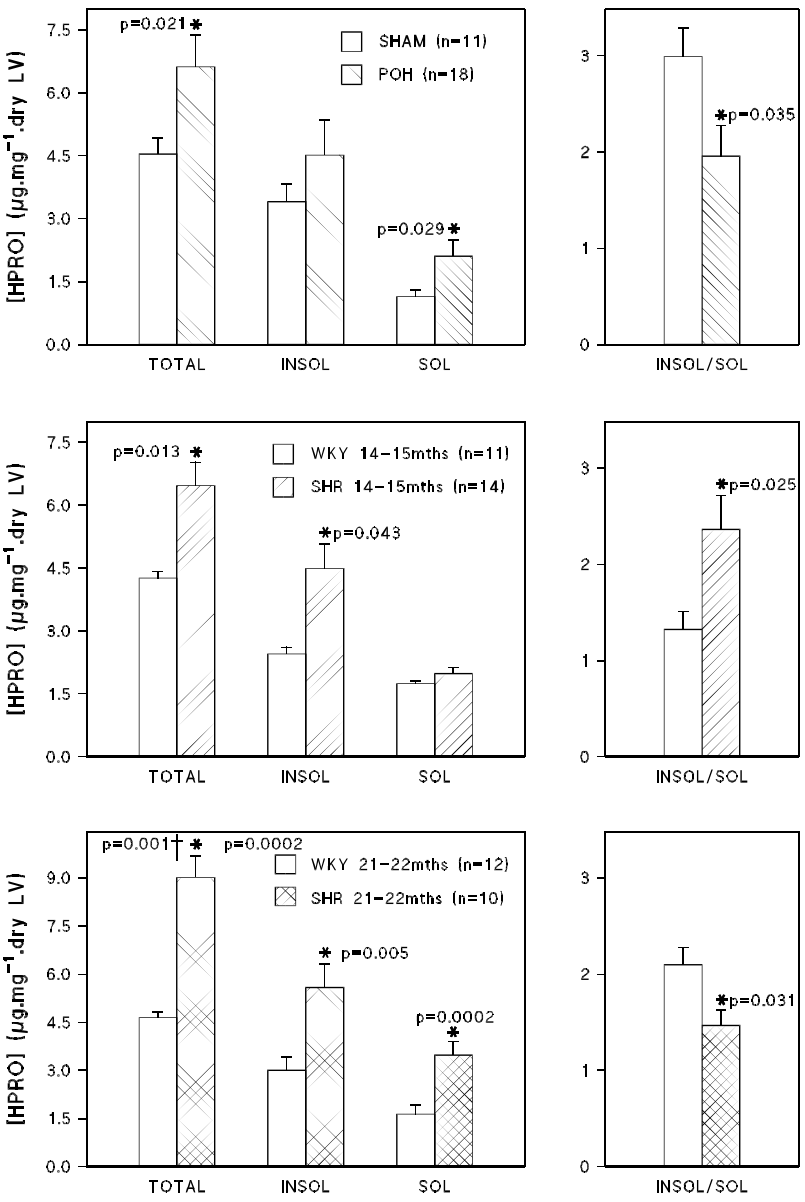


Figure 36. Myocardial collagen characteristics in aortic-banded rats with pressure overload hypertrophy (POH) and their sham-operated controls (SHAM) as well as in spontaneously hypertensive rats (SHR) and their normotensive Wistar Kyoto controls (WKY). [HPRO], hydroxyproline concentrations; INSOL [HPRO], collagen concentrations insoluble to cyanogen bromide (CNBr) digestion; SOL [HPRO], collagen concentrations soluble to CNBr digestion; INSOL/SOL, ratio of insoluble to soluble collagen concentrations. * $p<0.05$ versus SHAM or versus age-matched WKY, † $p<0.05$ versus SHR at 14-15 months of age.

collagen) was noted, and hence an increase in the ratio of insoluble to soluble collagen occurred in SHR at 14-15 months of age (Figure 36). In contrast, rats with POH had an increase in % myocardial collagen soluble to CNBr digestion (POH=39±7, SHAM=23±3, $p<0.05$), and hence an enhanced soluble myocardial collagen concentration, without a significant increase in the concentration of the insoluble portion (Figure 36). Consequently the ratio of insoluble to soluble collagen was decreased in the rats with POH. Although 21-22 month old SHR also had an increase in % myocardial collagen soluble to CNBr digestion (SHR=42±2, WKY=34±3, $p<0.05$), the marked degree of fibrosis resulted in concomitant increases in cross-linked and non-cross-linked collagen concentrations (Figure 36). The ratio of insoluble to soluble collagen was decreased in concordance with the increment in the percentage of collagen soluble to CNBr digestion.

Myocardial stiffness and myocardial collagen. In SHR and WKY, myocardial collagen concentration (HPRO) clearly correlated with myocardial stiffness, whereas in rats with POH and in SHAM operated rats, no association was evident (Figure 37). Increments in insoluble collagen but not soluble collagen were associated with increases in myocardial stiffness in SHR and WKY rats. Consequently the ratio of insoluble to soluble collagen was correlated with myocardial stiffness (Figure 37).

DISCUSSION

In the present study SHR with increases in myocardial stiffness and without evidence of LV remodeling had increments in myocardial cross-linked collagen, but not non-cross-linked collagen concentrations. In contrast, in rats with POH and LV remodeling without increases in myocardial stiffness, myocardial non-cross-linked, but not cross-linked collagen concentrations were enhanced. With advanced myocardial fibrosis in SHR, where concomitant LV remodeling and an increase in myocardial

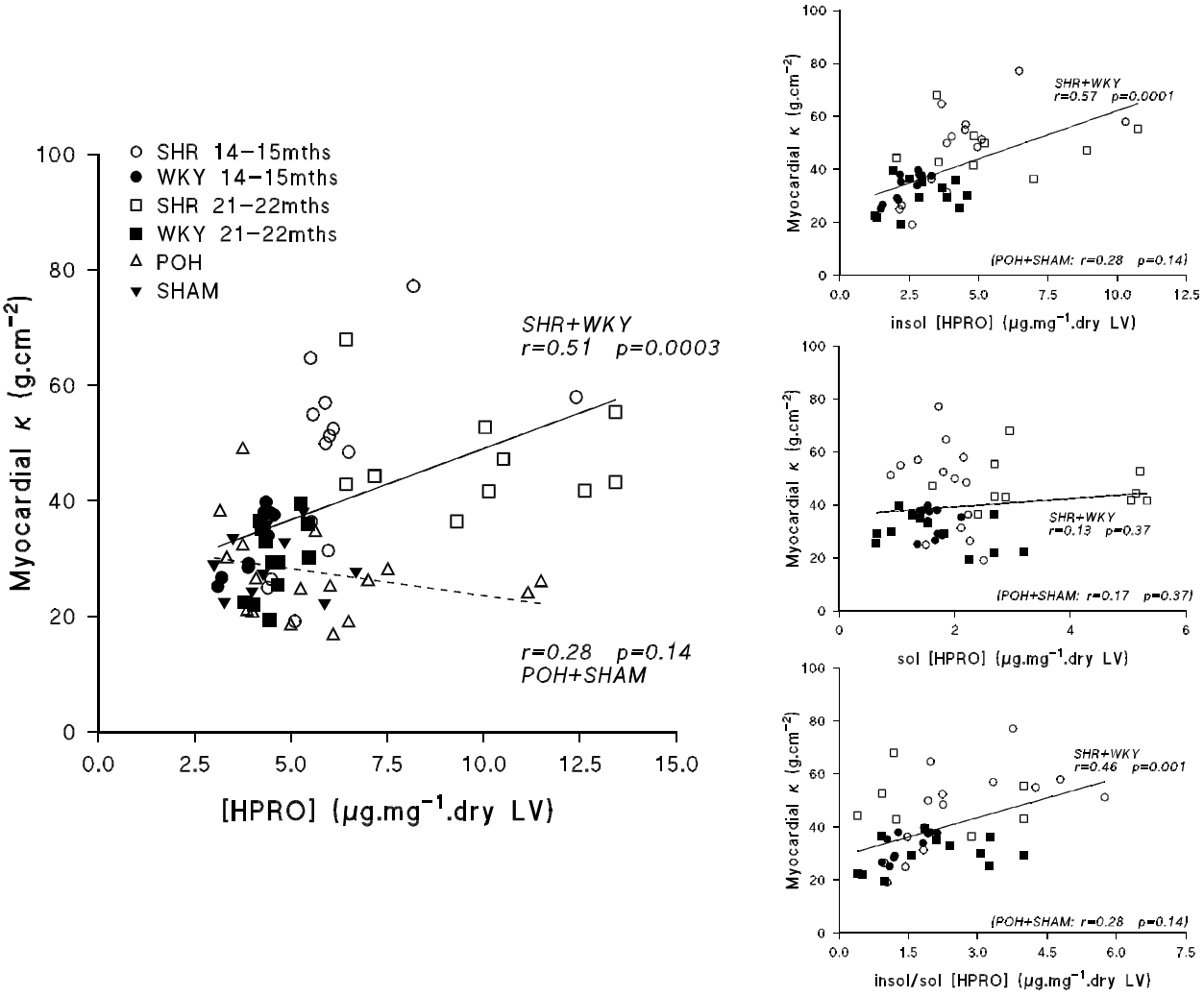


Figure 37. Correlations between myocardial stiffness (myocardial k) and myocardial collagen characteristics in spontaneously hypertensive rats (SHR) and their normotensive Wistar Kyoto controls (WKY). Correlation coefficients (r) and their p-values are shown for SHR and WKY. Correlation coefficients and their p-values for aortic-banded rats with pressure overload hypertrophy (POH) and their sham-operated controls (SHAM) are included for comparison. For other abbreviations refer to Figure 36.

stiffness was noted, increments in both cross-linked and non-cross-linked myocardial collagen concentrations were observed.

The variable effect of increases in myocardial collagen concentrations on material properties may be attributed to alterations in either collagen phenotypes, or cross-linked characteristics. Although type I collagen is thought to be a stiffer phenotype, only modest changes in myocardial stiffness may follow increases in type I-to-III collagen ratios in hypertensive heart disease (Woodiwiss et al 2001; Norton et al 2002). Moreover, type I-to-III myocardial collagen ratios are decreased in older as compared to younger SHR (Tsotetsi et al 2001), when myocardial stiffness is increased (Bing et al 1995; Brilla et al 1996). Consequently, alterations in myocardial collagen phenotypic ratios cannot explain disparate effects of fibrosis on material properties. In contrast, an augmented myocardial collagen cross-linking and enhanced concentrations of collagen in the cross-linked form accompany increments in myocardial stiffness (Norton et al 1997). In this regard, in the present study hypertensive rats without significant increases in myocardial cross-linked collagen concentrations (aortic-banded rats) failed to exhibit changes in myocardial material properties, whereas hypertensive rats with increases in myocardial cross-linked collagen concentrations (14-15 and 21-22 month old SHR) were noted to have an augmented stiffness. Although our group have previously shown the importance of collagen cross-linking in mediating myocardial stiffness in younger SHR (Norton et al 1997), we have provided no data to show that increases in total, but not cross-linked myocardial collagen concentrations fail to translate into a stiffer myocardium in POH, or that myocardial cross-linked but not non-cross-linked collagen concentrations parallel stiffness in older SHR.

Although part of the reason for the enhanced myocardial cross-linked collagen concentrations in 14-15 month old SHR was because of an augmented cross-linking (as evidenced by the decreases in collagen soluble to CNBr digestion), the same

mechanism does not explain increases in myocardial cross-linked collagen concentrations in 21-22 month old SHR. In the latter group, collagen solubility was enhanced which would tend to reduce cross-linked collagen concentrations. Hence increases in myocardial cross-linked collagen concentrations in older SHR are attributed to an augmented synthesis (Boluyt et al 1994), and not to alterations in cross-linked properties. Importantly, increments in myocardial cross-linked collagen concentrations in older SHR also cannot be attributed to reduced collagen degradation, as an increased collagenase activity has been shown to occur in SHR at this age (Brilla et al 1996).

The role of myocardial fibrosis as a determinant of chamber remodeling is controversial. The interstitial changes that are apparently more closely related to the development of chamber remodeling seem to be an increased activity of MMP (Mann and Spinale 1998) and a decrease in collagen cross-linking (Li et al 2001; Woodiwiss et al 2001) (which is likely to increase the susceptibility of myocardial collagen to MMP-induced degradation). These changes are thought to lead to breaks or tears in the myocardial collagen matrix and subsequently to produce myocyte slippage. Indeed, in the present study in rats with LV remodeling (aortic-banded rats and 21-22 month old SHR) increases in myocardial non-cross-linked collagen concentrations were noted. Although our group have previously shown that LV remodeling accompanies increments in myocardial non-cross-linked collagen concentrations in cardiac hypertrophy (Woodiwiss et al 2001), the present study provides the first data to show how these changes simultaneously impact on myocardial material properties as well as on LV remodeling. The present data indicate that LV remodeling without increments in myocardial stiffness accompanies increases in myocardial collagen concentrations if the collagen that accumulates is predominantly of the non-cross-linked phenotype. In contrast, if both cross-linked and non-cross-linked collagen accumulates, both LV remodeling and enhanced myocardial stiffness occur.

Importantly, in the present study, increases in non-cross-linked myocardial collagen concentrations in rats with POH and 21-22 month old SHR were mediated by changes in collagen cross-linking (as evidenced by the enhanced solubility to CNBr digestion) as well as through an accumulation of myocardial collagen. These data support a previous proposal that myocardial fibrosis could contribute to LV remodeling through increases in myocardial collagen susceptible to the effects of MMP (non-cross-linked phenotype) (Woodiwiss et al 2001). Indeed, pharmacological agents known to inhibit the synthesis of myocardial collagen are capable of attenuating the progression of LV remodeling (Greenberg et al 1995) and to contribute to reverse LV remodeling (Levine et al 1997) in human heart failure. Moreover, in SHR the use of an antihypertensive agent that decreases myocardial collagen concentrations (including non-cross-linked collagen concentrations) has been shown to prevent the development of LV remodeling, despite being ineffective at regressing LV hypertrophy (Tsotetsi et al 2001), and reverse remodeling in human heart failure (following the use of an LV assist device) is associated with an enhanced collagen cross-linking (Li et al 2001).

A possible limitation of the present study is the use of indirect methods to determine collagen concentrations and the amount of collagen cross-linking. However, both of these methods are well established techniques (Stegemann and Stalder 1967; Brownlee et al 1986). Furthermore with respect to alterations in cross-linking in association with LV remodeling, similar findings to ours have been reported by others (Gunja-Smith et al 1996; Spinale et al 1996; Li et al 2001) using alternative indirect techniques to determine collagen content and the extent of collagen cross-linking.

In summary, the data in the present study suggest that the cross-linked characteristics of collagen influence whether increments in myocardial collagen concentration impact on stiffness and chamber remodeling in hypertensive heart disease. These data provide a potential explanation for the variable effects of fibrosis on

myocardial material properties and chamber remodeling. Hence, increases in myocardial collagen concentration may play a role in both myocardial stiffness and dilatation, however, these effects are modified by alterations in collagen quality. Preferential accumulation of non-cross-linked myocardial collagen, as described in chapter 3, following chronic β-AR activation, will increase the susceptibility of myocardial collagen to MMP degradation. The consequence could be cardiomyocyte slippage and hence cardiac dilatation.

CHAPTER 5

Impact of β_2-Adrenoreceptor Gene Variants on Cardiac Cavity Size and Systolic Function in Idiopathic Dilated Cardiomyopathy.

ABSTRACT

In heart failure the Arg16Gly and Gln27Glu polymorphisms of the β_2-adrenoreceptor (β_2-AR) gene are associated with exercise-capacity, clinical outcomes and response to β-AR blocker therapy. Whether β_2-AR gene variants mediate these effects in-part through an impact on cardiac structural remodeling and pump function independent of the effects of β-blockers is uncertain. I evaluated whether the Arg16Gly and Gln27Glu variants of the β_2-AR gene predict left ventricular ejection fraction (LVEF) and LV end diastolic diameter (LVEDD) in patients with idiopathic dilated cardiomyopathy (IDC) before and 6 months after receiving standard medical therapy other than β-AR blockers. 394 patients with IDC and 393 age and gender-matched controls were genotyped for the β_2-AR gene variants using restriction-fragment length polymorphism-based techniques. LVEF and dimensions were determined in 132 patients (of whom 71 were newly diagnosed) both at baseline and after 6 months. Genotype of neither variant was associated with the presence of IDC. Moreover, β_2-AR genotype did not determine LVEF or LV dimensions prior to initiating therapy. After 6 months of therapy, LVEF increased by 7.1±1.0 absolute units ($p<0.0001$) and LVEDD decreased by 0.27±0.06 cm ($p<0.02$). After adjusting for baseline values as well as gender, age, and type of angiotensin-converting enzyme inhibitor therapy received, β_2-AR genotype was associated with neither final LVEF and LVEDD, nor change in LVEF and LVEDD. In conclusion, these data suggest that in heart failure, the functional Arg16Gly and Gln27Glu variants of the β_2-AR gene have no independent effect on adverse structural remodeling and pump function.

INTRODUCTION

Through a multitude of cardiac effects, including apoptosis (Communal et al 1999) and necrosis (Mann et al 1992), persistent β-adrenoreceptor (β-AR) activation determines progressive heart failure (Esler et al 1997; Bristow 2000). Although there is substantial evidence to implicate the β_1-AR subtype in evolving cardiac dysfunction (Molenaar and Parsonage 2005), the role of the β_2-AR is uncertain (Bristow et al 1986). As selective β_1-AR downregulation occurs in heart failure, the β_2-AR may play an increasingly important role in cardiac disease (Engelhardt et al 1999). Like the β_1-AR, the β_2-AR mediates contractile effects (Bristow et al 1986; Bristow 2000), but in contrast to the β_1-AR, which is pro-apoptotic, the β_2-AR is antiapoptotic (Communal et al 1999). In transgenic animals, although a 15-fold increase in β_1-AR expression results in heart failure (Molenaar and Parsonage 2005), a substantially greater increase (60-fold) in β_2-AR expression does not produce the same effects (Liggett et al 2000). Hence, it may be argued that increased β_2-AR activation in heart failure could be protective.

The activity of β_2-ARs is determined in part by functional variants within the gene encoding the receptor (Green et al 1994; McGraw et al 1998; Drysdale et al 2000; Dishy et al 2001). Both an Arg16Gly and a Gln27Glu polymorphism of the β_2-AR gene influence the degree of agonist-stimulated receptor downregulation (Green et al 1994). The potential role that β_2-AR activation has in heart failure (Bristow et al 1986; Communal et al 1999; Bristow 2000) has prompted a number of investigators to explore the impact of Arg16Gly and a Gln27Glu polymorphisms in cardiac disease (Liggett et al 1998; Wagoner et al 2000; Kaye et al 2003; Covolo et al 2004; Forleo et al 2004; de Groote et al 2005; Terra et al 2005). In this regard, β_2-AR genotypes resulting in enhanced agonist-stimulated receptor downregulation (Green et al 1994) are associated with, or show trends for an association with the progression to hospitalization, death or transplantation (Liggett et al 1998; Forleo et al 2004), a reduced exercise capacity

(Wagoner et al 2000), and left ventricular (LV) functional responses to β-blocker therapy (Kaye et al 2003). However, the mechanisms of β_2-AR genotype effects in heart failure (Liggett et al 1998; Wagoner et al 2000; Kaye et al 2003; Forleo et al 2004) are uncertain.

β_2-AR genotype-dependent anti-apoptotic actions (Communal et al 1999) could produce distinct clinical outcomes through the well recognized impact of apoptosis on structural remodeling and cardiac function (Narula et al 1996; Yarbrough et al 2003). Although some studies have reported on a lack of impact of β_2-AR genotypes on baseline LV dimensions and pump function (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004), the data are difficult to interpret for a number of reasons. These studies were cross-sectional rather than prospective analyses, conducted in patients with diverse cardiac pathologies and performed in patients of whom a proportion were receiving β-AR blocker therapy (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004). In contrast, some studies have shown β_2-AR genotype-specific effects on LV structure and function after β-AR blocker therapy (Wagoner et al 2000; Kaye et al 2003). However, whether the genetic effects noted in these studies (Wagoner et al 2000; Kaye et al 2003) were determined by independent actions of β_2-AR genotype, or interactions between genotype and the well known effects of β-AR blockers on β-AR function (Esler et al 1997; Bristow 2000), is not clear. Therefore, in the present study, to assess whether β_2-AR genotypes determine LV dimensions and pump function independent of β-AR blockers, I evaluated the relationship between Arg16Gly and Gln27Glu polymorphisms of the β_2-AR gene and LV dimensions and systolic function in patients with idiopathic dilated cardiomyopathy (IDC) before and 6 months after receiving medical therapy other than β-AR blockers.

METHODS

Study groups. This study was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand (approval number: M951122). The clinical component of the study was conducted between 1995 and 2001 when the use of β-AR blockers was not considered standard therapy for heart failure in South Africa. All patients gave informed consent before study entry. To ensure that β_2-AR gene polymorphisms were not associated with IDC in this population, a case-control study was performed in which 394 patients with IDC and 393 age-matched control subjects of the same ethnic origins (African ancestry) were recruited. Subjects in the control group had a greater body mass index, higher blood pressures and consisted of more females (Table 8). Patients were recruited if they were $\geq$ 18 and $\leq$ 70 years of age, in stable New York Heart Association (NYHA) functional class I to IV heart failure of unknown etiology, had a left ventricular ejection fraction (LVEF) < 40% as determined by radionuclide ventriculography, and had high quality echocardiographic images with a LV end diastolic diameter (LVEDD) > 5.5 cm. Exclusion criteria included evidence of another cause of heart failure and the presence of arrhythmias that could alter LVEF.

After initial presentation, and following a diagnosis by clinical examination and echocardiography (screening visit), 176 of the 394 patients agreed to participate in a prospective study assessing the impact of β_2-AR gene polymorphisms on LV dimensions and function. During the 6 month period of follow-up 24 patients died and 20 were lost to follow-up. Of the remaining 132 patients who were followed prospectively, 71 were newly diagnosed. This subgroup of IDC patients on whom follow-up LV structure and function was assessed were comparable in their demography and clinical characteristics to those of the total group of IDC patients assessed (Table 8).

Table 8. Demographic and clinical characteristics of patients with idiopathic dilated cardiomyopathy (IDC) and controls.

	Control (n=393)	IDC (n=394)	IDC (n=132)
		All	Prospective analysis
Age (years)	52.0±0.4	51.8±0.8	52.6±1.0
Gender (male/female [%])	149/244(62)	233/161 (41)*	82/50 (38)*†
Body mass index ($kg.m^{-2}$)	28.0±1.4	25.0±0.3*	25.1±0.5*
Systolic BP (mm Hg)	128±1	121±1*	122±2*
Diastolic BP (mm Hg)	78±1	79±1	81±1

BP, blood pressure; * $p<0.05$ versus controls; † for β_2-adrenoreceptor genotyping the gender distribution was 83/49 (37), other demographic and clinical characteristics are unchanged.

These 132 patients who were followed prospectively received treatment with digoxin and diuretics (furosemide) for 7 days and then angiotensin-converting enzyme inhibitors (ACEI) were added to their therapy. These patients were followed for 6 months. Monthly visits were scheduled for clinical assessment and evaluation of patient's adherence to therapeutic agents. Clinical examinations, echocardiographic assessments and radionuclide studies were performed at baseline, and then repeated at 6 months. The primary end-points were LVEF determined using radionuclide ventriculography and LV end diastolic diameter (LVEDD) determined using echocardiography. Radionuclide ventriculography as opposed to echocardiography was used as the method of preference to assess the impact of β_2-AR genotype on LV systolic function as this measurement is not subject to observer bias. To show a 10-point difference in radionuclide LVEF between groups with 80% power after 6 months of therapy required a sample size in each group of 21 patients.

*Functional class, echocardiography and radionuclide studies***.** A physician assessed the NYHA functional class of the patients during the baseline and follow-up visits. The same physician evaluated all patients. A multiple gated equilibrium cardiac blood pool scintigraphic technique was used to measure LVEF (Elscint Apex 409)(Pitt and Strauss 1977). Imaging was performed in the left anterior oblique projection providing the best septal separation of the ventricles with a 0-10^{o} caudal angulation. Calculations of LV performance were made as previously described (Reiber 1985) using an automatic edge detection algorithm for the determination of LV borders. A single observer interpreted all studies. Two-dimensional targeted M-mode echocardiography with doppler colour flow mapping was performed using a Hewlett Packard Sonos 5500 echocardiograph attached to a 2.5 or 3.5 MHz transducer. All studies were performed and interpreted by the same operator and recorded on videotape. Left ventricular dimensions were measured according to the American Society of Echocardiography

guidelines (Sahn et al 1978). Measurements of LV dimensions and function were determined on an average of ≥ 3 beats. The investigators that performed and interpreted the radionuclide and echocardiographic studies were unaware of the treatment assigned to patients.

Genotyping. Blood for genetic studies was obtained during the initial screening period. Deoxyribonucleic acid (DNA) was extracted from whole blood using standard techniques as previously described (Candy et al 1999). Genotyping was performed by an investigator (Danelle Badenhorst) unaware of the identity of the patient's from whom DNA was obtained. Genotyping was undertaken after the clinical component of the study was complete. Genotyping of the Arg16Gly and Gln27Glu variants of the β_2-AR gene was undertaken using polymerase chain reaction (PCR)-restriction fragment length polymorphism-based techniques employing the appropriate primer pairs and restriction enzymes. DNA was amplified using 5'-GCCTTCTTGCTGGCACCCCAT-3' and 5"-CAGACGCTCGAACTTGGCCATG-3' forward and reverse primers respectively as previously described (Martinez et al 1997). PCR was carried out in a total volume of 20 μl containing ~50 ng DNA, 1 x PCR buffer (Takara), 2 mM MgCl2, 0.2 mM dNTP, 2.5 mM forward and reverse primers, 3% dimethylsulfoxide, 1 $\mu g.ml^{-1}$ bovine serum albumin and 1 unit Taq polymerase (Takara). The PCR conditions were as follows: 94^{o}C for 4 minutes, followed by 30 cycles of denaturation (94^{o}C for I minute per cycle), annealing (63.8^{o}C for 45 seconds) and extension (72^{o}C for 1 minute) with a final extension step at 72^{o}C for 4 minutes. PCR resulted in a 168 base pair (bp) product. Genotyping of the Arg16Gly polymorphism was performed after 7 μl of PCR product was digested with 2.8 units of Nco*I* (Roche) at 37^{o}C overnight. Nco*I* cleaves 22 bp from the 3' end of both alleles and 18 bp from the 5' end of the Gly16 allele. Hence, homozygotes for the Gly16 allele were identified by the presence of a 128 bp product, homozygotes for the Arg16

allele by the presence of a 146 bp product and heterozygotes by the presence of both a 128 and 146 bp product. Genotyping of the Gln27Glu polymorphism was performed after 7 μl of PCR product was digested with 555.5 units of Bbv*I* (New England Biolabs) at 37^{o}C for 2 hours. Bbv*I* cleaves the Gln27 allele to produce a 63 and a 105 bp fragment. Homozygotes for the Glu27 allele were identified by the presence of a 168 bp product alone. Heterozygotes were identified by the presence of a 168, 105 and 63 bp fragment. Electrophoresis was performed on 4% agarose gels and banding patterns visualized with ethidium bromide staining and ultraviolet illumination (Figure 38). To avoid misgenotying as a consequence of failure of restriction enzyme digestion, a known heterozygous sample for each of the polymorphisms was included in each PCR, digestion procedure and gel. Furthermore, all samples that were homozygous Glu27 were re-analysed. Despite repeated attempts one patient could not be genotyped for the Gln27Glu polymorphism.

Analysis. Data are presented as mean ± SEM. Case and control group mean values were compared with the use of a two-sample Student's t test or a Mann-Whitney test (depending on whether variables were nominal or ordinal [Bartlett's test]). To test for Hardy-Weinberg equilibrium the expected genotype numbers were calculated from the allele frequencies and deviation from the observed genotype numbers determined using a χ^2 test. Haplotypes of the Arg16Gly and Gln27Glu polymorphisms were assigned in individuals who were homozygous for both polymorphisms or heterozygous for only one of the polymorphism (controls: n=323; IDC all: n=332; IDC prospective analysis: n=114). In haplotype analysis subjects homozygous for the Gly16 and Gln27 alleles were compared with the other haplotypes present as the Gly16Gly+Gln27Gln haplotype has previously been shown to be associated with differences in exercise performance (Wagoner et al 2000). Effects of alleles on the presence of IDC were evaluated using a χ^2 test. Genotype effects on the presence of IDC were assessed with logistic regression

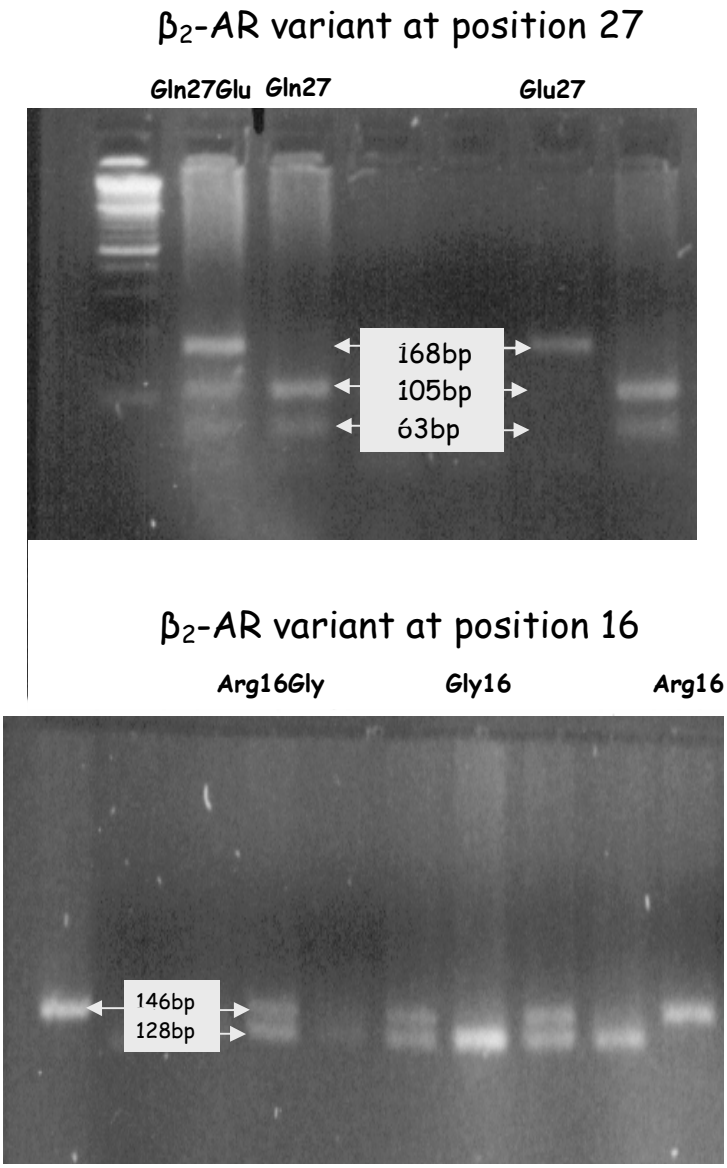


Figure 38. Representative samples of agarose gel electrophoresis showing the banding patterns obtained for β_2-adrenoreceptor genotyping. The upper panel is for the variant at position 27 and the lower panel is for the variant at position 16. See text for description of genotyping.

analysis with age, gender and body mass index (BMI) included as covariates. To assess the effect of genotype on either LVEF, or cardiac dimensions, a MANCOVA was performed with age, gender, disease duration and BMI included in the regression model. A paired Student's t test was used to detect changes from baseline. Analysis of covariance adjusting for baseline data, age, gender, disease duration and type of ACEI was employed to determine differences in changes in LV cavity size and function, and final LV cavity size and function between genotype groups. Genotype effects on final LV cavity size and function were assessed using MANCOVA with age, gender, BMI, disease duration and baseline data included as covariates. All probability values were adjusted for multiple genotyping.

RESULTS

Demographic and general clinical data. No significant differences were noted in the demographic or general clinical data in genotype-specific subgroups of patients on whom LV structure and function was determined (Table 9), except for an increase in the mean duration of disease in the Glu27 versus Gln27Glu and Gln27 groups (Table 9).

Association between β_2-AR genotype and IDC. Both the case and the control groups were estimated to be in Hardy-Weinberg equilibrium. The χ^2 values when comparing expected and actual genotype numbers were 5.53 and 4.48 for the case and the control groups respectively for the Arg16Gly variant of the β_2-AR gene, and 1.51 and 0.12 respectively for the Gln27Glu variant of the β_2-AR gene. Four of the eight possible haplotypes were common in our subjects (Table 10).

No differences were noted in the allele or haplotype frequencies between case and control groups (Table 10). β_2-AR genotype was not an independent predictor of IDC when considering the impact of Arg16Gly and Gln27Glu polymorphisms independently (logistic

Table 9. Demographic and clinical characteristics of patients with idiopathic dilated cardiomyopathy (IDC) prospectively studied, grouped according to β_2-adrenoreceptor genotype.

Gene variant	Arg16Gly			Gln27Glu		
Genotype group	Arg16 (CC)	Arg16Gly (GC)	Gly16 (GG)	Gln27 (CC)	Gln27Glu (GC)	Glu27 (GG)
n=	(21)	(75)	(36)	(91)	(34)	(6)
Age (years)	51.9±2.0	51.7±1.3	54.9±2.0	51.7±1.2	55.0±1.7	51.3±6.7
Gender (male/female [%])	10/11(52)	50/25(33)	22/14(39)	54/37(41)	23/11(32)	4/2(33)
Body mass index ($kg.m^{-2}$)	22.8±1.0	25.5±0.5	25.3±0.9	25.5±0.6	24.2±0.8	24.0±0.8
Systolic BP (mm Hg)	121±4	122±2	124±3	124±2	120±4	122±7
Diastolic BP (mm Hg)	80±3	82±1	81±2	82±1	80±2	79±4
Functional class (I/II/III/IV)	0/9/12/0	2/36/35/2	2/17/16/1	3/42/46/0	1/14/16/3	0/5/1/0
Disease duration (months)	7.1±4.0	14.2±2.1	7.5±3.0	12.5±2.0	7.4±3.3	30.8±7.9*
Perindopril/enalapril/trandolapril (%)	38/43/19	34/46/20	54/32/14	38/41/21	52/36/12	17/50/33

See Table 8 for abbreviations. *$p<0.05$ versus Gln27 and Gln27Glu.

Table 10. β_2-adrenoreceptor genotype and allele frequencies of patients with idiopathic dilated cardiomyopathy (IDC) and controls.

	Genotype			Allele	
	Arg16 (CC)	Arg16Gly (GC)	Gly16 (GG)	Arg 16 (C)	Gly16 (G)
Control (n=393)	75 (19)	219 (56)	99 (25)	369 (47)	417 (53)
IDC (n=394)	78 (20)	217 (55)	99 (25)	373 (47)	415 (53)
IDC* (n=132)	21 (16)	75 (57)	36 (27)	117 (44)	147 (56)

β_2-adrenoreceptor gene Arg16Gly variant

	Genotype			Allele	
	Gln27 (CC)	Gln27Glu (GC)	Glu27 (GG)	Gln27 (C)	Glu27 (G)
Control (n=393)	273 (69)	113 (29)	7 (2)	659 (84)	127 (16)
IDC (n=393)	275 (70)	107 (27)	11 (3)	657 (84)	129 (16)
IDC* (n=131)	91 (69)	34 (26)	6 (5)	216 (82)	46 (18)

β_2-adrenoreceptor gene Gln27Glu variant

	Gly16Gly +Gln27Gln	Arg16Arg +Gln27Gln	Arg16Gly +Gln27Gln	Gly16Gly +Gln27Glu	Gly16Gly +Glu27Glu
Control (n=323)†	52 (16)	72 (22)	149 (46)	40 (13)	7 (2)
IDC (n=332)†	47 (14)	80 (24)	149 (45)	46 (14)	9 (3)
IDC* (n=114)†	16 (14)	23 (20)	53 (46)	16 (14)	5 (4)

β_2-adrenoreceptor gene haplotypes

Numbers represent sample numbers (%). * represents patients studied prospectively. No relationship between genotype (logistic regression analysis adjusting for age, gender and body mass index) or allele (χ^2 analysis) and the presence of IDC was noted. † 3, 1 and 1 subjects in the control, IDC and IDC* groups respectively were Arg16Arg+Gln27Glu haplotype.

regression: Arg16Gly variant: GG vs CC; β-coefficient=-0.23±0.28, p=0.41; GC vs CC; β-coefficient=-0.09±0.25, p=0.71: Gln27Glu variant: CC vs GG; β-coefficient=0.81±0.56, p=0.14; GC vs GG; β-coefficient=0.91±0.57, p=0.11) or when constructing haplotypes for the two polymorphisms (logistic regression of Gly16Gly+Gln27Gln haplotype versus other haplotypes: β-coefficient=-0.17±0.22, p=0.45).

Association between β_2-AR genotype and LV function and cavity dimensions in IDC. A similar number of patients in each β_2-AR genotype-specific group died or were lost to follow-up (data not shown). All genotype groups received similar doses and type of drug therapy (type of angiotensin-converting enzyme inhibitor is indicated in Table 9). Neither the Arg16Gly nor the Gln27Glu polymorphisms of the β_2-AR gene predicted baseline LV function or cavity dimensions (Table 11). Moreover, haplotypes for the two polymorphisms (Gly16Gly+Gln27Gln haplotype versus other haplotypes) were not associated with baseline LV function or cavity dimensions (Table 12). Following 6 months of therapy, LVEF (MUGA) increased by 7.1±1.0 absolute units ($p<0.0001$), LVEDD decreased by 0.27±0.06 cm ($p<0.02$), and LV end systolic diameter decreased by 0.39±0.07 cm ($p<0.001$) in all patients considered together. The increase in LVEF and decrease in LVEDD and LVESD were the same in β_2-AR genotype-specific groups irrespective of whether the Arg16Gly and the Gln27Glu polymorphisms of the β_2-AR gene were considered separately (Table 11) or as haplotypes (Table 12). At the end of the study, β_2-AR genotype failed to predict LV function and cavity dimensions irrespective of whether the Arg16Gly and the Gln27Glu polymorphisms of the β_2-AR gene were considered separately (Table 11) or as haplotypes (Table 12).

DISCUSSION

The main findings of the present study are that the Arg16Gly and the Gln27Glu polymorphisms of the β_2-AR gene, failed to predict LV systolic performance and cavity

Table 11. Left ventricular chamber dimensions and function in patients with idiopathic dilated cardiomyopathy (IDC) prospectively studied, grouped according to β_2-adrenoreceptor genotype.

Gene variant	β_2-adrenoreceptor Arg16Gly								
Genotype group	Arg16 (CC) (n=21)			Arg16Gly (GC) (n=75)			Gly16 (GG) (n=36)		
	Baseline	Final	Change	Baseline	Final	Change	Baseline	Final	Change
LVEDD (cm)	6.44±0.18	6.32±0.25	-0.17±0.21	6.50±0.11	6.27±0.12	-0.32±0.09	6.59±0. 09	6.30±0.11	-0.20±0.10
LVESD (cm)	5.59±0.19	5.41±0.29	-0.24±0.23	5.69±0.10	5.31±0.13	-0.47±0.09	5.78±0.10	5.40±0.14	-0.31±0.13
LVEF (%)	24.1±1.3	33.2±2.7	9.1±2.7	24.1±0.9	30.9±1.5	6.8±1.3	23.5±1.1	30.5±2.1	7.0±2.0
Gene variant	β_2-adrenoreceptor Gln27Glu								
Genotype group	Gln27 (CC) (n=91)			Gln27Glu (GC) (n=34)			Glu27 (GG) (n=6)		
LVEDD (cm)	6.53±0.08	6.29±0.10	-0.28±0.0 8	6.54±0.14	6.32±0.13	-0.24±0.11	6.28±0. 18	5.87±0.20	-0.42±0.33
LVESD (cm)	5.68±0.08	5.33±0.12	-0.39±0.09	5.77±0.14	5.41±0.15	-0.39±0.13	5.54±0.2 4	5.05±0.31	-0.49±0.40
LVEF (%)	24.6±0.8	31.8±1.3	7.2±1.2	23.4±1.3	30.8±2.2	7.4±2.0	22.8±1.8	26.0±5.4	3.2±5.0

LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVEF, left ventricular ejection fraction.

Table 12. Left ventricular chamber dimensions and function in patients with idiopathic dilated cardiomyopathy (IDC) prospectively studied, grouped according to β_2-adrenoreceptor gene haplotypes.

Haplotype group	Gly16Gly +Gln27Gln (n=16)			Arg16Arg +Gln27Gln (n=23)			Arg16Gly +Gln27Gln (n=53)		
	Baseline	Final	Change	Baseline	Final	Change	Baseline	Final	Change
LVEDD (cm)	6.65±0.13	6.33±0.20	-0.28±0.14	6.68±0.17	6.38±0.22	-0.21±0.17	6.58±0.12	6.33±0.13	-0.27±0.09
LVESD (cm)	5.67±0.13	5.14±0.23	-0.53±0.18	5.90±0.18	5.46±0.25	-0.34±0.18	5.77±0.11	5.33±0.15	-0.44±0.11
LVEF (%)	24.2±1.5	32.3±3.2	8.0±3.0	22.6±1.45	31.2±2.3	8.0±2.6	22.7±1.1	30.1±1.7	6.8±1.5

Haplotype group	Gly16Gly +Gln27Glu (n=16)			Gly16Gly (GC) +Glu27Glu (n=5)		
	Baseline	Final	Change	Baseline	Final	Change
LVEDD (cm)	6.44±0.11	6.30±0.15	-0.14±0.14	6.50±0.38	6.24±0.46	-0.26±0.39
LVESD (cm)	5.65±0.14	5.46±0.18	-0.20±0.15	5.77±0.45	5.36±0.59	-0.41±0.50
LVEF (%)	23.8±2.6	27.3±2.7	3.6±2.4	21.3±1.3	28.3±5.9	7.0±5.0

LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVEF, left ventricular ejection fraction

dimensions before and after 6 months of standard medical therapy in patients with IDC not receiving β-AR blockers.

These data are the first to provide insight into the independent effect of β_2-AR genotypes on LV dimensions and systolic function in chronic heart failure. Previous data suggesting a lack of impact of β_2-AR genotypes on LV dimensions and systolic function in chronic heart failure (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004; de Groote et al 2005) are difficult to interpret, as these studies were confined to cross-sectional analyses (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004), conducted in patients with diverse cardiac pathologies (Liggett et al 1998; Wagoner et al 2000), and performed in patients of whom a proportion were receiving β-AR blocker therapy (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004). In contrast, in the present study, patients in whom β_2-AR genotypic effects on LV function and dimensions were determined, suffered from IDC only and were prospectively assessed for a 6 month period during which time they were not receiving β-blocker therapy.

Some previous studies have demonstrated a relationship between β_2-AR genotype and either exercise performance (Wagoner et al 2000), clinical outcomes (Liggett et al 1998; Forleo et al 2004) and LV functional responses to β-blocker therapy (Kaye et al 2003). Alternatively, one previous study reported no association between β_2-AR genotype and LV functional responses to β-blocker therapy (de Groote et al 2005). The lack of an independent effect of β_2-AR genotype on LV structure and function, as observed in the present study, would suggest that the β_2-AR genotype-specific effects reported on in prior studies (Liggett et al 1998; Wagoner et al 2000; Kaye et al 2003; Forleo et al 2004) cannot be attributed to an independent effect of β_2-AR genotype on LV structure and hence function. Alternative mechanisms must therefore be sought to explain these data (Liggett et al 1998; Wagoner et al 2000; Kaye et al 2003; Forleo et al 2004). If not attributed to LV

structural remodeling and hence pump function; an impact of β_2-AR genotype on exercise performance (Wagoner et al 2000), could be attributed to β_2-AR-mediated vascular or inotropic responsiveness; and an association or trend for an association between β_2-AR genotypes and clinical outcomes including death, hospitalization and transplantation (Liggett et al 1998; Forleo et al 2004), may be related to arrhythmic effects (Billman et al 1997), or β_2-AR-mediated vascular responsiveness (action on LV afterload). β_2-AR genotype-dependent LV functional responses to β-blocker therapy (Kaye et al 2003) may be through an impact of β_2-AR genotype on the ability of β-blocker therapy to produce beneficial cardiac effects.

The Arg16Gly and Gln27Glu polymorphisms frequently occur together, hence in the present study I examined the possible effects that these polymorphisms may have in combination. Haplotypes were constructed and patients with the Gly16Gly+Gln27Gln haplotype (ie. patients expressing receptors in which both are highly sensitive to downregulation) were compared to other haplotypes. Previously a decreased exercise capacity has been reported in patients who are homozygous Gly16 and Gln27 compared to other homozygous combinations (Wagoner et al 2000). However, in the present study haplotype analysis did not reveal an association between β_2-AR genotype and LV function and cavity dimensions at either baseline or follow-up***.*** Post hoc analysis of the change in LVEF of 8.0% (n=16) versus 6.6% (n=97) revealed that sample numbers of 1154 would be required in each haplotype group in order to show that this difference in LVEF between the haplotype groups is significant ($p<0.05$) with 80% power. Hence this small difference in LVEF between the two haplotype groups is unlikely to be of clinical relevance.

The potential limitations of the present study are as follows. First, prospective follow-up was for only 6 months. However, this period was selected as beyond 6 months mortality and morbidity related to heart failure would have limited the ability to appropriately

assess LV structural and functional changes (The SOLVD Investigators 1991). Second, the time course of the history of the disease varied considerably between genotype groups. However, those patients with the risk allele (Gln27) had the shorter duration of disease and tended to have the higher final LVEF. Moreover, in the data analysis duration of disease was included as a potential confounder. Third, LV structure and function were only assessed at rest rather than during exercise. It is therefore possible that I could have missed an impact of β_2-AR genotype on systolic function during exercise. However, the present hypothesis was based on the anti-apoptotic effects of the β_2-AR (Communal et al 1999) as apoptosis results in a loss of myocardial units, with subsequent cardiac dilatation and pump dysfunction, even at rest (Yarbrough et al 2003). As cardiac dilatation influences systolic function at rest, the lack of a relationship between genotype and systolic function in the present study, supports the null hypothesis of the present study. Last, although I assessed two functional polymorphisms of the β_2-AR gene, previous studies have shown that the functional Thr164Ile polymorphism of the β_2-AR gene is also associated with exercise capacity (Wagoner et al 2000); and the progression to death and transplantation (Liggett et al 1998). However, based on the distribution of the alleles previously reported on for the Thr164Ile polymorphism (Liggett et al 1998) I would have required 539 patients to show a 10 point difference in radionuclide LVEF between groups with 80% power after 6 months of therapy. Consequently, this was not a polymorphism that could be assessed in a single-centre study such as the present study.

In conclusion, the present study indicates that the functional Arg16Gly and Gln27Glu variants of the β_2-AR gene have no independent effect on adverse structural remodeling and pump function in patients with IDC. The relationship between these genotypes and progression to hospitalization, death or transplantation (Liggett et al 1998; Forleo et al 2004); a reduced exercise capacity (Wagoner et al 2000), and left ventricular (LV) functional responses to β-blocker therapy (Kaye et al 2003) as previously

described is therefore unlikely to be attributed to an independent effect of genotype on cardiac chamber dimensions and pump function.

CHAPTER 6

Gly389Arg β_1-Adrenoreceptor and Del322-325 α_2 - Adrenoreceptor Gene Variants as Predictors of Idiopathic Dilated Cardiomyopathy and its Progression in Subjects of African Descent.

ABSTRACT

The utility of β_1-adrenoreceptor (AR) Gly389Arg and α_{2C}-AR Del322-325 genotyping in predicting the development and progression of heart failure is uncertain. In conjunction with the α_{2C}-AR Del322-325 variant, the Gly389Arg polymorphism may predict an increased risk of the development of heart failure in subjects of African ancestry. To further assess the utility of β_1-AR Gly389Arg and α_{2C}-AR Del322-325 variant genotyping in heart failure in this population group, I therefore assessed the relationship between these gene variants and the presence, severity and progression of idiopathic dilated cardiomyopathy (IDC) in black South Africans. For this purpose 403 black South African patients with IDC and 429 control subjects of the same ethnic ancestry were recruited. Genotype for the Gly389Arg and Del322-325 variants were assessed using a restriction fragment length polymorphism (RFLP)-based technique and automated sequencing respectively. Left ventricular ejection fraction (LVEF) and dimensions were determined at baseline and after 6 months of standard medical therapy. β-AR blockers were not indicated as standard care at the time of completing this study and hence were excluded from therapy. All patients (n=50) and controls (n=50) genotyped for the α_{2C}-AR variant were homozygous for the Del322-325 allele. In this population group homozygous for the risk allele of the α_{2C}-AR variant, the Gly389Arg polymorphism was not associated with IDC (odds ratio for Arg389 allele=1.03, confidence limits=0.84-1.27; odds ratio for homozygosity for the Arg389 allele=1.03, confidence limits=0.78-1.35), nor did it predict left ventricular function and cavity dimensions either before or after therapy. In conclusion, these data suggest that genotyping of the β_1-AR Gly389Arg and α_{2C}-AR Del322-325 variants are of little use in predicting the risk, severity or progression of IDC in patients of African descent.

INTRODUCTION

Persistent β-adrenoreceptor (β-AR) activation promotes progressive heart failure (Esler et al 1997; Bristow 2000). As such, a cornerstone of therapy in heart failure is the use of blockers of β-ARs (Waagstein et al 1993; Packer et al 1996; CIBIS-II 1999; MERIT-HF 1999; Packer et al 2001; Gerson et al 2002; Poole-Wilson et al 2003; Waagstein et al 2003). With respect to the type of β-AR involved, there is substantial evidence to implicate the β_1-AR subtype in the pathogenesis of progressive heart failure (Molenaar and Parsonage 2005). The activity of β_1-AR is nevertheless determined in part by functional variants within the gene encoding the receptor. A common polymorphism of the β_1-AR gene – a substitution of glycine (β_1-Gly389) for arginine (β_1-Arg389) at amino acid 389 – occurs within a Gs-coupling domain (Mason et al 1999). As such, β-ARs with the β_1-Arg389 variant have a much greater ability to couple to adenylyl cyclase than do those receptors with the β_1-Gly389 variant (Mason et al 1999). The increased ability of the β_1-Arg389 receptor polymorphism to activate adenylyl cyclase may therefore determine the natural history of progressive heart failure or its response to β-AR blocker therapy.

Although some studies have suggested that the response to β-AR-blockers in patients with heart failure depends on the position 389 genotype of the β_1-AR gene (Johnson et al 2003; Liu et al 2003; Mialet Perez et al 2003), there is no clear independent association between this variant and heart failure (Tesson et al 1999; Iwai et al 2002; Small et al 2002; Mialet Perez et al 2003; Covolo et al 2004; Forleo et al 2004). Nevertheless, the β_1-Arg389 variant, when present with a polymorphic α_{2c}-AR gene variant, has been reported to increase the risk for developing heart failure in African-Americans (Small et al 2002). α_2-ARs operate as presynaptic inhibitory receptors that control the release of norepinephrine and influence the progression of heart failure

(Brede et al 2002). A common coding polymorphism of the gene for the α_{2C}-AR – the deletion of four consecutive amino acids (Del322-325) – results in a substantial loss of agonist-mediated receptor function in transfected cells (Small et al 2000). Since α_{2C}-AR activation inhibits norepinephrine release (Hein et al 1999; Aggarwal et al 2001), the presence of the α_{2C}-AR Del322-325 polymorphism may result in enhanced norepinephrine release and hence increase the risk for heart failure (Cohn et al 1984). Although it appears that the β_1-AR variant, when present with a polymorphic α_{2c}-AR gene variant increases the risk for heart failure in a group of African-Americans, this relationship was identified in a small study sample (n=78 cases and n=84 controls)(Small et al 2002). Moreover, in groups of European descent an interaction between these two gene variants has not been confirmed (Metra et al 2006). Thus, the utility of genotyping for the β_1-AR Gly389Arg and α_{2C}-AR Del322-325 variants in predicting the development, severity or progression of heart failure is uncertain.

The aim of the present study was therefore to assess the utility of genotyping for the β_1-AR Gly389Arg and α_{2C}-AR Del322-325 variants in predicting the presence, severity or progression of idiopathic dilated cardiomyopathy (IDC) in a relatively large sample of black South Africans.

METHODS

Study groups, follow-up, clinical assessments and cardiac measurements. This study was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand (approval number: M951122). The clinical component of the study was conducted between 1995 and 2001 when the use of β-AR blockers was not considered standard therapy for heart failure in South Africa. All patients gave informed consent before study entry. To assess the relationship between the β_1-AR and α_{2C}-AR gene polymorphisms and IDC in this population, a case-control study was

performed in which 403 patients with IDC and 429 age-matched control subjects of the same ethnic origins (African ancestry) were recruited. To obtain this sample the same patients and control subjects were utilized as described in chapter 5, with the exception that the present study was terminated after the study described in chapter 5. Consequently, a few more patients and controls were included in the present study. The inclusion and exclusion criteria for recruitment have been described in chapter 5. The demographic and clinical characteristics of the case and control groups were largely as described in chapter 5, Table 8. Subjects in the control group had a greater body mass index and consisted of more females.

After initial presentation, and following a diagnosis by clinical examination and echocardiography (screening visit), 176 of the 403 patients agreed to participate in a prospective study assessing the impact of the β-AR gene polymorphisms on LV dimensions and function. As indicated in chapter 5, during the 6 month period of follow-up 24 patients died and 20 were lost to follow-up. Of the remaining 132 patients who were followed prospectively, 71 were newly diagnosed. The demographic and clinical characteristics of the subgroup of IDC patients on whom follow-up LV structure and function was assessed were comparable in their demography and clinical characteristics to those of the total group of IDC patients assessed and were the same as described in chapter 5, Table 8. The treatment and the measurements performed on the 132 patients who were prospectively studied have been described and justified in chapter 5. The primary end-points were LVEF determined using radionuclide ventriculography and LV end diastolic diameter (LVEDD) determined using echocardiography. To show a 10-point difference in radionuclide LVEF between groups with 80% power after 6 months of therapy required a sample size in each group of 21 patients. The approach to determine heart failure functional class, echocardiography and radionuclide studies has also been described in chapter 5.

Genotyping. Blood for genetic studies was obtained during the initial screening period. Deoxyribonucleic acid (DNA) was extracted from whole blood using standard techniques as previously described (Candy et al 1999). Genotyping was performed by an investigator (Danelle Badenhorst) unaware of the identity of the patient's from whom DNA was obtained. Genotyping was undertaken after the clinical component of the study was complete. Genotyping of the Gly389Arg variant of the β_1-AR gene was undertaken using a polymerase chain reaction (PCR)-restriction fragment length polymorphism-based technique employing the appropriate primer pairs and restriction enzymes. DNA was amplified using 5'-CGCTCTGCTGGCTGCCCTTCTTCC-3' and 5'-TGGGCTTCGAG TTCACCTGCTATC-3' forward and reverse primers respectively as previously described (Maqbool et al 1999). PCR was carried out in a total volume of 20 µl containing ~50 ng DNA, 1 x PCR buffer (Takara), 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM forward and reverse primers, 6% dimethylsulfoxide, 1 $\mu g.ml^{-1}$ bovine serum albumin, and 1 unit Taq polymerase (Takara). The PCR conditions were as follows: 94^oC for 4 minutes, followed by 30 cycles of denaturation (94^oC for I minute per cycle), annealing (60^oC for 45 seconds) and extension (72^oC for 1 minute) with a final extension step at 72^oC for 4 minutes. The Arg389 allele PCR product contains a unique site for restriction by 1333 units of Bcg*I* (3 hours at 37℃). Cleavage of the 530bp product in to 342 and 154bp fragments confirms the presence of this allele. Bcg*I* (New England Biolabs) cleaves twice to excise its recognition site accounting for the 34bp discrepancy in the fragments generated. The restriction digests were electrophoresed on 3% agarose gels and visualised with ethidium bromide staining and ultraviolet illumination (Figure 39). To avoid misgenotying as a consequence of failure of restriction enzyme digestion, a known heterozygous sample for each of the polymorphisms was included in each PCR, digestion procedure and gel and all samples were genotyped in duplicate.

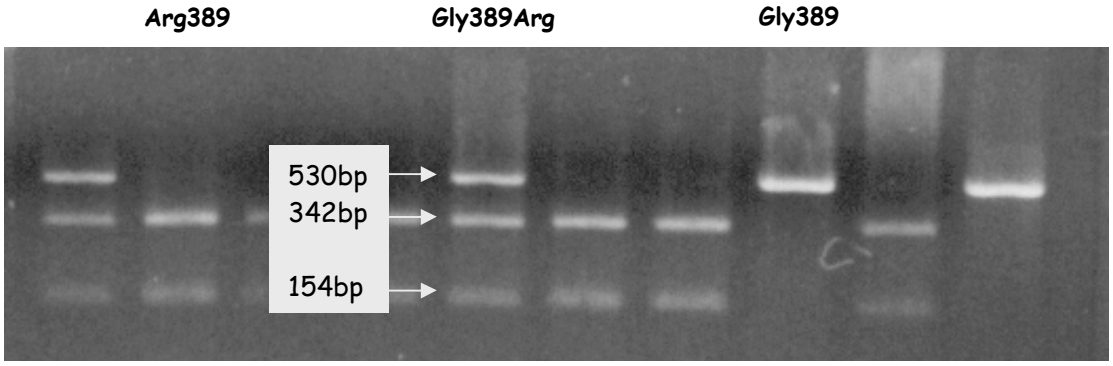


Figure 39. A representative sample of agarose gel electrophoresis showing the banding patterns obtained for β_1-adrenoreceptor genotyping. See text for description of genotyping.

Genotyping of the Del322-325 variant of the α_{2c}-AR gene was undertaken using PCR-sequencing techniques in 50 patients and 50 control subjects. DNA was amplified using 5'-AGCCCGACGAGAGCAGCGCA-3' and 5'-AGGCCTCGCGGCAGATGCCGTA CA-3' forward and reverse primers respectively as previously described (Small et al 2000). PCR was carried out in a total volume of 20 µl containing ~50 ng DNA, 1 x PCR buffer (Takara), 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM forward and reverse primers, 12% dimethylsulfoxide and 1 unit Taq polymerase (Takara). The PCR conditions were as follows: 94^{o}C for 4 minutes, followed by 40 cycles of denaturation (94^{o}C for 40 seconds per cycle), annealing (59^{o}C for 30 seconds per cycle) and extension (72^{o}C for 30 seconds per cycle) with a final extension step at 72^{o}C for 7 minutes. PCR products were purified using shrimp alkaline phosphatase and *E. coli* exonuclease I (Fermentas). Samples were processed using the *BigDye version 3.1 Dye Terminator Cycle Sequencing Kit* (Applied Biosystems) according to the manufacturer's instructions on the Genetic analysis system SCE2410 (SpectruMedix LLC, Pennsylvania, USA). Analysis was performed using BaseSpectrum V2.1.1 software (SpectruMedix LLC, Pennsylvania, USA). Figure 40 shows a typical example of the sequencing pattern obtained in all patients and subjects genotyped. All patients and controls genotyped for the Del322-325 variant of the α_{2c}-AR gene were homozygous for the Del322-325 allele.

Analysis. Data are presented as mean $\pm$ SEM. Case and control group mean values were compared with the use of a two-sample Student's t test or a Mann-Whitney test (depending on whether variables were nominal or ordinal [Bartlett's test]). To test for Hardy-Weinberg equilibrium the expected genotype numbers were calculated from the allele frequencies and deviation from the observed genotype numbers determined using a χ^2 test. Effects of alleles on the presence of IDC were evaluated using a χ^2 test. Genotype effects on the presence of IDC were assessed with logistic regression analysis with age, gender and body mass index (BMI) included as covariates. To assess

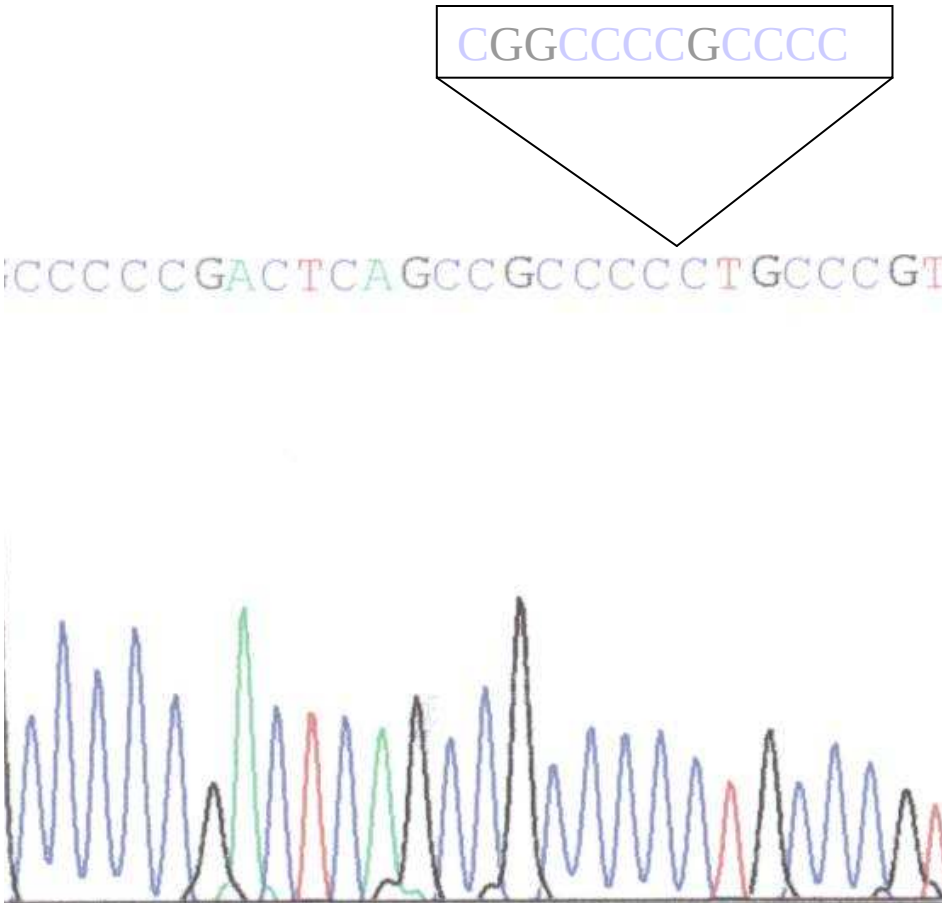


Figure 40. Typical example of a sequencing electrophoresis pattern of the α_{2c}-adrenoreceptor gene, showing the homozygous pattern for the Del322-325 variant noted in all control subjects and patients with idiopathic dilated cardiomyopathy genotyped. The insertion sequence is indicated in the box.

the effect of genotype on either LVEF, or cardiac dimensions, a MANCOVA was performed with age, gender, disease duration and BMI included in the regression model. A paired Student's t test was used to detect changes from baseline. Analysis of covariance adjusting for baseline data, age, gender, disease duration and type of ACEI was employed to determine differences in changes in LV cavity size and function, and final LV cavity size and function between genotype groups. Genotype effects on final LV cavity size and function were assessed using MANCOVA with age, gender, BMI, disease duration and baseline data included as covariates.

RESULTS

Demographic and general clinical data. No significant differences were noted in the demographic or general clinical data in genotype-specific subgroups of patients on whom LV structure and function was determined (Table 13).

Association between β_1-AR genotype and IDC. Both the case and the control groups were estimated to be in Hardy-Weinberg equilibrium. The χ^2 values when comparing expected and actual genotype numbers were 2.72 and 1.84 for the case and the control groups respectively (Table 14).

No differences were noted in the allele frequencies between case and control groups (Table 14). β_1-AR genotype was not an independent predictor of IDC in this population who are homozygous for the α_{2C}-AR Del322-325 allele (logistic regression: CC vs GG, β-coefficient=-0.07±0.32, p=0.83; CC vs GC, β-coefficient=-0.11±0.21, p=0.60; CC vs GC+GG, β-coefficient=-0.10±0.19, p=0.61).

Association between β_1-AR genotype and LV function and cavity dimensions in IDC. A similar number of patients in each β_1-AR genotype-specific group died or were lost to follow-up (data not shown). All genotype groups received similar doses and type of drug therapy (type of angiotensin-converting enzyme inhibitor is indicated in Table 13). The

Table 13. Demographic and clinical characteristics of patients with idiopathic dilated cardiomyopathy (IDC) prospectively studied, grouped according to β_1-adrenoreceptor genotype.

Gene variant	Gly389Arg		
Genotype group	Arg389 (CC)	Gly389Arg (GC)	Gly389 (GG)
n=	(70)	(47)	(15)
Age (years)	52.7±1.4	50.8±1.7	52.9±2.7
Gender (male/female [%])	44/26 (37)	27/20 (43)	12/3 (20)
Body mass index ($kg.m^{-2}$)	25.2±0.5	25.2±0.7	25.0±1.2
Systolic BP (mm Hg)	121±2	123±3	123±5
Diastolic BP (mm Hg)	81±2	80±2	81±3
Functional class (I/II/III/IV)	2/32/34/2	1/23/22/1	0/8/7/0
Disease duration (months)	12.1±2.2	12.2±3.1	9.3±4.9
Perindopril/enalapril/trandolapril (%)	40/46/14	34/43/23	53/20/27

BP, blood pressure.

Table 14. β_1-adrenoreceptor genotype and allele frequencies of patients with idiopathic dilated cardiomyopathy (IDC) and controls.

	B_1-adrenoreceptor gene Gly389Arg polymorphism				
	Genotype			Allele	
	Arg389 (CC)	Gly389Arg (GC)	Gly389 (GG)	Arg389 (C)	Gly389 (G)
Control (n=429)	210 (49)	172 (40)	47 (11)	592 (69)	266 (31)
IDC (n=403)	200 (50)	161 (40)	42 (10)	561 (70)	245 (30)
IDC* (n=132)	70 (53)	47 (36)	15 (11)	187 (71)	77 (29)

Numbers represent sample numbers (%). *represents patients studied prospectively. No relationship between genotype (logistic regression analysis adjusting for age, gender and body mass index) or allele (χ^2 analysis) and the presence of IDC was noted (see text for values).

Gly389Arg polymorphism of the β_1-AR gene failed to predict baseline LV function or cavity dimensions (Table 15). Following 6 months of therapy, LVEF (MUGA) increased by 7.0±1.0 absolute units ($p<0.0001$), LVEDD decreased by 0.27±0.06 cm ($p<0.02$), and LV end systolic diameter decreased by 0.38±0.07 cm ($p<0.01$) in all patients considered together. The increase in LVEF and decrease in LVEDD and LVESD were the same in β_1-AR genotype-specific groups (Table 15). At the end of the study, β_1-AR genotype failed to predict LV function and cavity dimensions (Table 15).

DISCUSSION

The main findings of the present study are as follows: A common coding polymorphism of the gene for the α_{2C}-AR – a deletion of four consecutive amino acids (Del322-325) – that results in a substantial loss of agonist-mediated receptor function in transfected cells (Small et al 2000) appears to exist in all black South Africans. In this population group homozygous for the risk allele of the α_{2C}-AR variant, the Gly389Arg polymorphism of the β_1-AR gene is not associated with IDC, nor does it predict the degree of systolic dysfunction and dilatation at baseline or after 6 months of standard medical therapy (excluding β-AR blockers) in IDC.

As with many genetic variants studied to-date, the reported relationships between the functional Gly389Arg polymorphism of the β_1-AR gene and heart failure have been inconsistent. Although studies conducted in transgenic mice have demonstrated that the β_1-Arg389 variant predisposes to a depressed ventricular function and pathological fibrosis (Mialet Perez et al 2003), several studies relating the Gly389Arg polymorphism of the β_1-AR gene with the risk for human heart failure or IDC and the progression of these diseases have produced inconsistent data (Tesson et al 1999; Iwai et al 2002; Small et al 2002; Mialet Perez et al 2003; Covolo et al 2004; Forleo et al 2004). One potential explanation for the inconsistencies in the reported

Table 15. Left ventricular chamber dimensions and function in patients with idiopathic dilated cardiomyopathy (IDC) prospectively studied, grouped according to β_1-adrenoreceptor genotype.

Gene variant	B1Gly389Arg								
Genotype group	Arg389 (CC) (n=70)			Gly389Arg (GC) (n=47)			Gly389 (GG) (n=15)		
	Baseline	Final	Change	Baseline	Final	Change	Baseline	Final	Change
LVEDD (cm)	6.67±0.09	6.35±0.11	-0.28±0.09	6.44±0.12	6.29±0.13	-0.21±0.11	6.33±0.22	5.99±0.28	-0.39±0.15
LVESD (cm)	5.84±0.09	5.41±0.13	-0.39±0.10	5.66±0.12	5.40±0.15	-0.34±0.13	5.43±0.22	4.98±0.34	-0.50±0.16
LVEF (%)	23.45±0.87	30.94±1.51	7.18±1.49	24.24±0.96	30.33±1.75	6.07±1.41	24.60±2.51	33.97±3.85	9.37±2.80

LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVEF, left ventricular ejection fraction.

relationships between the Gly389Arg polymorphism of the β_1-AR gene and heart failure is that an α_{2C}-AR gene variant influences the impact of the β_1-AR gene variant. Indeed, the presence of an α_{2C}-AR Del322-325 polymorphism has been shown to increase the chances that the β_1-Arg389 variant confers an increased risk for heart failure in 79 African-Americans with heart failure and 84 control subjects (Small et al 2002). This interactive effect between the β_1-Arg389 and α_{2C}-AR Del322-325 polymorphisms has also been examined in a much larger group of individuals of European descent, but the interaction was not reproduced (Metra et al 2006). The present study is the first to assess the role of the β_1-AR and the α_{2C}-AR gene variants in a relatively large study sample of patients (n=403) and controls (n=429) of African descent. In the present study I was unable to show a relationship between the β_1-Arg389 gene variant and IDC in black South Africans all of whom were apparently homozygous for the risk α_{2C}-AR Del322-325 allele. Moreover, in a prospective study I was unable to show a relationship between the β_1-Arg389 variant and either the degree of systolic dysfunction or the extent of cardiac dilatation in black South Africans with IDC all of whom were apparently homozygous for the risk α_{2C}-AR Del322-325 allele.

Differences between the outcomes of the present study and that showing an increased risk of heart failure related to the β_1-Arg389 and α_{2C}-AR Del322-325 gene variants in 79 African-Americans with heart failure and 84 control subjects (Small et al 2002) may include the following: First, the significant interactive effect previously reported on between the α_{2C}-AR Del322-325 and the β_1-Arg389 variant in African-Americans was between subjects homozygous for both the Del322-325 and Arg389 alleles versus subjects with at least one Gly389 and one α_{2C}-AR wild type allele (Small et al 2002). The absence of the α_{2C}-AR wild type allele in black South Africans prevented me from making this comparison. However, although this failed to reach statistical significance because of sample size limitations, homozygosity for the β_1-Arg389 variant

in African-American subjects also homozygous for the Del322-325 allele produced an odds ratio for heart failure of 3.46 (confidence intervals 0.68-17.6) (Small et al 2002). In contrast, in the present study, homozygosity for the β_1-Arg389 variant in subjects homozygous for the Del322-325 allele produced an odds ratio for heart failure of 1.03 (confidence intervals 0.78-1.35). Therefore, alternative factors are likely to account for differences in the present and a previous study (Small et al 2002). As with many genetic association studies, although studies with small sample sizes, such as that conducted by Small et al (2002) may show associations with disease phenotypes, there is a reduced chance of showing an effect with increasing sample sizes as in the study presently described. Moreover, in order to maintain consistency, I studied a sample of patients with IDC rather than recruiting patients with mixed forms of heart failure, such as IDC and ischaemic dilated cardiomyopathies, as reported on by Small et al (2002). Whether genetic effects in IDC and ischaemic dilated cardiomyopathy are similar is unknown. Last, it is possible that although groups of African descent were studied by both Small et al (2002) and our group, different environmental and genetic factors may contribute to heart failure in African-Americans and black South Africans.

Although, as the present study indicates, it is unlikely that the β_1-AR gene variant at position 389 has a pathophysiological role to play in IDC in groups of African descent, the α_{2C}-AR Del322-325 polymorphism may nevertheless still have an important role to play in this ethnic group. Previous studies have demonstrated a substantially increased risk of heart failure in African-Americans with the α_{2C}-AR Del322-325 allele (Small et al 2002). Moreover, Regitz-Zagrosek et al (2006) have demonstrated an association between the presence of the deletion polymorphism and reduced event and death rates in patients with IDC. In the present study the presence of homozygosity for the α_{2C}-AR Del322-325 allele in all patients (n=50) and controls (n=50) genotyped, precluded me from studying the impact of this genetic variant in isolation on IDC in this population

group. Nevertheless, this does not detract from the importance of the finding that genotyping for the α_{2C}-AR Del322-325 allele in black South Africans is of little practical use.

The limitations of the present study are as follows: First, I did not genotype all cases and controls for the α_{2C}-AR Del322-325 polymorphism. However, in the 100 individuals genotyped (50 cases and 50 controls), all individuals were homozygous for the Del322-325 allele. Second, as with all case-control studies, I did not account for population stratification. However, the selection of controls was from the same ethnic group and geographic location (Soweto) as the cases, and the study sample was relatively large. Third, as for the study described in chapter 5 prospective follow-up was only for 6 months. However, this period was selected as beyond 6 months mortality and morbidity related to heart failure would have limited the ability to appropriately assess LV structural and functional changes (SOLVD 1991). Fourth, LV structure and function were only assessed at rest rather than during exercise. It is therefore possible that I could have missed an impact of β_1-AR genotype on systolic function during exercise. However, the main hypothesis of the present study was related to the risk for IDC rather than the impact of β_1-AR genotype on LV structure and function.

In conclusion the present study demonstrates that black South Africans all appear to be homozygous for the α_{2C}-AR Del322-325 variant, and that the Gly389Arg polymorphism of the β_1-AR gene does not predict an increased risk for IDC or determine disease severity or progression of IDC in this population group homozygous for the risk allele of the α_{2C}-AR variant. These data do not support a role for α_{2C}-AR Del322-325 and β_1-AR Gly389Arg genotyping in predicting the development or severity of IDC in black South Africans. Whether β_1-AR Gly389Arg genotyping predicts response to β-AR blocker therapy in IDC in this population group still requires further study.

CHAPTER 7

Summary and conclusions

As reviewed in chapter 1, although there is a substantial body of evidence to implicate sympathetic nervous system activation in progressive heart failure, the role of the sympathetic nervous system as a causal factor in the development of heart failure has only recently begun to be studied. As part of the present thesis I provide evidence to further substantiate the notion that chronic sympathetic activation contributes toward the development of heart failure, at least in pressure overload hypertrophy (chapter 2). Having demonstrated that sympathetic activation can contribute toward the development of heart failure, in the present thesis I then focussed my efforts on two questions. First, I studied the potential mechanisms by which sympathetic activation can contribute toward the development of heart failure (chapters 3 and 4). Second, I attempted to clarify the potential role of gene variants that modulate the impact of sympathetic activation on the heart in the development and the progression of heart failure (chapters 5 and 6). The present discourse summarizes the evidence reported on in the present thesis and the place of this evidence in the context of the current scientific literature. The flow diagram in Figure 41 provides a summary of the hypotheses posed in the present thesis.

Cardiac hypertrophy has for many years been recognized as a precursor for heart failure (Kannel et al 1972). However, the exact mechanisms responsible for the progression from compensated cardiac hypertrophy to pump dysfunction and systolic heart failure have remained elusive. In chapter 2 I hypothesized that chronic sympathetic activation in cardiac hypertrophy contributes toward systolic cardiac dysfunction and heart failure in pressure overload hypertrophy (pathway A in Figure 41). This hypothesis was derived from a number of lines of evidence including the following: First, increased myocardial norepinephrine concentrations are measured in the coronary sinus in patients with hypertensive hypertrophy prior to the development of heart failure (Agabiti-Rosei et al 1987; Kelm et al 1996; Schlaich et al 2003). Second, transgenic animal models with decreased adrenergic activation are protected against the development of

dilatation and heart failure when exposed to pressure-overloads (Esposito et al 2002). Third, in compensated hypertensive hypertrophy excessive adrenergic activation is associated with downregulation of β-adrenergic systems (Limas and Limas 1978; Castellano et al 1993; Böhm et al 1994a; Böhm et al 1995), changes that could promote the development of contractile dysfunction. Last, blockade of β-adrenoreceptors (AR) prevents the transition from cardiac hypertrophy to heart failure in hypertension independent of blood pressure effects (Chan et al 2004). As it is difficult to perform prospective human studies when assessing the transition from compensated LVH to heart failure, I assessed this hypothesis in an animal model of pressure overload hypertrophy known to progress to heart failure, namely the spontaneously hypertensive rat (SHR). In chapter 2 and published in the journal *Hypertension* (Badenhorst et al 2003b), I provide the first evidence to indicate that excessive β-adrenergic activation produces the transition from compensated LVH to pump dysfunction in pressure overload states. Together with evidence to support the notion that sympathetic activation precedes heart failure in pressure overload states and that genetic and pharmacological-induced decreases in sympathetic effects inhibit the transition to heart failure in pressure-overload states, the evidence reported on in chapter 2 provides strong support for an important role of sympathetic activation in the transition to heart failure in pressure-overload states.

The relative role of myocardial contractility and adverse chamber remodeling (chamber dilatation) as causes of pump dysfunction in heart failure has been controversial (Figure 40, pathway B versus C). Prior studies performed in animal models of myocardial infarction have shown both reduced (Capasso and Anversa 1992; Cheung et al 1994; Li et al 1995) and normal (Lefroy et al 1996; Melillo et al 1996; Anand et al 1997) contractile function of the remaining viable myocardium or myocytes. Moreover, in pressure-overload states pump dysfunction is more closely related to adverse chamber

remodeling than with myocardial dysfunction (Norton et al 2002). Similarly, as reported in chapter 2 and published in the journal *Hypertension* (Badenhorst et al 2003b) I have shown that chronic sympathetic activation promotes the progression from concentric left ventricular hypertrophy to left ventricular dilatation and pump dysfunction in hypertension, without mediating intrinsic myocardial contractile dysfunction (chapter 2). This has subsequently been substantiated in a further study conducted in older SHR by our group and reported on in the journal *Hypertension* (Veliotes et al 2005). These data lend further support for the notion that in pressure overload states adverse chamber remodeling is more important as a cause of the transition to cardiac failure associated with pump dysfunction than are intrinsic myocardial contractile abnormalities (pathways C rather than B in Figure 41).

The ability to maintain intrinsic myocardial function despite chronic β-AR activation, as described in chapter 2, is perhaps counterintuitive to the ability of β-AR activation to promote apoptosis and decrease β-AR-induced inotropic responses (see chapter 1 for a review), both of which are expected to decrease myocardial function. Indeed as described by our group, chronic β-AR activation with the doses of isoproterenol described in chapter 2, has been shown by our group to promote apoptosis and reduce β-AR-induced inotropic responses (Osadchii et al 2005; Osadchii et al 2007). Our group has more recently provided an explanation for the ability to maintain intrinsic myocardial function following chronic and sustained β-AR activation despite β_1- and β_2-AR-mediated inotropic down-regulation and β-AR-induced apoptosis. In these studies either alterations in β-AR responsiveness occurred at supraphysiological norepinephrine concentrations only (Osadchii et al 2005) or in conjunction with marked up-regulation of α-AR mediated contractile responsiveness as well as an increased myocardial norepinephrine release (Osadchii et al 2007). The increase in α-AR mediated contractile responsiveness was sufficiently large as to maintain norepinephrine-induced inotropic

responses, despite profound decreases in both β_1- and β_2-AR-mediated contractile responses (Osadchii et al 2007).

Having demonstrated that chronic β-AR activation promotes the progression from compensated LVH to pump dysfunction, and that this effect is largely through alterations in chamber remodeling as opposed to myocardial contractile dysfunction, I subsequently studied the potential mechanisms of β-AR-induced left ventricular remodeling. In the present thesis I evaluated whether chronic β-AR activation could promote necrosis (pathway D in Figure 41), apoptosis (pathway D in Figure 41), activation of matrix metalloproteinases (pathway E in Figure 41) or alterations in the quality of myocardial collagen (pathway F in Figure 41). These alterations could all be responsible for side-to-side slippage of cardiac myocytes and thus an increase in cardiac chamber dimensions (see section 2.1.2.5.1).

In chapter 3 I describe a lack of cardiomyocyte apoptotic effects induced by chronic β-AR activation in compensated hypertrophy despite marked pro-apoptotic effects noted in normotensive rats without cardiac hypertrophy. These data would suggest that β-AR-induced cardiac dilatation is unlikely to be mediated through cardiomyocyte pro-apoptotic effects. In chapter 3 I have also described changes in the activity of proteins that influence the integrity of the collagen matrix following chronic β-AR activation. Activation of β-ARs increased matrix metalloproteinase (MMP) activity, but did not significantly alter MMP-2 or tissue inhibitor of MMP-2 expression. Although stimulation of cardiac adrenoreceptors has previously been shown to induce MMP expression (Figure 40, pathway E) (Briest et al 2001; Menon et al 2005), only trend effects were noted in the present study. In addition to alterations in MMP, additional interstitial effects produced by chronic β-AR activation are also described in chapter 3. These include qualitative changes in myocardial collagen, where collagen of the non-cross-linked form accumulates in response to chronic β-AR activation (pathway F of

Figure 41). A reduction in myocardial collagen cross-linking has previously been shown to accompany sympathetic nervous system activation (Woodiwiss et al 2001). Collagen of the non-cross-linked form is thought to be susceptible to MMP degradation (Figure 40, pathway E). The consequent breaks or tears in the myocardial collagen matrix may lead to myocyte slippage and subsequent cardiac dilatation (Figure 40, pathway C). Increases in soluble collagen concentrations may also account for increases in MMP activity (Tomasek et al 1997; Ruangpanit et al 2001), and thus for cardiomyocyte slippage.

Although previous studies have demonstrated that qualitative changes in the cross-linked properties of myocardial collagen are important in the progression to cardiac dilatation and pump dysfunction, in contrast to data provided in chapter 3, these changes are associated with decreases and not increases in myocardial collagen concentrations (Woodiwiss et al 2001). In chapter 3 and as published in the journal *Hypertension* (Badenhorst et al 2003b), I have demonstrated increases in myocardial collagen concentrations (myocardial fibrosis) following chronic β-AR activation despite a change in the qualitative characteristics of myocardial collagen that would increase the susceptibility to digestion (a decrease in cross-linking). Increases in myocardial collagen concentrations are better recognized as contributing toward left and not right (dilatation) shifts in left ventricular diastolic pressure-volume relations, through alterations in myocardial stiffness. Thus, whether β-AR-induced changes in the cross-linked properties of myocardial collagen contribute toward chamber dilatation is in question. In order to clarify this issue, in chapter 4 I therefore hypothesized that the diverse relationship that exists between the degree of myocardial fibrosis and both stiffness and chamber remodeling, could in-part be explained by a modulating influence of alterations in myocardial collagen cross-linking on the impact of fibrosis on cardiac diastolic properties. In order to examine this hypothesis I evaluated myocardial interstitial

changes in rat models of hypertensive heart disease with varying functional abnormalities, including increased stiffness without remodeling, remodeling without increased stiffness, and both remodeling and increased stiffness. This chapter, published in the journal *Cardiovascular Research* (Badenhorst et al 2003a) provided clear evidence to support the notion that fibrosis contributes to myocardial stiffness as well as left ventricular dilatation in left ventricular hypertrophy, albeit an effect that is modulated by the quality of myocardial collagen. These data therefore support an important role for pathway F in Figure 41.

With respect to the role of genetic variants that modulate the impact of sympathetic activation on the heart in the development and the progression of heart failure, I restricted my studies to those variants that modulate the function of β-ARs. The Arg16Gly and Gln27Glu polymorphisms of the β_2-AR gene influence the degree of agonist-stimulated receptor downregulation (Green et al 1994) and are hence implicated in heart failure. These polymorphisms are associated with, or show trends for an association with the progression to hospitalization, death or transplantation (Liggett et al 1998; Forleo et al 2004), a reduced exercise capacity (Wagoner et al 2000), and left ventricular functional responses to β-blocker therapy (Kaye et al 2003) (pathway G of Figure 41). However, the mechanisms of β_2-AR genotype effects in heart failure (Liggett et al 1998; Wagoner et al 2000; Kaye et al 2003; Forleo et al 2004) are uncertain. β_2-AR genotype-dependent anti-apoptotic actions (Communal et al 1999) could produce distinct clinical outcomes through the well recognized impact of apoptosis on structural remodeling and cardiac function (Narula et al 1996; Yarbrough et al 2003). Although some studies have reported on a lack of impact of β_2-AR genotypes on baseline LV dimensions and pump function (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004), these data are difficult to interpret for a number of reasons. These studies were cross-sectional rather than prospective analyses, conducted in patients with diverse

cardiac pathologies and performed in patients of whom a proportion were receiving β-AR blocker therapy (Liggett et al 1998; Wagoner et al 2000; Forleo et al 2004). In contrast, some studies have shown β_2-AR genotype-specific effects on LV structure and function after β-AR blocker therapy (Wagoner et al 2000; Kaye et al 2003). However, whether the genetic effects noted in these studies (Wagoner et al 2000; Kaye et al 2003) were determined by independent actions of β_2-AR genotype, or interactions between genotype and the well known effects of β-AR blockers on β-AR function (Esler et al 1997; Bristow 2000), is not clear. As described in chapter 5 of this thesis and as will soon be published in the journal *Pharmacogenomics* (Badenhorst et al *in press*), I found a lack of impact of β_2-AR genotype on left ventricular dimensions and pump function at either baseline or after 6 months of standard medical therapy (but without β-AR blocker therapy) in patients with idiopathic dilated cardiomyopathy (IDC). These data therefore indicate that the relationship between these genotypes and progression to hospitalization, death or transplantation (Liggett et al 1998; Forleo et al 2004); a reduced exercise capacity (Wagoner et al 2000), and left ventricular (LV) functional responses to β-blocker therapy (Kaye et al 2003) as previously described is unlikely to be attributed to an independent effect of genotype on cardiac chamber dimensions and pump function (pathway G of Figure 41).

A common polymorphism of the β_1-AR gene – a substitution of glycine (β_1-Gly389) for arginine (β_1-Arg389) at amino acid 389 – occurs within a Gs-coupling domain (Mason et al 1999). β_1-Arg389 has a much greater ability to couple to adenylyl cyclase than does β_1-Arg389 (Mason et al 1999) and hence may either predispose to heart failure (Figure 41, pathway H) or contribute toward the progression of heart failure (Figure 41, pathway I). Indeed, transgenic mice with the β_1-Arg389 genotype develop depressed ventricular function and pathological fibrosis (Mialet Perez et al 2003).

However, the relationship between the Gly389Arg polymorphism of the β_1-AR gene and human heart failure has been inconsistent (Tesson et al 1999; Iwai et al 2002; Small et al 2002; Mialet Perez et al 2003; Covolo et al 2004; Forleo et al 2004). Nevertheless, the β_1-Arg389 variant, when present with a polymorphic α_{2c}-AR gene variant, has been reported to increase the risk for developing heart failure in African-Americans (Small et al 2002). A common coding polymorphism of the gene for the α_{2C}-AR – a deletion of four consecutive amino acids (Del322-325) – results in a substantial loss of agonist-mediated receptor function in transfected cells (Small et al 2000). The presence of the α_{2C}-AR Del322-325 polymorphism may therefore result in enhanced norepinephrine release and hence increase the risk for heart failure. Although it appears that the β_1-AR variant, when present with a polymorphic α_{2c}-AR gene variant increases the risk for heart failure in a group of African-Americans, this relationship was identified in a small study sample (n=78 cases and n=84 controls)(Small et al 2002). Moreover, in groups of European descent an interaction between these two gene variants has not been confirmed (Metra et al 2006). Thus, the utility of genotyping for the β_1-AR Gly389Arg and α_{2C}-AR Del322-325 variants in predicting the development, severity or progression of heart failure, particularly in groups of African ancestry is uncertain. In chapter 6 I report on the relationship between the β_1-AR Gly389Arg and α_{2C}-AR Del322-325 variants and the presence, severity and progression of IDC in black South Africans. In this study I demonstrated that black South Africans all appear to be homozygous for the α_{2C}-AR Del322-325 variant, and that the Gly389Arg polymorphism of the β_1-AR gene neither predicts an increased risk for IDC (pathway H of Figure 41), nor is associated with disease severity or progression (pathway I of Figure 41) of IDC in this population group. These data therefore do not support a role for α_{2C}-AR Del322-325 and β_1-AR Gly389Arg genotyping in predicting the development or severity of IDC in black South Africans.

In conclusion therefore, data described in the present thesis provide evidence that supports the notion that chronic β-AR activation promotes the progression from compensated left ventricular hypertrophy to heart failure; that the principle mechanism mediating this effect is through chamber dilatation; and that the potential mechanisms responsible for this adrenergic-induced chamber dilatation include activation of enzymes that degrade myocardial collagen, including MMP-2, and increases in the amount of myocardial collagen of a subtype that is more susceptible to digestion (non-cross-linked collagen). Further, this thesis provides evidence obtained in a prospective study in patients with IDC to indicate that that the relationship between functional β_2-AR genotypes and the progression to hospitalization, death or transplantation (Liggett et al 1998; Forleo et al 2004); a reduced exercise capacity (Wagoner et al 2000), and left ventricular functional responses to β-blocker therapy (Kaye et al 2003), is unlikely to be attributed to an independent effect of genotype on cardiac chamber dimensions and pump function. Moreover, this thesis provides evidence to indicate that genotyping black South Africans for α_{2C}-AR Del322-325 and β_1-AR Gly389Arg variants is of little use when predicting the development or severity of IDC in this population group.

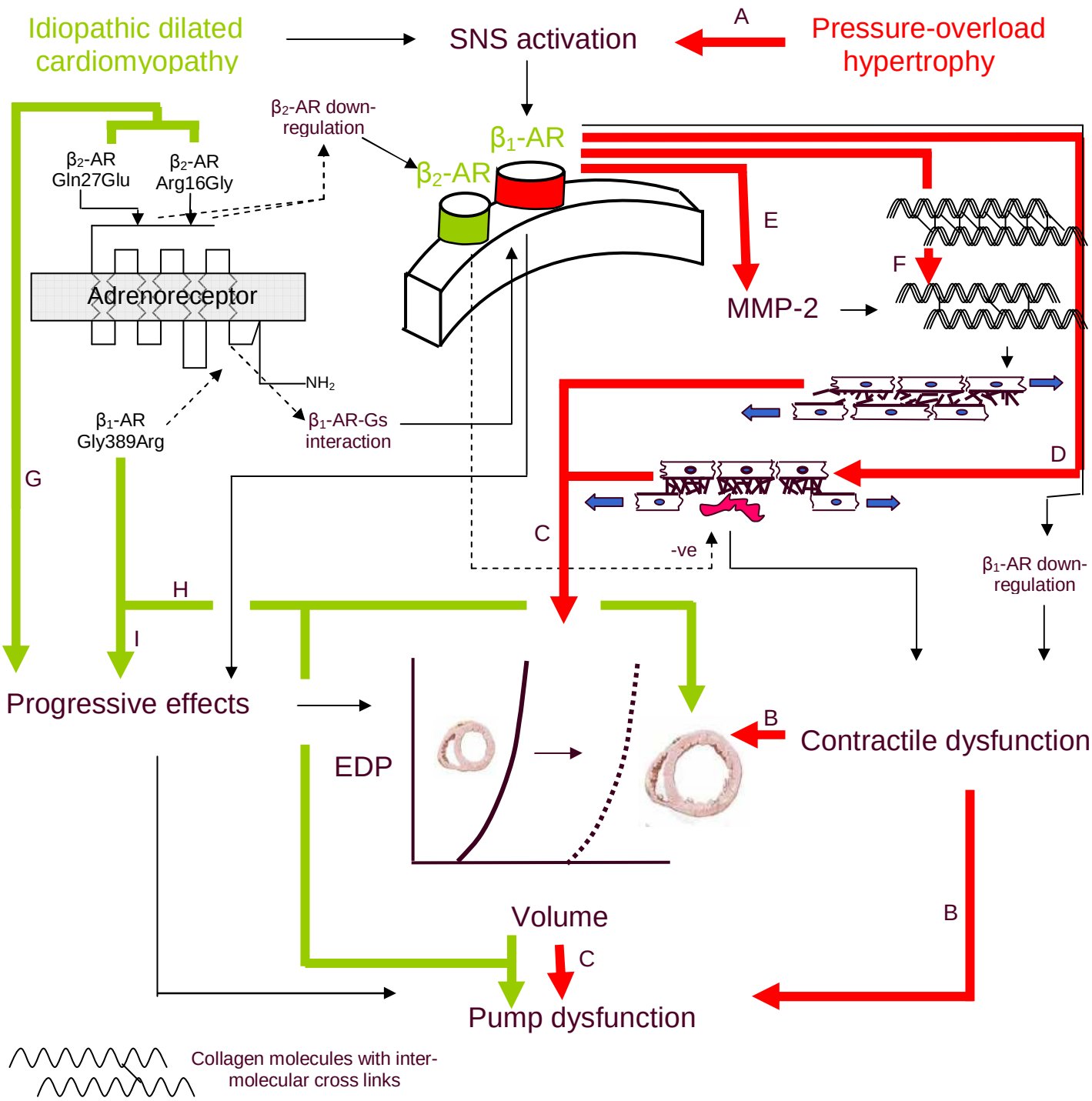


Figure 41. Summary of critical cellular pathways activated with chronic sympathetic nervous system stimulation in cardiac pathology and the hypotheses tested in the present thesis (dark coloured lines). The thick red lines show hypotheses tested in pressure overload hypertrophy and the thick green lines the hypotheses tested in idiopathic dilated cardiomyopathy. A-to-I are used to explain the diagramme in the text. The meaning of cartoons are provided in chapter 1, figures 2-to-6. SNS activation, sympathetic nervous system activation; MMP-2, matrix metalloproteinase-2; EDP, end diastolic pressure; $\beta_{1/2}$-AR, $\beta_{1/2}$-adrenoreceptor; Gln27Glu, substitution of glutamine for glutamic acid at amino acid position 27 of the β_2-AR gene; Arg16Gly, substitution of arginine for glycine at amino acid position 16 of the β_2-AR gene; Gly389Arg, substitution of glycine for arginine at amino acid position 389 of the β_1-AR gene.

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