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IMPACT OF OBESITY ON THE SUSCEPTIBILITY OF THE MYOCARDIUM TO HYPERTENSIVE AND ADRENERGIC-INDUCED APOPTOSIS

Leanda Vengethasamy

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg 2009

DECLARATION

I, Leanda Vengethasamy declare that this dissertation is my own work with all assistance acknowledged. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Leanda Vengethasamy

……. day of …… 2009

I certify that the studies in this dissertation have been approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg. The ethics approval numbers are 2006/59/04 and 2006/99/03.

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Leanda Vengethasamy

……. day of …… 2009

…….. .....……..

Professor Angela Woodiwiss (supervisor) Dr Olebogeng Majane (co-supervisor)

……. day of …… 2009 ……. day of …… 2009

PRESENTATIONS

The following is a list of presentations offered in support of this dissertation.

Vengethasamy L, Majane OHI, Woodiwiss AJ and Norton GR. An obesity-inducing diet promotes cardiomyocyte apoptosis in cardiac pathology. Poster presented at the 36th Annual Congress of the Physiology Society of Southern Africa (PSSA), University of Pretoria, 16-19 September, 2008.

Vengethasamy L, Majane OHI, du Toit E, Badenhorst M, Woodiwiss AJ and Norton GR. A diet that promotes obesity potentiates adrenergic-induced cardiomyocyte apoptosis in rats. Presented at the 35th Annual Congress of the Physiology Society of Southern Africa (PSSA), Muldersdrift, Gauteng, 9-12 September, 2007.

ABSTRACT

Excess adiposity may increase the risk of heart failure through interactions with conventional risk factors. As cardiomyocyte apoptosis may be an important mechanism responsible for the development of heart failure the aim of the present study was to determine whether obesity enhances a) the increased cardiomyocyte apoptosis that accompanies pressure-overload hypertrophy and b) sympathetic-induced cardiomyocyte apoptosis. The impact of dietary-induced obesity on cardiomyocyte apoptosis was studied in elderly spontaneously hypertensive rats (SHR) and age-matched (8-9 months of age at the beginning of the study) Wistar Kyoto rats (WKY) after a 5 month feeding period and in young WKY rats (1 month of age at the beginning of the study) receiving either isoproterenol (ISO) or the vehicle (saline) for 5 days at the end of the feeding period. To induce obesity rats were fed a diet that promotes hyperphagia. At the end of the feeding period echocardiography was performed. Cardiac myocyte apoptosis was assessed using a TUNEL staining technique. Rats receiving the obesity-inducing diet had increases in body weight and visceral fat content. No further changes in systolic blood pressure were observed in rats during the feeding period. SHRs on the obesity-inducing diet had an increased left ventricular end-diastolic diameter and a decreased endocardial fractional shortening. As compared to lean rats, dietary-induced obesity resulted in an increase in the percentage of cardiomyocytes that were apoptotic in SHRs ($3.4\pm0.5\%$, $p<0.005$ vs all other groups) and in WKYs receiving ISO ($0.35\pm0.05\%$, $p<0.05$ vs Control-ISO and $p<0.01$ vs Control-saline and Diet-saline groups). In conclusion, obesity was associated with cardiomyocyte apoptosis through an interaction with pressure-overload hypertrophy

and excessive sympathetic activation. These findings provide insights into the potential mechanisms through which obesity may promote the development of heart failure.

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TABLE OF CONTENTS

Page

DECLARATION……ii

PRESENTATIONS……iii

ABSTRACT……...iv

ACKNOWLEDGEMENTS……...vi

TABLE OF CONTENTS……..vii

LIST OF FIGURES……. viii

LIST OF TABLES..... x

LIST OF ABBREVIATIONS……xi

PREFACE…….……... xiv

Chapter 1: Obesity and the heart: Critical review of the potential role of obesity in promoting the development of heart failure…...……....1

Chapter 2: Methods…….30

Chapter 3: Results……...50

Chapter 4: Discussion……74

Conclusion……88

References…….89

LIST OF FIGURES

2.1 Study designs employed to assess the impact of excess adiposity on the adverse effects of pressure-overload hypertrophy and sympathetic activation……...32

2.2 Photographs of the experimental setup used to measure tail-cuff systolic blood pressure and an example of a recording obtained…….37

2.3 Photographs of the echocardiogram used to assess left ventricular structure and function and a representative M-mode image……...39

2.4 Images of cross-sections of myocardial tissue stained with van Gieson's stain showing tissue fibrosis and normal portions of the heart…….….44

2.5 Images of cross-sections of myocardial tissue stained for apoptotic nuclei……...48

3.1 Changes in body weight over time (5 months) in young WKY rats……...52

3.2 Visceral fat weight in young WKY rats……..53

3.3 Changes in body weight over time (5 months) in elderly SHR and age-matched WKY control rats……..55

3.4 Visceral fat weight and body weight normalized to tibial length in elderly SHR and age-matched WKY control rats…….56

3.5 Systolic blood pressures in elderly SHR and age-matched WKY rats……...59

3.6 Left ventricular weight normalized to tibial length in elderly SHR and age-matched WKY control rats…….64

3.7 Effects of acute isoproterenol administration on left ventricular systolic chamber and myocardial function as well as heart rate in young WKY rats……..67

3.8 Left ventricular end diastolic diameter and systolic chamber function on elderly SHR and age-matched WKY rats……...68

3.9 Percentage cardiomyocyte apoptotic nuclei and pathological score after 5 days of daily isoproterenol or saline administration in young WKY rats……71

3.10 Percentage cardiomyocyte apoptotic nuclei and pathological score in elderly SHR and age-matched WKY control rats……..73

LIST OF TABLES

3.1 Comparison of body morphometry in young WKY rats……54

3.2 Comparison of cardiac morphometry in young WKY rats subsequently receiving ISO or the saline vehicle…….61

3.3 Comparison of cardiac morphometry in elderly SHR and age-matched WKY rats……...62

3.4 Left ventricular dimensions in young WKY rats……65

3.5 Left ventricular dimensions and myocardial function in elderly SHR and age-matched WKY rats……....69

LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
Ang II	angiotensin II
ANOVA	analysis of variance
ATP	adenosine triphosphate
β	beta
BMI	body mass index
BW	body weight
Ca^{2+}	calcium ion
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
ED	end diastolic
ES	end systolic
FS_{end}	endocardial fractional shortening
FS_{mid}	midwall fractional shortening
GTP	guanosine triphosphate
HbA_{1c}	glycosylated haemoglobin
H & E	haemotoxylin and eosin
h/r	wall thickness to radius ratio
HR	heart rate
HRP	horseradish peroxidase
HW	heart weight

ISO	isoproterenol
kg/m	kilogram per metre
kJ	kilojoule
LV	left ventricle
LVEDD	LV end diastolic diameter
LVESD	LV end systolic diameter
LVM	LV mass
LVW	LV weight
mg	milligram
mm Hg	millimeters of mercury
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
PBS	phosphate buffered saline
PWT	posterior wall thickness
RAAS	renin-angiotensin-aldosterone system
ROS	reactive oxygen species
SBP	systolic blood pressure
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
SSC	saline-sodium citrate
TdT	terminal deoxynucleotidyl transferase
TUNEL	terminal uridine deoxynucleotidyl transferase dUTP nick end-labeling
WKY	Wistar Kyoto

ZDF	Zucker Diabetic fatty rat

PREFACE

The prevalence of obesity is increasing in both developed and developing countries. There is therefore an urgent need to determine the mechanisms related to obesity-induced cardiovascular disease. Although epidemiological studies have shown that obesity may have adverse effects on the myocardium independent of conventional cardiovascular risk factors, whether these effects are truly independent of the myocardial effects of hypertension and sympathetic nervous system activation, which coincide with obesity, needs further elucidation.

The present dissertation was designed to determine whether obesity enhances the increased cardiomyocyte apoptosis that accompanies pressure-overload hypertrophy and sympathetic activation. In this dissertation I report on the effects of a 5 month exposure to an obesity-inducing diet in the spontaneously hypertensive rat on cardiomyocyte apoptosis. I also explored the interaction between obesity and enhanced adrenergic activity induced by isoproterenol, a beta-adrenergic agonist administered for 5 days to obese rats. The outcomes of the current dissertation suggest that although dietary-induced obesity has no impact on systolic blood pressure or glycosylated haemoglobin, it could enhance the detrimental effects of hypertension and sympathetic activation on the heart. These data therefore provide insights into the mechanisms of the deleterious effects of obesity on cardiac structure and function.

In the introductory chapter to this dissertation I have described the evidence that supports an adverse effect of obesity on cardiac structure and function in clinical and animal studies. Furthermore, I have reviewed the literature on hypertension and

sympathetic nervous system activation as risk factors associated with obesity and the potential detrimental effects associated with hypertensive hypertrophy and sympathetic activation including cardiomyocyte apoptosis. I have subsequently described the methodology employed and the results of this dissertation in chapters 2 and 3 respectively. In the final chapter of this dissertation I have summarized the findings of the present research and outlined the study limitations. Furthermore I have suggested potential mechanisms which may play a role in the outcomes of the present findings.

Chapter 1

Obesity and the heart: Critical review of the potential role of obesity in promoting the development of heart failure.

1.0 Introduction

Traditionally, heart failure is considered to occur as a consequence of myocardial ischaemia and/or infarction, sustained haemodynamic overloads (through afterload [hypertension] or preload [renal failure, thiamine deficiencies, etc] effects), myocardial inflammation (myocarditis), valvular heart disease, pericardial disease, endocrine disorders, as well as a variety of hereditary disorders affecting the myocardium and ultimately producing a decline in the pump capacity (Kannel 1989) or an impaired filling (diastolic heart failure) (Setaro et al 1990, Davies et al 1992, Kono et al 1992). Heart failure is a disorder that can affect up to 20/1000 people in the general population (Schocken et al 1992) and 80/1000 people older than 65 years of age (Mair et al 1996). It is a progressive condition that contributes to a substantial proportion of morbidity and mortality (Cowie et al 2000, Mosterd et al 2001) and from the time of diagnosis, survival rates are comparable with those of malignancies (Lenfant 1994, Stewart et al 2001, Hobbs 2004). Heart failure is a condition that impairs quality of life more than any other chronic disease (Hobbs et al 2002). Thus, there is no question that novel mechanisms responsible for heart failure should continue to be sought and as a consequence novel therapeutic approaches that reduce morbidity, mortality and improve the quality of life of patients with heart failure will continue to emerge.

The past decade has heralded an impressive increase in research attempting to elucidate the pathophysiological mechanisms responsible for the progressive decline in cardiac function in heart failure, mechanisms which have culminated in novel therapeutic approaches (Hunt et al 2001). However, there are still many aspects of heart failure and

causes of heart failure where the pathophysiological mechanisms still require further elucidation. One potential cause of heart failure, the pathophysiological effects of which remain obscure, is obesity. In this regard there is now substantial evidence to indicate that independent of conventional cardiovascular risk factors, and in the absence of obvious causes of myocardial damage, such as myocardial infarction; excess adiposity, as indexed by body mass index (BMI), is associated with an increased risk of heart failure (Hubert et al 1983, Kenchaiah et al 2002). These data are of great concern, as the prevalence of obesity has achieved epidemic proportions in both developed and in developing countries (Bourne et al 2002, Flegal et al 2002, Ogden et al 2006). In the United States of America, the prevalence of obesity in 2004 was ~32% and in South Africa, the prevalence of obesity prior to 2002 was estimated to be 7.5% in men and 30.0% in women (Puoane et al 2002, Ogden et al 2006). Although these figures may not represent trends at the present time, it is likely that prevalence rates are increasing rather than decreasing and hence that these figures may under- rather than overestimate the prevalence of obesity. Consequently, the emerging epidemic of obesity could translate into an increasing prevalence of obesity-mediated heart failure. In response to this, it may be argued that there is an urgent need to better understand the mechanisms responsible for the relationship between obesity and heart failure.

In the present dissertation I have hypothesized that the adverse effects of excess adiposity may increase the risk of heart failure through myocardial effects that occur through interactions, or synergistic effects with conventional cardiovascular risk factors. In this dissertation, I explored whether obesity interacts with hypertensive left ventricular hypertrophy to promote further hypertrophy and hence excessive cardiomyocyte

apoptosis, a well recognized mechanism which may be responsible for cardiac dysfunction in heart failure (Cheng et al 1996, Abbate et al 2003, Olivetti et al 1997, Frustaci et al 1999, Kocher et al 2001, Li et al 1997). Thus, in the introductory chapter to the present dissertation, I will first critically review the evidence to suggest that obesity promotes myocardial dysfunction in-part independent of conventional cardiovascular risk factors. I will subsequently describe the evidence that supports a hypothesis that interactions between excess adiposity and hypertensive cardiac hypertrophy may promote excessive myocardial damage.

2.0 Can obesity-associated heart failure be attributed to adverse effects on the myocardium that are independent of conventional cardiovascular risk factors?

From an epidemiological perspective, although BMI is associated with heart failure independent of conventional cardiovascular risk factors such as hypertension, diabetes mellitus or dyslipidaemia or independent of coronary artery events (Kenchaiah et al 2002), a role for these risk factors or for coronary artery disease is not excluded by this evidence. Indeed, assessments of these risk factors or of coronary artery events in an epidemiological context does not necessarily account for temporal variations in risk factors over time or for sub-clinical coronary artery changes.

Importantly, there is substantial evidence to indicate that excess adiposity is a risk factor for the development of hypertension, diabetes mellitus or dyslipidaemia (Colditz et al 1995, Perry et al 1995, Mokdad et al 2003, Skarfors et al 1991, Huang et al 1998) and

hence when obesity occurs, it is difficult to segregate the impact of these risk factors from direct myocardial effects of obesity when assessing the role of obesity in heart failure. Indeed, hypertension and diabetes mellitus may produce primary cardiomyopathies that may progress to congestive heart failure (Tsotetsi et al 2001, Norton et al 1996, Norton et al 1997, Norton et al 2002, Forcheron et al 2009). Alternatively obesity-induced risk factors may promote the development of coronary artery disease, which in-turn could result in an underlying cardiomyopathy of ischaemic origin. In this regard, the role of hypertension, diabetes mellitus and dyslipidaemia in mediating coronary atheroma and ischaemic heart disease is well recognized (Megnien et al 1996, Jensen et al 2000, Jeppesen et al 1998, Galla et al 2007, Moreno et al 2000, Barrett-Connor et al 1991). Hence obesity-induced coronary atheromatous changes may promote the development of an ischaemic cardiomyopathy through a number of cardiovascular risk factors. Alternatively, obesity may promote the development of coronary heart disease independent of blood pressure and cholesterol effects (Lakka et al 2002, Kim et al 2006) by increasing the chances of endothelial dysfunction, a major pathophysiological change responsible for coronary atherosclerosis (Al Suwaidi et al 2001). The mechanisms of this effect remain obscure. Importantly, however the risk of myocardial infarction and ischaemic heart disease increases with an increasing BMI (Yusuf et al 2005, Chen et al 2006).

Despite the difficulties in segregating the potential role of conventional cardiovascular risk factors or sub-clinical coronary artery disease from obesity-induced direct myocardial effects, there is nevertheless emerging evidence to indicate that obesity is capable of producing myocardial changes that are independent of either conventional

cardiovascular risk factors or underlying coronary artery disease. Hence obesity-induced myocardial changes could promote the development of heart failure. This evidence shall be reviewed in subsequent sections.

2.1 Obesity induces cardiac enlargement which may promote the development of heart failure.

There is unequivocal clinical evidence to indicate that obesity promotes cardiac chamber enlargement and hypertrophy. In this regard, there is also some evidence to suggest that cardiac chamber enlargement and hypertrophy may antedate the development of cardiac dysfunction or heart failure. The following describes the evidence to support these notions.

With respect to the impact of excess adiposity on heart size, body size is the most consistent factor associated with left ventricular mass (LVM) (Lauer et al 1991, Lauer et al 1992, de Simone et al 1992, Gottdiener et al 1994, Urbina et al 1995, Gardin et al 1995, Sherif et al 2000, Lorber et al 2003, Fox et al 2004). The prevailing hypothesis is that increases in body size augment heart size through changes in loading conditions on the heart. Indeed, increases in blood volume associated with excess adiposity result in an enhanced cardiac output and stroke volume (Stoddard et al 1992, Messerli et al 1983). The chronic volume overload mediated by obesity results in an increase in LV filling volumes and thus an enhanced preload and myocardial oxygen demand on the heart (Peterson et al 2004a). The compensatory change that occurs is thus cardiomyocyte growth which is intended to reduce wall stress and myocardial oxygen demand by

increasing the wall thickness of the heart. If wall thickness does not increase in keeping with increments in filling volumes, LV wall stress may increase (Alpert et al 1995a, Berkalp et al 1995, Zarich et al 1991, Nakajima et al 1985), the consequence potentially being a failing myocardium.

There is no doubt that severe obesity is associated with a cardiomyopathy attributed to chronic volume overload which is characterized by LV dilatation, an increased LV wall stress, and compensatory LV hypertrophy (Alpert et al 2001). Nevertheless, there is considerable dispute as to whether the cardiac enlargement noted in less severe forms of obesity is associated with increases in wall thickness which are considered to be out of keeping or in keeping with the dimension changes in the heart. This is an important concept as excessive increases in wall thickness may also contribute to an increased myocardial oxygen demand and thus predispose to excessive cardiovascular events. Indeed, an increased wall thickness to internal dimension ratio, which is called concentric hypertrophy, is associated with a greater mortality than cardiac hypertrophy where the increased wall thickness change is in-keeping with the internal dimension change (eccentric hypertrophy) (Krumholz et al 1995, Koren et al 1991). What is the evidence to support the notion that concentric or eccentric LV hypertrophy occurs in obesity?

Earlier studies suggest that the predominant effect of obesity on LV structure is eccentric LV hypertrophy (LV end diastolic diameter increases in proportion to wall thickness with no change in relative wall thickness) (Messerli et al 1983, Messerli et al 1982, Lauer et al 1992, Gottdiener et al 1994, de Simone et al 1994). This notion has been supported by some recent studies also showing a lack of impact of body size on

relative wall thickness despite an increase in LVM (Kizer et al 2004, Fox et al 2004). However, other recent studies indicate that adiposity may be associated with a relatively greater increase in LV wall thickness as compared to LV end diastolic diameter with an increased relative wall thickness (concentric LV remodeling and concentric LV hypertrophy) being the primary consequence (Mensah et al 1999, Gutin et al 1998, Avelar et al 2007, Peterson et al 2004, Wong et al 2004, Woodiwiss et al 2008). The uncertainty as to whether overweight or obesity promotes primarily eccentric or concentric LV hypertrophy in these studies may relate in some studies to the use of small study samples (Messerli et al 1983, Messerli et al 1982, Mensah et al 1999, Gutin et al 1998, Peterson et al 2004, Wong et al 2004), non-random recruitment approaches (Messerli et al 1983, Messerli et al 1982, Gottdiener et al 1994, de Simone et al 1994), the assessment of study groups with a limited blood pressure range (Lauer et al 1991, Lauer et al 1992), the use of a high proportion of previously treated participants (~44-90% of participants) (Gottdiener et al 1994, Kizer et al 1994, Fox et al 1994), the assessment of adiposity indices employed as discrete traits (Messerli et al 1983, Messerli et al 1982, Lauer et al 1991, Lauer et al 1992, Gottdiener et al 1994, de Simone et al 1994, Fox et al 2004, Avelar et al 2007, Peterson et al 2004, Wong et al 2004), and the recruitment of patients with severe obesity (body mass index>35 kg/m^2) only (Avelar et al 2007). Further, in some studies showing relations between excess adiposity and relative wall thickness, adjustments were not made for confounders (Mensah et al 1999, Gutin et al 1998, Peterson et al 2004). Thus, there are many reasons to explain the uncertainty that exists regarding the impact of obesity on LV geometry. Nevertheless, more recent evidence obtained by our group, evidence which addresses all of the

preceding concerns indicates that obesity is indeed associated with concentric LV remodeling and hypertrophy (Woodiwiss et al 2008). However, it is also possible that obesity promotes the progression from concentric LV hypertrophy to chamber dilatation and increases in LV chamber diameters. What is the evidence that either LV hypertrophy or chamber dilatation could promote the development of heart failure?

There is now some evidence to indicate that an increase in cardiac chamber dimensions (cardiac dilatation) in people without clinical evidence of heart failure is a precursor of LV dysfunction and clinical heart failure (Gaudron et al 1993, Pfeffer et al 1993, Vasan et al 1997). These data are based on the notion that cardiac dilatation may increase myocardial wall stress and reduce pump function if appropriate increases in wall thickness do not accompany the increases in chamber dimensions. Furthermore, there is some evidence to suggest that LV hypertrophy is a risk factor for heart failure. Indeed, in the Cardiovascular Health Study, LV hypertrophy at baseline was associated with a decrease in LV systolic chamber function as indexed by ejection fraction, and a decrease in LV diastolic function, as indexed by transmitral velocity measurements. Importantly, these associations were independent of conventional cardiovascular risk factors, including conventional blood pressure, as assessed over a 5 year follow-up period (Drazner et al 2004). Thus, LV hypertrophy predicts the transition to cardiac dysfunction. What is not clear from any of these studies (Gaudron et al 1993, Pfeffer et al 1993, Vasan et al 1997, Drazner et al 2004) nevertheless, is to what extent the increases in cardiac chamber dimensions and LV hypertrophy could be attributed to obesity. Indeed, if obesity is associated with concentric LV hypertrophy then it is unlikely that obesity-induced increases in LV chamber dimensions will promote the transition to heart failure.

However, if concentric LV hypertrophy prevails, the possibility that excessive increases in wall thickness predispose to increases in myocardial oxygen demand need to be considered. Thus, at present it is uncertain whether obesity-induced increases in LV chamber dimensions or LV hypertrophy can promote the progression to cardiac dysfunction. Nevertheless, there is some direct evidence to indicate that obesity can promote chamber or myocardial dysfunction independent of conventional cardiovascular risk factors. Both clinical and pre-clinical evidence is available to support these notions.

2.2 Obesity and cardiac dysfunction in clinical studies

The question of whether excess adiposity promotes cardiac dysfunction independent of conventional cardiovascular risk factors has attracted considerable interest. A number of clinical studies have reported on the adverse effects of obesity on diastolic cardiac function (Pascual et al 2003, Scaglione et al 1992, Chakko et al 1998, Zarich et al 1991, de Divitiis et al 1981, Stoddard et al 1992, Alpert et al 1995a, Alpert et al 1995b, Sasson et al 1996, Mureddu et al 1996, Mureddu et al 1998). In addition diastolic dysfunction has been reported to occur in uncomplicated obesity i.e. independent of diabetes, hypertension and dyslipidaemia (Iacobellis et al 2002, Iacobellis et al 2004). Evidence suggests that one potential cause for diastolic dysfunction in obesity may be an increase in epicardial fat (Iacobellis et al 2007). Obesity has been shown to be associated with an increase in epicardial fat thickness and weight loss may significantly reduce epicardial fat thickness (Iacobellis et al 2003, Iacobellis et al 2007, Iacobellis et al 2008). Thus epicardial fat may be a cardiovascular risk factor and has indeed been

associated with impaired diastolic filling (Iacobellis et al 2007). However the role of epicardial fat is not supported by all studies (Fox et al 2009). Furthermore these authors suggest that visceral adipose tissue (and other anthropometric measures of obesity) may be a more precise correlate of cardiac structure and function (Fox et al 2009).

However, with the exception of a few studies (Alpert et al 1995a, Scaglione et al 1992), early studies using load-dependent assessments of cardiac chamber function, failed to report on an adverse effect of excess adiposity on cardiac systolic chamber function. In this regard, a number of studies, using indices of chamber rather than myocardial function, indicate that systolic cardiac function is either preserved in obesity (Pascual et al 2003, Zarich et al 1991, Stoddard et al 1992, de Simone et al 1996, Mureddu et al 1996, Berkalp et al 1995), or reduced only as a consequence of increases in cardiac loading conditions (Alpert et al 1995a). Furthermore, weight loss may have no apparent impact on measures of systolic chamber function (Willens et al 2005), although some studies suggest otherwise (Karason et al 1998).

However, more recent clinical studies employing sophisticated technology to study myocardial function (load-independent tissue Doppler indices of myocardial function), have reported on reductions in both systolic and diastolic myocardial function in overweight people even after adjustments for blood pressure, age, gender and LV mass (Peterson et al 2004, Wong et al 2004). Moreover, weight loss mediated by either gastric bypass surgery (Willens et al 2005) or lifestyle intervention (Wong et al 2006) has been demonstrated to result in improvements in myocardial function as assessed using tissue Doppler measurements of myocardial function. However, the use of sophisticated technology to study the effects of weight loss on myocardial function have revealed

discrepant data with respect to the impact of weight loss on cardiac systolic function (Wong et al 2006, Skilton et al 2007). Whilst one study demonstrated beneficial effects of weight loss on systolic myocardial function (Wong et al 2006), the other failed to show similar outcomes (Skilton et al 2007).

2.3 Obesity and cardiac dysfunction in pre-clinical studies

A number of pre-clinical studies have provided evidence to suggest that cardiomyocyte dysfunction occurs in insulin-resistant or obese states. Indeed, the function of the sarcoplasmic reticular Ca^{2+} ATPase enzyme, which is responsible for Ca^{2+} sequestration during relaxation, is impaired in insulin resistant animals (Wold et al 2005). These data provide an explanation for the impaired diastolic function which may occur in obese patients without diabetes mellitus, hypertension or underlying coronary artery disease. Further, decreases in myocardial adrenergic-induced contractile responsiveness may occur in dietary-induced obese animals (Carroll et al 1997). Reduced cardiomyocyte contractile function may occur in dietary-induced obese animals (Relling et al 2006), and in leptin deficient animals with obesity (Dong et al 2006). A decreased cardiomoycyte contractile response to insulin and insulin-like growth factor-1 has been reported on in genetically obese rats (Ren et al 2000). All of these studies would support potential mechanisms for obesity-induced myocardial contractile disturbances. Despite these data however, when assessed *in vivo*, animal models of obesity nevertheless have a preserved pump function (Carroll et al 2006, du Toit et al 2008) even if the same model of obesity produces an impaired pump function as assessed *ex vivo*.

What could explain the impaired contractile function as determined *ex vivo* in animal models of excess adiposity whilst in the same animal models systolic function may be maintained *in vivo*? One potential explanation is that obesity may indeed be associated with myocardial alterations that impair contraction, but at least in the early stages of obesity, compensatory myocardial changes may occur that preserve function. In this respect, obesity is associated with increased circulating fatty acid concentrations and an elevated myocardial fatty acid oxidation (Aasum et al 2008, Mazumder et al 2004). An increased fatty acid availability in the presence of an enhanced fatty acid oxidation, may improve myocardial performance. Indeed, the inclusion of fatty acids in the perfusion media has been shown to improve cardiac performance in the *ob/ob* mice model of obesity (Mazumder et al 2004). This finding may explain a reduced systolic function noted in isolated cardiac myocytes or hearts when assessed in the absence of fatty acids as an energy source in animal models of obesity without hypertension or diabetes mellitus (Dong et al 2006, Relling et al 2006, Ren et al 2000, du Toit et al 2005), although cardiac systolic function as assessed *in vivo* in the presence of circulating fatty acids may be preserved (Carroll et al 2006).

2.4 Obesity and cardiomyocyte apoptosis

There is increasing evidence that cardiomyocyte apoptosis (programmed cell death in the myocardium) may be an important change that contributes toward cardiac dysfunction and heart failure. In this regard, cell loss may precede an impaired ventricular pump function (Olivetti et al 1994). Further, in pressure-overload

hypertrophy, an increased cardiomyocyte apoptosis occurs in decompensated as compared to compensated hearts (Condorelli et al 1999). Evidence of myocyte cell loss (Olivetti et al 1994) and cardiomyocyte apoptosis (Yamamoto et al 2000) is noted in hypertensive patients and an increased cardiomyocyte apoptosis occurs in transgenic models of heart failure (Sarkar et al 2004). Moreover, inhibitors of the caspase enzyme, an enzyme that is central to mediating the apoptotic process, have been demonstrated to reduce cardiomyocyte apoptosis and attenuate the reduction in left ventricular pump dysfunction that accompanies the transition from compensated LV hypertrophy to heart failure (Hayakawa et al 2003, Engel et al 2004).

What is the evidence to suggest that obesity could promote cardiomyocyte apoptosis? In this regard, an increase in the percentage cardiomyocyte apoptosis has been observed in the myocardium of the Zucker Diabetic Fatty (ZDF) rat and in the *ob/ob* mouse (Zhou et al 2000, Barouch et al 2006). However, whether obesity *per se* or other factors mediate excessive cardiomyocyte apoptosis in these animal models of obesity (Zhou et al 2000, Barouch et al 2006), is unclear. First, as these are genetic models of excess obesity, it is difficult to assess whether the genetic modifications increase the susceptibility of the myocardium to excessive cardiomyocyte apoptosis or whether obesity *per se* is responsible for these changes (Zhou et al 2000, Barouch et al 2006). Second, the role of blood pressure changes in mediating excessive cardiomyocyte apoptosis in the ZDF rat needs to be considered. In this respect, it is well recognized that hypertension is associated with an increased cardiomyocyte apoptosis in both animal models (Li et al 1997, Diez et al 1997, Liu et al 2000, Hamet et al 1995, Fortuno et al 1998) and in human hypertension (Gonzalez et al 2002) and that the ZDF rat has an

increased blood pressure (Carlson et al 2000, Nagao et al 2003). Although the *ob/ob* mouse does not have elevated blood pressures and hence excessive cardiomyocyte apoptosis cannot be attributed to blood pressure effects in this model (Mark et al 1999b, Christoffersen et al 2003), both the ZDF and the *ob/ob* mouse models of obesity are associated with diabetes mellitus (Coleman 1978, Corsetti et al 2000). As with hypertension, diabetes mellitus is also associated with increases in cardiomyocyte apoptosis (Bäcklund et al 2004, Ghosh et al 2005, Cai et al 2006). In summary therefore, it is uncertain whether an increased cardiomyocyte apoptosis in animal models of obesity is related to the genetic defects and associated changes produced by these defects, or the effects of blood pressure or diabetes mellitus rather than obesity *per se*. Importantly, no study has reported on the effects of dietary-induced obesity on cardiomyocyte apoptosis. Moreover, no study has excluded the potential role of conventional cardiovascular risk factors in mediating obesity-associated increases in cardiomyocyte apoptosis. Consequently, as part of the present dissertation I assessed the impact of dietary-induced obesity on cardiomyocyte apoptosis in a model of obesity that our group has previously shown to produce no increase in blood pressure and no effect on glycaemic control (du Toit et al 2008).

3.0 Interactions between excess adiposity and conventional cardiovascular risk factors could account for obesity-induced adverse effects on the heart.

As indicated in the introduction to this chapter, obesity is a major determinant of an increased blood pressure and an increased blood pressure could still account for the

relationships between obesity and heart failure in epidemiological studies. What is the evidence to suggest that obesity is a major role player in influencing blood pressure and what evidence could favour a role for hypertension in promoting obesity-induced cardiac dysfunction?

With respect to a role for obesity in mediating increases in blood pressure, there is now substantial evidence from population-based studies, with large study samples (n=10969-15063) in favour of obesity being a major determinant of conventional blood pressure and the development of hypertension (Zhu et al 2005, Harris et al 2000). Indeed, the odds of developing hypertension are ~1.7-3.4 times greater in obese individuals as compared to lean individuals (Harris et al 2000). Further, there is substantial evidence to indicate that weight reduction results in decreases in blood pressure. Indeed, in a meta-analysis of a number of weight reduction studies, with a total sample size of 4874 participants, it is estimated that a decrease in 4.4 mmHg of systolic blood pressure and 3.6 mmHg of diastolic blood pressure will occur for every 5.1 kg of weight loss over 16.5 months (Neter et al 2003). Furthermore, in an alternative study involving 3245 participants, a ~1.4-2.0 fold chance of hypertension remission was reported to occur for every 1 kg decrease in body weight over 9 years (Juhaeri et al 2003).

What evidence could favour a role for hypertension in promoting obesity-induced cardiac dysfunction? Although the majority of studies describing an adverse impact of obesity on myocardial function have attempted to exclude the impact of blood pressure or hypertension by selecting normotensive participants or accounting for blood pressure effects in multivariate adjusted regression models, these approaches still do not necessarily exclude a role for hypertension in mediating obesity-induced myocardial

dysfunction. One possibility that has not been given due consideration is whether obesity interacts with hypertension to promote myocardial dysfunction. Under these circumstances much lower levels of blood pressure may be required to promote adverse effects on the heart in patients whom are overweight or obese, effects which may not be accounted for in multivariate adjusted models. Is there evidence that obesity interacts with hypertension to promote adverse effects on the heart?

Recent evidence in severe obesity (body mass index>35 kg/m^2) suggests that LV hypertrophy is mediated by an enhanced impact of blood pressure on LV growth (Avelar et al 2007). Furthermore, in a large cross-sectional population study conducted in South Africa, the first evidence has emerged to indicate that even mild-to-moderate forms of excess adiposity enhance the impact of conventional or ambulatory blood pressure, and of arterial stiffness (considered as continuous traits) on LV mass index (Norton et al 2009, in press). In contrast to these studies (Avelar et al 2007, Norton et al 2009, in press), in a predominantly middle-aged population sample (Fox et al 2004), and in a randomly selected Framingham sample (Lauer et al 1992), interactions between discrete categories of BMI (lean, overweight and obese) and blood pressure (normal, high-normal and hypertensive) failed to predict LV mass index. In this regard the assessment of blood pressure as a discrete trait could potentially reduce the sensitivity of detecting an interactive effect. Furthermore, in contrast to the South African study (Norton et al 2009, in press), where ~22% of subjects were receiving antihypertensive therapy and ~19% were hypertensive but not receiving therapy, in one study (Fox et al 2004) ~50% of subjects (the majority of hypertensives) were receiving antihypertensive therapy, thus potentially modifying the effects of blood pressure on LV mass index. Further, in the

Framingham Heart Study, by excluding treated hypertensives from the analysis, a very narrow range of blood pressure values was obtained in the participants (Lauer et al 1992), thus potentially limiting the sensitivity of detecting an interactive effect. Thus, there is clinical evidence to suggest that obesity could, at least enhance the adverse impact of blood pressure on cardiac size. Is there evidence to indicate that obesity could promote the deleterious effects of an increased blood pressure on cardiac function?

In this regard although this hypothesis has previously been posed with respect to changes in diastolic function in the heart (Lavie et al 1987, Grossman et al 1991) and for the risk of developing congestive heart failure and cardiac arrythmias (Zhang et al 2000, Morse et al 2005), few studies with small study sample sizes have addressed this question. In this regard, one study has demonstrated that obese hypertensives may have a lower left ventricular pump function (endocardial fractional shortening) than non-obese hypertensives (Grossman et al 1991), but another study suggests that the presence of obesity does not worsen myocardial function associated with hypertension (de Simone et al 1996). Thus, considerably more evidence is required to test the hypothesis that obesity enhances the adverse impact of conventional cardiovascular risk factors on the myocardium. In this regard, in the present dissertation, based on the findings that obesity enhances the impact of blood pressure on cardiac hypertrophy, I hypothesized that obesity could potentiate the development of LV hypertrophy and thus increase the deleterious effects of hypertensive hypertrophy that ultimately mediate the progression to heart failure. In this regard, in the present dissertation I studied the impact of obesity on myocardial apoptosis in hypertensive hypertrophy. Thus, in the subsequent discussion I will review the evidence to indicate that hypertensive cardiac hypertrophy is associated

with excessive cardiomyocyte apoptosis, and the evidence to indicate that cardiomyocyte apoptosis is an important mediator of the progression to heart failure.

4.0 Cardiomyocyte apoptosis in hypertensive hypertrophy: Presence and potential role in contributing to heart failure.

There is ample evidence to suggest that excessive cardiomyocyte apoptosis occurs in pressure-overload hypertrophy. In this regard, it is well recognized that hypertension is associated with an increased cardiomyocyte apoptosis in both animal models (Li et al 1997, Diez et al 1997, Liu et al 2000, Hamet et al 1995, Fortuno et al 1998) and in human hypertension (Gonzalez et al 2002, Yamamoto et al 2000) even prior to the onset of heart failure. Left ventricular hypertrophy is accompanied by cardiomyocyte upregulation of the proapoptotic gene *bax* in chronic pressure-overload in rats (Condorelli et al 1999) and is associated with Gq activation (a class of GTP-binding proteins) which promotes hypertrophic growth and has been shown to induce cardiomyocyte apoptosis (Hirotani et al 2002, Adams et al 1998, Adams et al 2000). As suggested in section 2.4 above, there is increasing evidence that cardiomyocyte apoptosis may be an important change that contributes toward cardiac dysfunction in hypertensive hypertrophy. Indeed, in pressure-overload hypertrophy, a greater degree of cardiomyocyte apoptosis occurs in decompensated as compared to compensated hearts (Condorelli et al 1999). Moreover, inhibitors of the caspase enzyme, an enzyme that is central to mediating the apoptotic process, have been demonstrated to reduce cardiomyocyte apoptosis and attenuate the reduction in left ventricular pump dysfunction that accompanies the transition from

compensated LV hypertrophy to heart failure (Hayakawa et al 2003, Engel et al 2004). What are the potential mechanisms that mediate increases in cardiomyocyte apoptosis in the hypertrophic heart in pressure-overload states? Furthermore, could obesity promote these mechanisms?

5.0 Potential mechanisms that mediate cardiomyocyte apoptosis in pressure-overload hypertrophy: Potential interactions with excess adiposity.

A number of mechanisms have been suggested to mediate excessive cardiomyocyte apoptosis in pressure-overload cardiac hypertrophy, mechanisms that could be enhanced by obesity. In this regard, from the perspective of the present dissertation there are two major hypotheses that need to be considered. First, pressure-overload states increase myocardial wall stress and thus activate a number of downstream pathways that mediate apoptosis. If obesity increases cardiac cavity dimensions in a manner that exceeds the compensatory increase in wall thickness, then these stress-activated pro-apoptotic pathways could be excessively activated and an interaction between hypertension and obesity may contribute toward cardiomyocyte apoptosis.

Second, a major mechanism that is hypothesized as being responsible for the transition to heart failure in pressure-overload hypertrophy is through sympathetic activation, and obesity is well recognized as promoting sympathetic effects. Thus, in the following discussion I will first highlight the cellular pathways responsible for stress-activated cardiomyocyte apoptosis. Second, in subsequent sections I will describe the evidence to suggest that sympathetic activation occurs in pressure-overload states and

that this change may mediate the progression to heart failure through cardiomyocyte apoptosis. Third, I will review the evidence to indicate that sympathetic activation may be further promoted by the presence of excess adiposity.

5.1 Stress-activated cardiomyocyte apoptosis.

Stress effects on the myocardium may be a key factor in the development of myocardial dysfunction in hypertensive heart disease, and this effect may be mediated through an impact on cardiomyocyte apoptosis. Systemic hypertension does indeed result in oxidative stress and the generation of reactive oxygen species (ROS) in the cardiovascular system including in the heart. This effect has been noted in both animal models (Dhalla et al 1996, Suzuki et al 1995, Schnackenberg et al 1999, Nakazono et al 1991, Dobrian et al 2001) and in human hypertension (Russo et al 1998). The generation of ROS in the myocardium may be an important determinant of the development of heart failure as the transition from compensated LV hypertrophy to heart failure is associated with an increase in oxidative stress (Li et al 2002, Singal et al 1982, Singal et al 1993). What is the evidence that the generation of ROS in the myocardium translates into increases in cardiomyocyte apoptosis? In this regard, mechanical stretch of papillary muscles, in association with an increase in superoxide anions (O_2^-), induces cardiomyocyte apoptosis (Cheng et al 1995). Moreover, an increased intracellular ROS in cardiomyocytes is associated with an increase in apoptosis (Siwik et al 1999).

5.2 Sympathetic activation occurs in pressure-overload states and may mediate the progression to heart failure through cardiomyocyte apoptosis.

As indicated in the aforementioned discussion, an alternative mechanism that could account for excessive cardiomyocyte apoptosis in pressure-overload hypertrophy is through activation of the sympathetic nervous system. Indeed there is now increasing evidence in support of a notion that the sympathetic nervous system is critical in the progression from compensated hypertrophy to heart failure and that this effect may be mediated by cardiomyocyte apoptosis.

What is the evidence to suggest that the sympathetic nervous system is critical in the progression from compensated hypertrophy to heart failure? First, increased myocardial norepinephrine concentrations are noted 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) and in spontaneously hypertensive rats prior to the development of heart failure (Veliotes et al 2005). The mechanism of excessive myocardial norepinephrine release in hypertensive hypertrophy appears to be because of a reduced norepinephrine re-uptake as well as an increased sympathetic nervous system activity (Esler et al 1986, Schlaich et al 2003, Rumantir et al 2000). Second, transgenic animal models with a decreased adrenergic activation are protected against the development of 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 1994, Böhm et al 1995), changes that could promote

the development of contractile dysfunction. Fourth, chronic sympathetic activation mediated by exogenous administration of a β-adrenoreceptor agonist promotes the transition from compensated cardiac hypertrophy to pump dysfunction in hypertension (Badenhorst et al 2003, Gibbs et al 2004, Veliotes et al 2005). Last, blockade of β-adrenoreceptors prevents the transition from cardiac hypertrophy to heart failure in hypertension, an effect that occurs independent of blood pressure changes (Chan et al 2004). Based on these lines of evidence it is apparent that hypertensive hypertrophy is associated with excessive myocardial or systemic sympathetic activation; and that attenuation of sympathetic activation in pressure-overload states prevents the transition to heart failure. In addition, further promotion of excessive sympathetic activation in pressure-overload states hastens the transition to pump dysfunction.

What is the evidence that activation of the sympathetic nervous system in pressure-overload states could promote cardiomyocyte apoptosis? Although there is no direct evidence to suggest that this change occurs in pressure-overload states, there is ample evidence to indicate that excessive cardiomyocyte apoptosis may occur in any situation associated with sympathetic activation. Indeed, sympathetic-induced cardiomyocyte apoptosis is a well recognized change (Communal et al 1998, Qin et al 2001, Osadchii et al 2007), an effect that occurs through β-adrenoreceptor activation (Shizukuda and Buttrick 2001, Communal et al 1998).

5.3 Sympathetic activation in obesity.

As indicated in the aforementioned discussion, in the present dissertation I have tested the hypothesis that obesity may interact with pressure-overload cardiac hypertrophy to promote cardiomyocyte apoptosis. This hypothesis is based in-part on the notion that obesity augments pressure-overload hypertrophy and hence potentially enhances the adverse effects on the myocardium that are often associated with pressure-overload states. One such adverse effect of pressure-overload hypertrophy is cardiomyocyte apoptosis. I have also hypothesized in the aforementioned discussion that this adverse effect in pressure-overload states is mediated in-part by sympathetic activation in these states and that obesity could thus, in-part, augment cardiomyocyte apoptosis in pressure-overload states by enhancing sympathetic effects. However, what is the evidence for an impact of obesity on the sympathetic nervous system?

There is now substantial evidence to indicate that an increased sympathetic nervous system activity accompanies either an increased energy intake, a well recognized cause of obesity, and that sympathetic nervous system activity increases in obesity itself irrespective of energy intake. Previously it was thought that sympathetic nervous system activity is reduced with increases in energy intake or with obesity, thus predisposing individuals to weight gain (Peterson et al 1988). However, other studies have dispelled this notion. Indeed, with respect to energy intake, Young and Landsberg (1977) have demonstrated that two days of fasting reduced and overfeeding increased sympathetic nervous system activity. Moreover, O'dea et al (1982) demonstrated that noradrenaline spillover rate and noradrenaline clearance rates increased with an increased energy

intake. Furthermore, muscle sympathetic nervous system activity may increase after oral carbohydrate administration to human subjects (Berne et al 1989). With respect to obesity, there is considerable evidence to suggest that the sympathetic nervous system is activated in this condition (Haynes et al 1997, Haynes et al 1998, Casto et al 1998, Wofford and Hall 2004, Tentolouris et al 2006, Yang and Barouch 2007, Mark et al 1999a). Importantly, noradrenaline spillover and microneurographic techniques (Vaz et al 1997, Grassi et al 1998a) as well as measurements of plasma concentrations and urinary excretion rates of noradrenaline (Troisi et al 1991, Masuo et al 2000) have clearly demonstrated that sympathetic nervous system activity is augmented in obese adults. Furthermore, dietary-induced weight gain is associated with an increase in sympathetic nervous system activity (Gentile et al 2007) and reductions in body weight have been shown to result in a decrease in sympathetic nervous system overactivity in obese subjects (Grassi et al 1998b, Straznicky et al 2005).

A number of mechanisms may explain obesity-related increases in sympathetic nervous system activity. Importantly, sympathetic nervous system activation in obesity may in-part be attributed to the compensatory hyperinsulinaemia that occurs in response to insulin resistance (Hall et al 1996). Because insulin-mediated glucose uptake within central hypothalamic neurons regulates sympathetic activity in response to dietary intake, hyperinsulinemia is thought to provoke sympathetic nervous system stimulation (Young & Landsberg 1980, Landsberg & Young 1982). Indeed, mean urinary noradrenaline excretion is positively correlated with serum insulin concentrations (Troisi et al 1991).

Alternatively, hyperleptinaemia associated with obesity may also mediate excessive sympathetic nervous system activation. Leptin is a peptide hormone produced

and secreted primarily by adipocytes. It is found in excess in obesity (Eikelis et al 2004, Van Dielen et al 2002, Frederich et al 1995) and is present in concentrations in the plasma in proportion to the percentage of body fat (Considine et al 2005, Schwartz et al 1996a). Leptin binds to receptors mainly in the hypothalamus to regulate body weight by reducing appetite and increasing metabolic rate (Mistry et al 1997, Schwartz et al 1996b, Mercer et al 1996). In addition to the role of leptin in the control of food intake and metabolic rate, leptin also increases sympathetic nervous system activity. In this regard, leptin infusion causes an increase in sympathetic nervous system activity via its actions on the ventromedial and dorsomedial hypothalamic regions (Marsh et al 2003). An infusion of leptin increases sympathetic nervous system activity to brown adipose tissue, the kidneys, the hindlimb and the adrenal gland in rats (Haynes et al 1997). In obesity, chronic leptin infusion also increases sympathetic nervous system activity (Rahmouni et al 2005, Corriea et al 2002). However, it is now widely accepted that obese individuals are resistant to the effects of leptin (Münzberg & Myers 2005), including the anorexic and body weight reducing effects of leptin (Rahmouni et al 2005). Although obesity is associated with leptin resistance, leptin may nevertheless induce increases in sympathetic nervous system activity in the obese state. This may be explained by selective leptin resistance i.e. leptin resistance to appetite or weight reducing effects (Rahmouni et al 2005, Correia et al 2002, Münzberg et al 2004, Rahmouni et al 2002), whilst sympathetic nervous system activation may be sustained (Dunbar et al 1997). Indeed, dietary-induced obese mice are resistant to the reductions in food intake and body weight induced by intraperitoneal and intra-cerebroventricular administration of leptin whilst leptin still increases sympathetic nervous system activity (Rahmouni et al 2005).

Although there is considerable evidence for a role for leptin in mediating an increased sympathetic nervous system activity, most of the studies have focused on sympathetic activation to areas such as the kidneys or the hindlimb. If obesity were to augment the adverse effects of pressure-overload hypertrophy on myocardial tissue through sympathetic effects, then evidence for obesity-induced cardiac sympathetic neuronal overactivation needs to be sought. Evidence in favour of this notion is that plasma leptin concentrations are positively correlated with heart rate (Hirose et al 1998) and that even in the presence of increased blood pressures, which would be expected to produce a compensatory decrease in heart rate, a chronic leptin infusion in rats increases heart rate (Carlyle et al 2002). Furthermore, adrenergic blockade abolishes the increase in heart rate that occurs in response to chronic leptin infusion (Carlyle et al 2002). Whether these changes in heart rate in association with leptin are because sympathetic neuronal activation to the heart increases, or whether sympathetic neuronal activation of the adrenal gland occurs, is uncertain. Irrespective of the mechanism involved, obesity is associated with cardiac effects which reflect an enhanced sympathetic nervous system stimulation of the myocardium, and hence through excessive sympathetic neuronal activation, could potentially promote the deleterious effects of pressure-overload hypertrophy on the heart.

6.0 Summary of hypothesis

As indicated in the aforementioned discussion the mechanisms responsible for the strong epidemiological evidence to indicate that obesity induces heart failure, are unclear.

Although some recent studies provide evidence to suggest that obesity may reduce cardiac systolic function independent of conventional cardiovascular risk factors, the possibility that conventional cardiovascular risk factors play a major role in mediating the relationship between obesity and heart failure cannot be excluded. As our group has recently demonstrated that even mild-to-moderate obesity enhances the impact of blood pressure on cardiac hypertrophy (Norton et al 2009, in press), a finding supported by published data in severe obesity (Avelar et al 2007), I hypothesized that obesity could enhance the extent of cardiac hypertrophy in hypertension, a change that ultimately mediates the progression to heart failure. In this regard, in the present dissertation I hypothesized that one potential detrimental effect of hypertensive hypertrophy that could be enhanced by obesity is cardiomyocyte apoptosis. I also hypothesized that obesity may augment the cardiomyocyte apoptosis that occurs in hypertensive hypertrophy by enhancing the impact of sympathetic-induced cardiomyocyte apoptosis.

7.0 Aims

The aim of the present dissertation was therefore to determine whether obesity enhances a) the increased cardiomyocyte apoptosis that accompanies pressure-overload hypertrophy and b) sympathetic-induced cardiomyocyte apoptosis.

Chapter 2

Methods.

2.1 Study groups

The studies described in the present dissertation were approved by the University of the Witwatersrand Animal Ethics Screening Committee (approval numbers: 2006/59/04 and 2006/99/03) and comply with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. To assist the reader figure 2.1 summarizes the overall study design employed to assess the impact of excess adiposity on the adverse effects of pressure-overload hypertrophy or adrenergic activation. These studies are described in sections 2.1.1 and 2.1.2.

2.1.1 Model to assess the impact of excess adiposity on cardiomyocyte apoptosis in pressure-overload hypertrophy.

To assess the impact of obesity on cardiomyocyte apoptosis in pressure-overload hypertrophy, I studied the effect of dietary-induced obesity on cardiomyocyte apoptosis in spontaneously hypertensive rats (SHR) (Figure 2.1). The SHR model was selected as an appropriate model of cardiomyocyte apoptosis associated with pressure-overload hypertrophy, as our group has previously demonstrated marked increases in cardiomyocyte apoptosis as well as norepinephrine concentrations in the coronary effluent of isolated, perfused hearts at 18.5 months of age in SHR as compared to normotensive Wistar Kyoto control rats (WKY) (Veliotes et al 2005). Furthermore, the SHR has previously been shown to be associated with marked cardiomyocyte apoptosis by alternative groups (Li et al 1997, Liu et al 2000, Hamet et al 1995, Diez et al 1997).

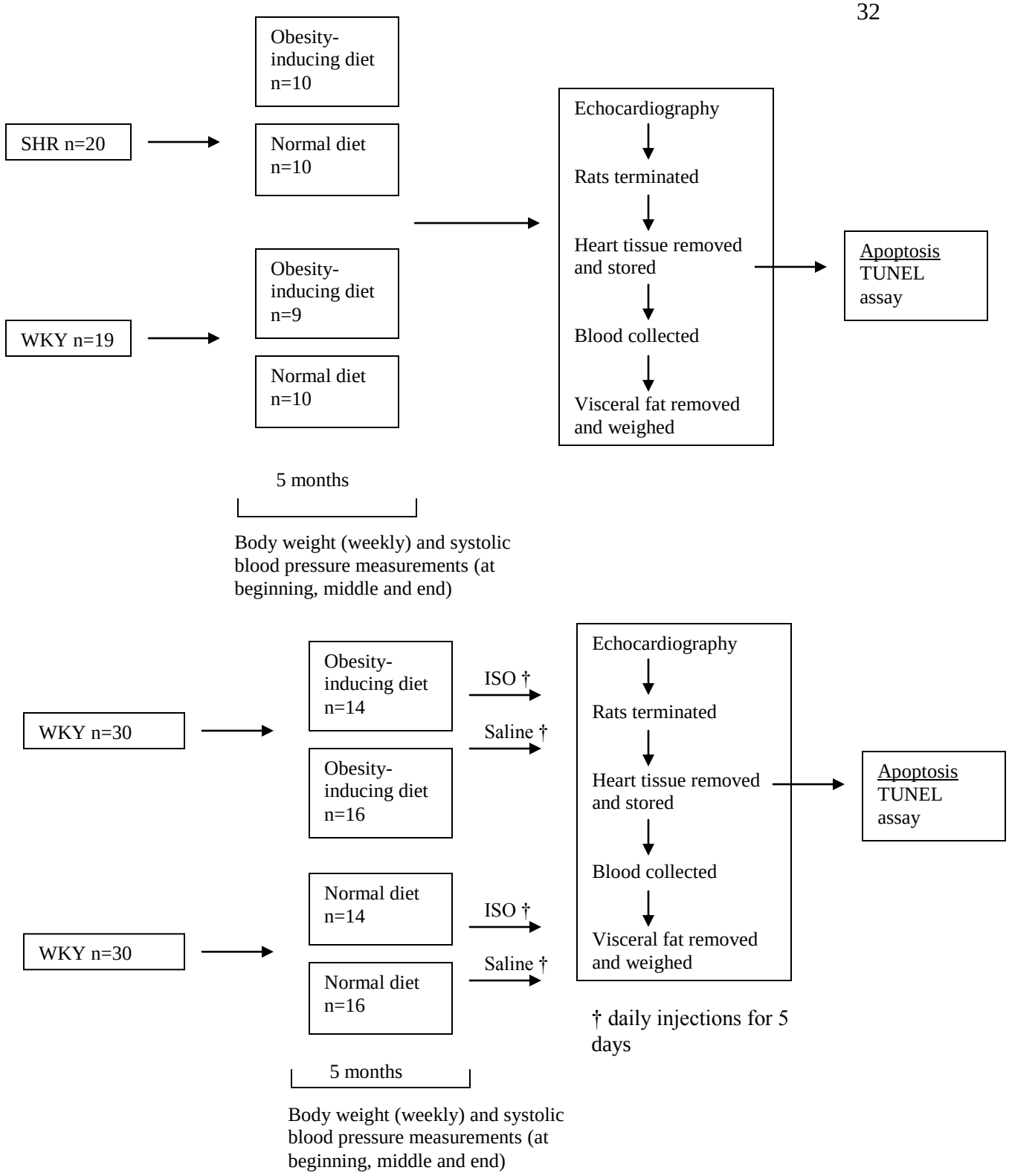


Figure 2.1. Study designs employed to assess the impact of excess adiposity on the adverse effects of pressure overload hypertrophy and sympathetic activation in rats. WKY- Wistar Kyoto; SHR-spontaneously hypertensive rat; ISO- isoproterenol

Thus, 8-9 month old SHR (an age when compensatory LV hypertrophy occurs)(Tsotetsi et al 2001) and age-matched WKY rats were assigned to receive either an obesity-inducing or a control diet for 5 months as described in a subsequent section (section 2.2)(du Toit et al 2008). The myocardium of rats was subsequently studied in rats at 13-14 months of age.

2.1.2 Model to assess the impact of excess adiposity on sympathetic-induced cardiomyocyte apoptosis.

In order to evaluate the impact of obesity on sympathetic (adrenergic)-induced cardiomyocyte apoptosis, I studied the effect of dietary-induced obesity on cardiomyocyte apoptosis provoked by five days of β-adrenoreceptor activation in intact normotensive WKY rats (Figure 2.1). This model of β-adrenoreceptor-mediated cardiomyocyte apoptosis has previously been established in our laboratory (Osadchii et al 2007). Importantly, excessive cardiomyocyte apoptosis does not occur with single doses of isoproterenol, but rather requires repeated administration to induce a measurable cardiomyocyte apoptotic effect (Osadchii et al 2007). We have also demonstrated that this model of β-adrenoreceptor activation, if continued for prolonged periods (five months or more), results in marked cardiac dilatation and pump dysfunction (Woodiwiss et al 2001, Osadchii et al 2007), without inducing cardiomyocyte necrosis (Woodiwiss et al 2001). In this model of cardiomyocyte apoptosis, chronic β-adrenoreceptor activation is induced by daily injections of the β-adrenoreceptor agonist, isoproterenol for five days (0.01mg/kg/day for 3 days and 0.02mg/kg/day for 2 days). Isoproterenol (Sigma-Aldrich,

South Africa) was constituted in sterile saline (0.9% NaCl) in order that each rat would receive subcutaneous injections of ~0.1-0.2 ml volumes daily. Control rats received daily injections of sterile saline at the same volume. In this study, dietary-induced obesity was produced by feeding 150-200 g WKY rats the diet described in a subsequent section (section 2.2) for five months. At the end of the five month feeding period, experimental diet-fed rats and age-matched dietary controls were subsequently assigned to receive either daily isoproterenol injections or the saline vehicle (see Figure 2.1).

2.2 Obesity-inducing diet and the assessment of the degree of adiposity

Rats were obtained from the Central Animal Services at the University of the Witwatersrand where they were in temperature controlled rooms with appropriate light-dark cycles. Animals were allowed free access to food and water 24 hours a day. Standard rat food was supplied by EPOL, South Africa and the experimental diet employed in the present study contained normal rat food, elevated carbohydrates and fats and resembles a Western-type diet (Cordain et al 2005). The experimental diet was produced by soaking 1.32 kg of normal rat food in 812 ml of water to which 280g of sugar and four tins (385g each) of condensed milk (Nestlé, South Africa) were added. The dietary ingredients were then mixed to form a paste and the diet was made up weekly and stored at 4^{o}C.

The experimental diet consisted of weight/weight, 65% carbohydrates derived from maize and simple carbohydrates (sugar), 19% protein derived from soya and fish, and 16% fat derived from milk products and fish. In comparison, the control diet

consisted of 60% carbohydrates derived from maize, 30% protein derived from soya and fish, and 10% fat derived from fish without milk products (du Toit et al 2008). The experimental diet consisted of weight/weight 7.8% saturated fats, 5.5% polyunsaturated fats and 2.6% monounsaturated fats. In contrast, the control diet consisted of weight/weight 1.9% saturated fats, 5.8% polyunsaturated fats and 2.6% monounsaturated fats. This diet is designed to induce hyperphagia (Pickavance et al 1999). Our group has previously shown that differences in micronutrient (vitamins and minerals) intake, produced by dilution of the diet by addition of carbohydrates and fats, does not modify either body size or cardiac function (du Toit et al 2008).

To assess the efficacy of the obesity-inducing diet, body weights were recorded in rats before the start of feeding and then once a week at the same time on the same day of each week until the end of the study. At the end of the study, visceral fat weight was determined post-mortem. To weigh visceral fat, fat was removed from the retroperitoneal space on the posterior wall of the abdominal cavity and from the omentum. To account for intrinsic differences in body size between WKY rats and SHRs in the model to assess the impact of excess adiposity on cardiomyocyte apoptosis in pressure-overload hypertrophy, body weight was also expressed per tibial length at the end of the study.

2.3 Blood pressure measurements

Tail artery systolic blood pressures were measured in SHR and WKY rats at the beginning of the study and at the end of the feeding period and were recorded at the same time of day for each rat on both occasions. A non-invasive tail-cuff technique was used to

measure systolic blood pressure in conscious, restrained rats (Norton et al 1993, Tsotetsi et al 2001). Rats were initially habituated to the restrainers and to the procedure (Norton et al 1993). In order to achieve habituation, rats were placed in restrainers for two hours a day on two separate days, their tails warmed and the tail-cuffs inflated to 200 mmHg for 30 seconds at regular periods five times per day. To obtain measurements of tail artery systolic blood pressures, rats were housed in restrainers for 20 minutes and the tail pre-heated to vasodilate the tail artery. A photoelectric diode model 29 pulse amplifier was used to detect a pulse in the rat tail artery (Figure 2.2). A tail-cuff, coupled to a pressure transducer was placed proximal to the photoelectric diode and inflated until the pulse disappeared. The cuff was then slowly deflated until the pulse reappeared. Whilst deflating the cuff, cuff pressures and the tail pulse were simultaneously recorded on a model R511A recorder, Beckman, USA (Figure 2.2). The cuff pressure at which the pulse was again detected on deflation of the tail-cuff was taken as systolic blood pressure (Figure 2.2). The tail artery systolic blood pressure was measured three times on each occasion and the mean of three measurements was used as the recording for that day.

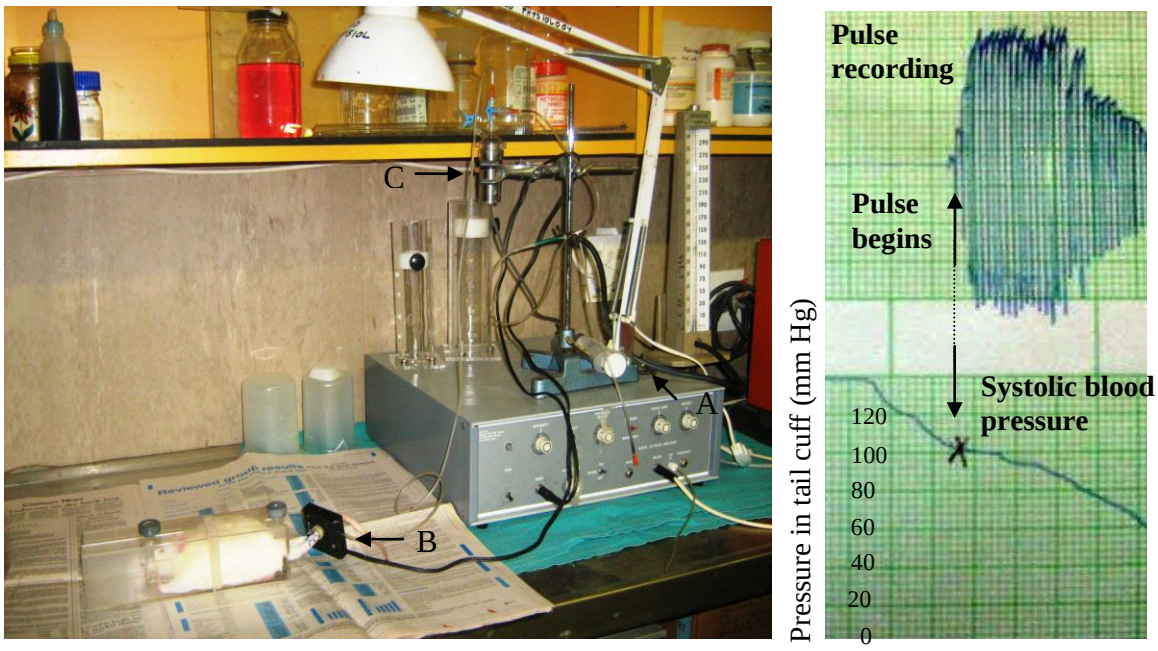


Figure 2.2. Photograph of the experimental setup used to measure tail artery systolic blood pressures in rats (left panel) and an example of a recording obtained (right panel). A - A syringe is coupled to the pressure transducer and tail-cuff. The syringe is used to inflate the tail-cuff; B - The photoelectric diode is used to detect the tail pulse (upper right) and tail-cuff to occlude the tail artery; C - The pressure transducer is used to record the pressure in the tail-cuff (lower right).

2.4 Echocardiography

To determine the impact of obesity on left ventricular dimensions and systolic chamber or myocardial function in anaesthetized SHR and WKY rats, echocardiography was performed at the end of the study using previously described methods (Norton et al 2002, Woodiwiss et al 2001). In rats receiving isoproterenol, echocardiography was performed immediately before and then again 15 minutes after the last dose of isoproterenol. The last dose of isoproterenol was administered 24-hours after the preceding dose. This approach was adopted to determine whether any differences in cardiomyocyte aopoptosis noted between groups could be attributed to differences in haemodynamic responses to isoproterenol. A pilot study established that the isoproterenol-induced increase in systolic function was no different at 5 minutes, 15 minutes or 30 minutes after subcutaneous isoproterenol administration.

In the study to assess the impact of obesity in young WKY rats, 8 rats from each of the saline-treated groups and 10 rats from each of the ISO-treated groups were randomly selected. To perform echocardiography rats were anaesthetized with 50mg/kg of ketamine (Bayer, Edms, Isando, South Africa) and 3mg/kg of xylazine (Bayer, Edms, Isando, South Africa) to ensure immobility during the procedure as previously described (Norton et al 2002). As ketamine increases sympathetic nervous system activity and xylazine decreases sympathetic nervous system activity the overall effect of the anaesthetic on echocardiography measurements is negligible. A high resolution ultrasonic probe (7 MHz paediatric probe) was used to obtain two-dimensional guided M-mode images of the rat left ventricle using an approach previously described (Chung et al 1998,

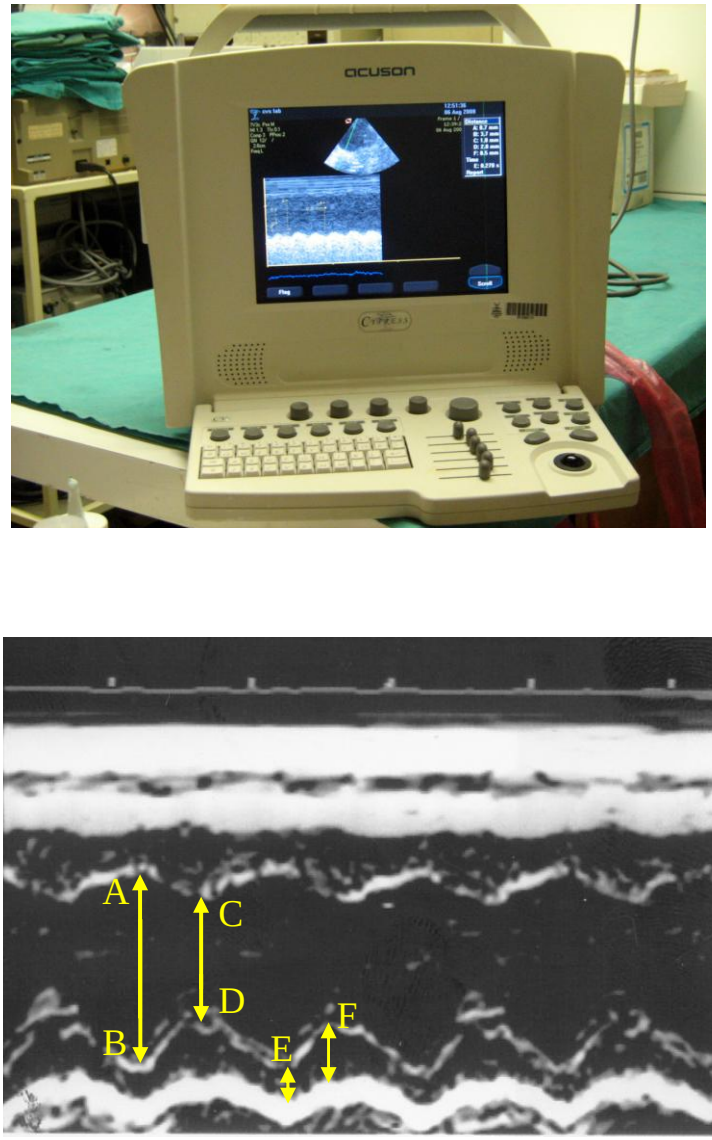


Figure 2.3. Upper panel illustrates the echocardiogram used to assess left ventricular structure and function in rats and the lower panel shows a representative M-mode image used to determine left ventricular structure and function. AB - left ventricular end diastolic diameter; CD - left ventricular end systolic diameter; E - left ventricular end diastolic posterior wall thickness; F - left ventricular end systolic posterior wall thickness.

Norton et al 2002) (Figure 2.3) on an Accuson echocardiograph (Siemens Medical Division USA, Inc). Care was taken to ensure that an M-mode image was obtained at the level of the papillary muscles. As the right ventricular cavity was difficult to visualize in concentrically remodelled SHR, septal wall thickness values were not determined. However, left ventricular end diastolic and end systolic internal diameter and posterior wall thickness values were determined (Figure 2.3). Left ventricular internal dimensions during systole and diastole were measured using the American Society for Echocardiography's (Sahn et al 1978) leading edge method (Norton et al 2002). Measurements were made from 3 consecutive beats. Left ventricular chamber and myocardial systolic function were determined from endocardial (FS_{end}) and midwall (FS_{mid}) fractional shortening respectively, as previously described (Norton et al 2002) using the following equations:

$$FS_{end} = EDD\text{-}ESD/EDD \times 100$$

$$FS_{mid} = \frac{[(EDD+ED\ PWT)/2-(ESD+ES\ PWT)/2]}{(EDD+ED\ PWT)/2} \times 100$$

where EDD = end diastolic diameter, ESD = end systolic diameter, ED PWT = end diastolic posterior wall thickness and ES PWT = end systolic posterior wall thickness. The calculation of midwall diameter at either end diastole or end systole is based on an assumption that PWT = septal wall thickness, and hence that half of PWT = half of septal wall thickness.

2.5 Blood analysis

Immediately after the last echocardiogram was obtained the thoracic cavity was opened under anaesthesia and the hearts immediately removed. To determine the impact of the model of obesity on percentage glycosylated haemoglobin (HbA_{1c}), blood samples were obtained from the thoracic cavity immediately after extirpation of the heart. HbA_{1c} measurements were determined immediately (using non-frozen blood) using HBA1C II Tina-quant kit (Cobas-Roche Diagnostics).

2.6 Assessment of the degree of ventricular hypertrophy

The heart was rinsed in saline and the large vessels and atria carefully dissected free from the ventricles. The ventricles were then weighed. The right ventricular free wall was then separated from the left ventricle and its septum and the right ventricular free wall weighed. The left ventricle with the septum intact was then weighed. The heart weight and left ventricular weight were then expressed as absolute weights as well as heart or ventricular weight per 100 g body weight or per tibial length. Heart and left ventricular weight were expressed per 100g body weight to account for differences in overall body size, including differences in growth between SHRs and WKY and differences on the degree of adiposity produced by dietary intervention. Heart and left ventricular weight were expressed per tibial length to account for differences in growth between SHRs and WKY without adjusting for the impact of adiposity on heart size.

2.7 Cardiomyocyte fibrosis and apoptosis

After weighing heart tissue, a longitudinal slice of the left ventricle from the apex to the base through the left ventricular free wall was obtained from all rats for histology. Left ventricular tissue was stored in 10% buffered formaldehyde for subsequent histology. Buffered formaldehyde consists of 10.4 ml of 37-40% formaldehyde (Associated Chemical Enterprises, South Africa) to which 89.6 ml phosphate buffered saline (PBS) is added. The solution is then buffered to pH 7.4. The PBS solution consists of sodium chloride (137mM), potassium chloride (2.68mM), potassium dihydrogen orthophosphate (1.47 mM) and disodium hydrogen orthophosphate (8.1mM) (Merck, South Africa) which are added to distilled water and then buffered to pH 7.4. Myocardial tissue was subsequently processed by dehydration in graded alcohol, cleared in chloroform, embedded in paraffin wax and cut into sections. Left ventricular tissue was processed routinely for light microscopy and 50 μm-thick sections of the long axis circumference were cut through the full thickness of the left ventricular wall. Ten slices were obtained at 1-mm intervals and stained with van Gieson's stain and with haemotoxylin and eosin (H & E). To stain with H & E sections were dewaxed, hydrated and stained with haemotoxylin (consisting of ammonium alum, haemotoxylin, chloral hydrate, citric acid, sodium iodate, distilled water). The slides were then washed in running tap water and stained with eosin (consisting of eosin, erythrosin, distilled water, calcium chloride). The sections were then rinsed in tap water, dehydrated and subsequently mounted with permanent mounting medium (Entellan, Merck KGaA, Germany). To stain with van Gieson's stain, sections were dewaxed, hydrated and stained

with Wigerts haemotoxylin (consisting of haemotoxylin, distilled water, hydrochloric acid and ferric chloride). After washing in running tap water the sections were stained with van Geison's solution (consisting of picric acid, acid fuchsin), blotted dry, dehydrated and subsequently mounted using permanent mounting medium (Entellan, Merck KGaA, Germany). The reagents used in H&E and van Geison's stain were obtained from Merck, South Africa. 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). The grading results were confirmed by a second observer. Representative slides are illustrated in Figure 2.4.

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

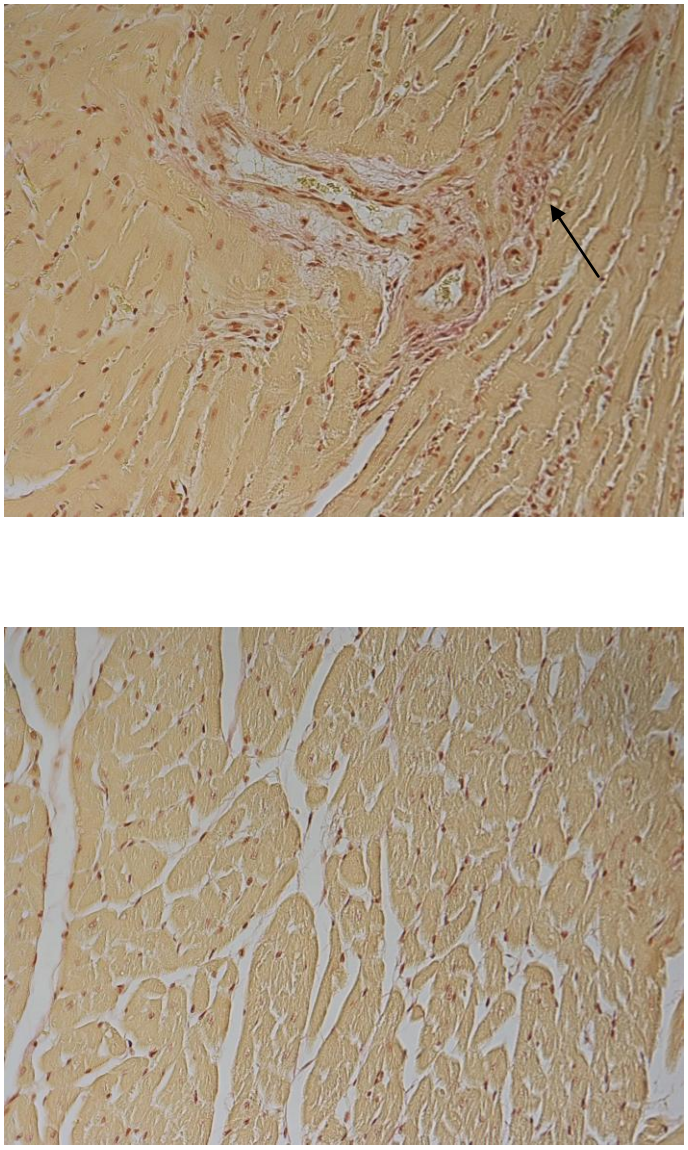


Figure 2.4. Histological images obtained using light microscopy from cross-sections of myocardial tissue stained with van Gieson's stain. The slides show portions of the heart with evidence of tissue fibrosis following cell death (upper panels) as compared to normal portions of the heart (lower panels). Arrow indicates fibrosis (stained pink).

DNase I activity from the positive control which may affect the experimental slides by staining non-apoptotic cells.

To identify apoptotic nuclei, all procedures were carried out at room temperature except where otherwise stated. Paraffin embedded sections were first immersed in xylene for 5 minutes to de-paraffinize the tissue sections. The tissue sections were then washed by immersing the slides in 100% ethanol for 5 minutes and again for 3 minutes. The sections were then rehydrated by immersing the slides through graded ethanol washes (95%, 85%, 70% and 50%) for 3 minutes each. The slides were then washed in 0.85% NaCl solution for 5 minutes and in PBS for 5 minutes. The tissue sections were then fixed by immersing the slides in 4% paraformaldehyde solution for 15 minutes. The slides were then immersed in PBS for 5 minutes. The liquid was then dried from the tissue sections and the slides were placed on a flat surface. A 20µg/ml protein kinase K solution was prepared from the 10µg/ml Proteinase K stock solution by diluting it with PBS. 100µl of the proteinase K solution was then added to the slides to cover each tissue section. The slides were then incubated for 30 minutes at room temperature to allow the proteinase K to increase the permeability of the cells. The tissue sections were then washed by immersing the slides in PBS for 5 minutes and re-fixed by immersing in 4% paraformaldehyde solution and washed again in PBS for 5 minutes. At this point the positive control slide was treated with DNase I to cause DNA fragmentation whilst the experimental slides remained in a PBS solution. 100µl of DNase I buffer was added to the positive control slide to cover the tissue sections and incubated at room temperature for 5 minutes. The DNase I buffer liquid was then tapped off the tissue sections and DNase I buffer containing DNase was added to cover the tissue sections. The slides were

then incubated for 10 minutes at room temperature. The excess liquid was removed by tapping the slides. The positive control slide was then washed 4 times in distilled water and in PBS for 5 minutes. After DNase treatment the positive control slide was again processed with the experimental slides. The excess liquid was removed by tapping the slides and the tissue sections were then covered with Equilibration Buffer for 8 minutes. Whilst the sections were equilibrating, 10µl of Biotinylated Nucleotide Mix and 10µl of rTDT Enzyme were added to 980µl of Equilibration Buffer for the reaction mix. A control incubation buffer was prepared for the negative control slide by adding 1µl of Biotinylated Nucleotide Mix and 1µl of distilled water to 98µl of Equilibration buffer. After equilibration the slides were blotted with tissue paper to remove excess liquid and 100µl of the rTDT reaction mix was then added to each tissue section. The sections were then covered with plastic cover slips and incubated at 37˚C for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur. After 60 minutes the slides were removed from the incubator and the plastic cover slips were removed. 20X saline-sodium citrate (SSC, supplied with the TUNEL kit) was diluted 1:10 with distilled water. The rTDT reaction was terminated by immersing the slides in 20X SSC solution for 15 minutes. This procedure was repeated. The tissue sections were subsequently washed in PBS twice for 5 minutes each to remove unicorporated biotinylated nucleotides. The slides were then immersed in 0.3% hydrogen peroxide for 5 minutes to block the endogenous peroxides and washed with PBS for 5 minutes. Streptavidin HRP was diluted in PBS. 100µl was added to each slide to cover the tissue sections and the slides were incubated at room temperature for 30 minutes. The slides were then washed with PBS for 5 minutes. 50µl of DAB Substrate 20X Buffer, 50µl of DAB 20X

Chromogen and 50µl of Hydrogen Peroxide 20X were added to 950µl of distilled water. 100µl of the DAB solution was then added to each slide to cover the tissue sections for 8 minutes at room temperature. The slides were then rinsed 4 times with distilled water, dehydrated by immersing the slides in graded ethanol washes (50%, 70%, 85% and 95%) and immersed in xylene. The slides were subsequently mounted using permanent mounting medium (Entellan, Merck KGaA, Germany).

The number of apoptotic cardiomyocyte nuclei and the total number of cardiomyocyte nuclei (haematoxylin and eosin stain) in each slide were counted on ten 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 2.5. All sections were coded and a single observer "blinded" to the identity of the rat from which the section was obtained recorded the number of apoptotic nuclei, and counted the total number of cardiac myocyte nuclei from the H & E slides.

2.8 Data analysis

As 5 days of ISO administration had no effect on heart or left ventricular weight, data for experimental-diet groups were pooled and compared to pooled data for control-diet fed groups. The effects of the experimental-diet on heart and left ventricular weight, and on left ventricular dimensions was then assessed using an unpaired t-test.

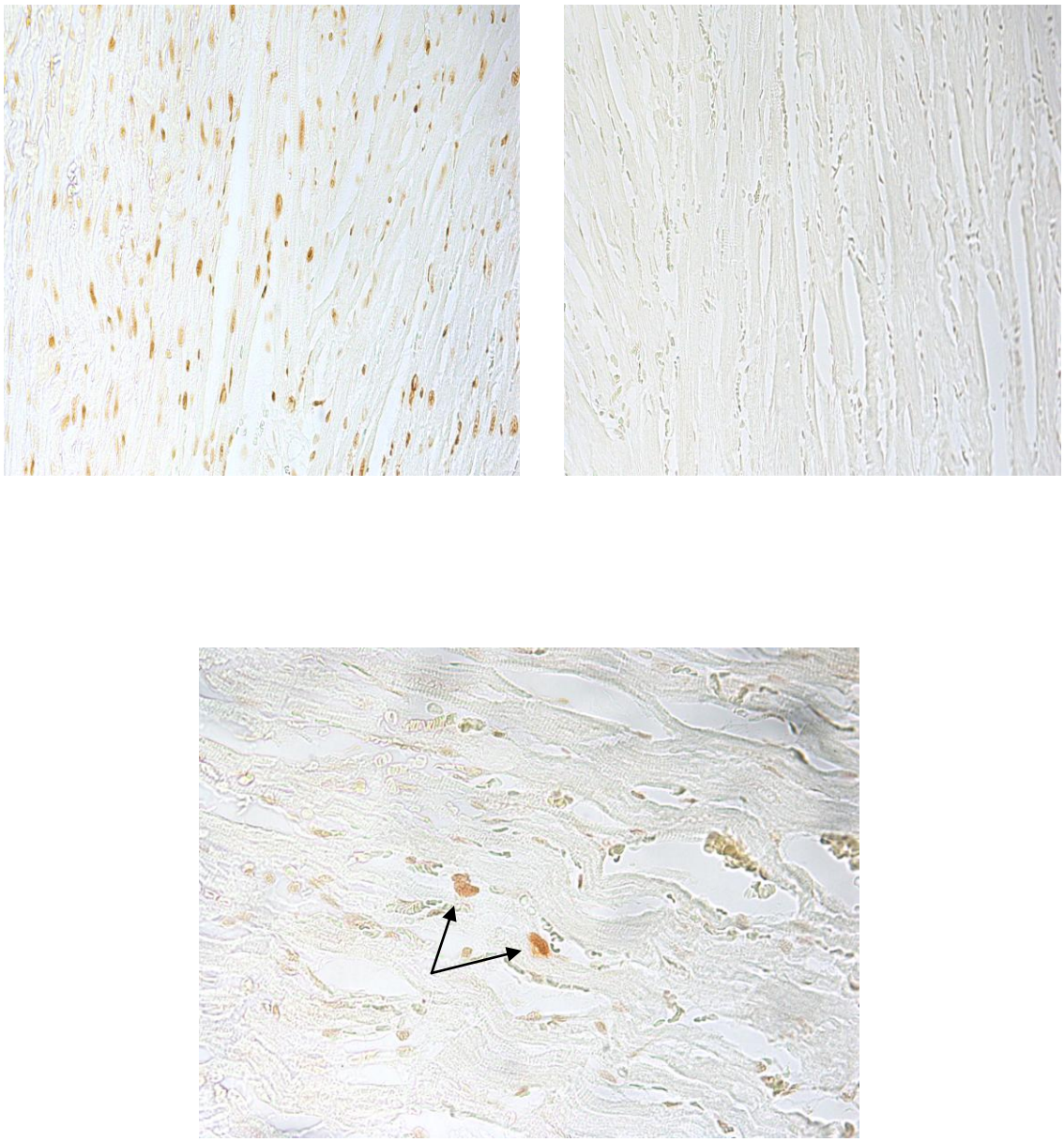


Figure 2.5. Histological sections of the myocardium stained for apoptotic nuclei. The upper panels illustrate sections obtained from a positive (left) and a negative (right) control (20X magnification) and the lower panel a section from the myocardium of a heart showing apoptotic cardiomyocyte nuclei (arrow) (40X magnification). Note the numerous apoptotic nuclei in the positive control section.

In the model to assess the impact of excess adiposity on cardiomyocyte apoptosis in pressure-overload hypertrophy, differences in body weight and tail-cuff systolic blood pressure between obese SHR, SHR and age-matched WKY control rats were assessed using a repeated measures two-way ANOVA. Cardiac morphometry, LV dimensions andfunction, glycosylated haemoglobin and the percentage of cardiomyocyte apoptosis between obese SHR, SHR and age-matched WKY control rats, were assessed using two-way ANOVA.

In the model to assess the impact of excess adiposity on sympathetic-induced cardiomyocyte apoptosis, differences in body weight, tail-cuff systolic blood pressure and LV function before and after acute ISO administration between young WKY diet-ISO, WKY diet-vehicle, WKY control-ISO and WKY control-vehicle were assessed using a repeated measures two-way ANOVA. Cardiac morphometry, LV dimensions, glycosylated haemoglobin and the percentage of cardiomyocyte apoptosis between young WKY diet-ISO, WKY diet-vehicle, WKY control-ISO and WKY control-vehicle were assessed using a two-way ANOVA.

The pathological score between groups was assessed using a Kruskal-Wallis test followed by Dunn post hoc tests (nonparametric ANOVA). All data are expressed as mean ± standard error of the mean (SEM).

Chapter 3

Results.

3.1 Food intake in diet versus control rats.

The experimental groups consumed a greater quantity of food and consequently, energy intake was enhanced in the experimental groups (31±1.28g/day; 570±23 kJ/day respectively) as compared to the control group (20±1.00g/day; 371±18 kJ/day respectively), $p<0.0001$.

3.2 Obesity in young Wistar Kyoto rats.

Figure 3.1 shows body weight changes over the 5 month study period and Figure 3.2 shows the visceral fat weight at the end of the study in young (1 month of age at the beginning of the study) WKY rats receiving either the experimental diet or the control diet. The experimental diet resulted in an increase in body and visceral fat weight as compared to control diet fed rats (Figures 3.1 and 3.2). The body weight in rats receiving the experimental diet was significantly greater than the body weight in rats receiving the control diet, from the fourth month of the study (Figure 3.1). Importantly, similar dietary effects on body weight and visceral fat were noted in rats assigned to receive either isoproterenol or the vehicle for 5 days at the end of the study period (Table 3.1).

3.3 Obesity in elderly Wistar Kyoto and Spontaneously Hypertensive rats.

Figure 3.3 shows body weight changes over the 5 month study period and Figure 3.4 shows the visceral fat weight and the body weight normalized to tibial length (as an

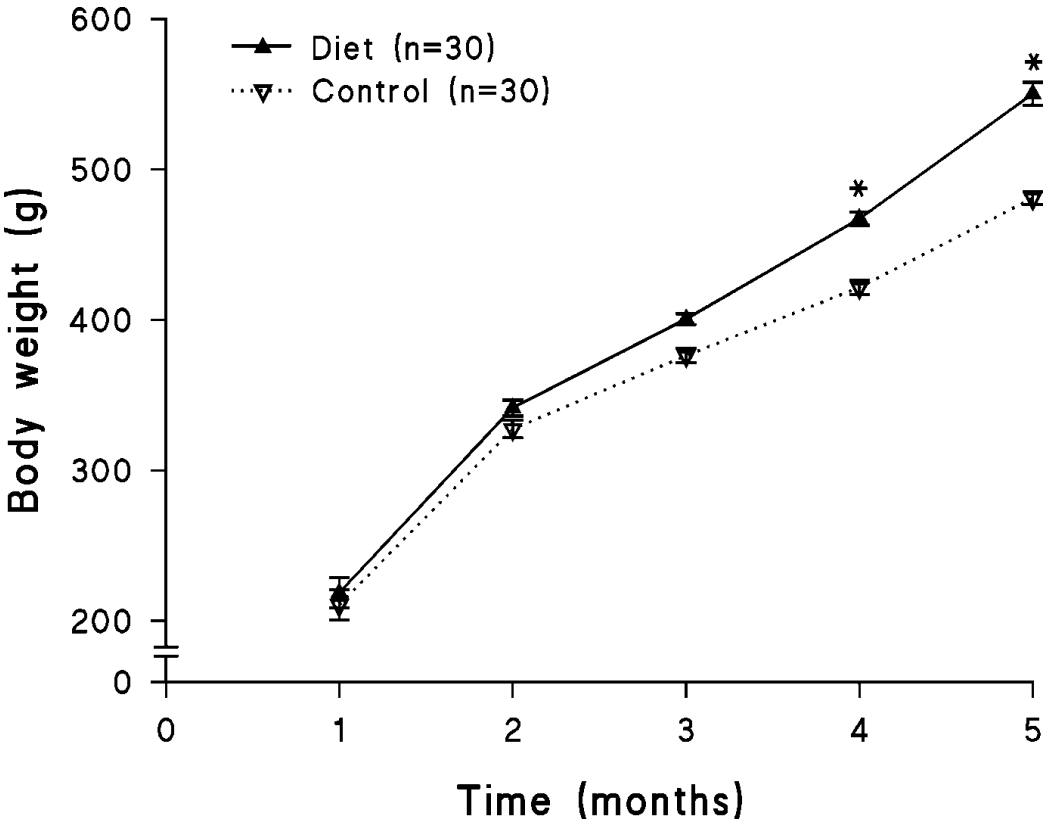


Figure 3.1 Changes in body weight over time in young Wistar Kyoto (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). Body weights in Diet-fed rats were significantly greater than Control rats from 4 months on the experimental diet. *$p<0.001$ vs Control group.

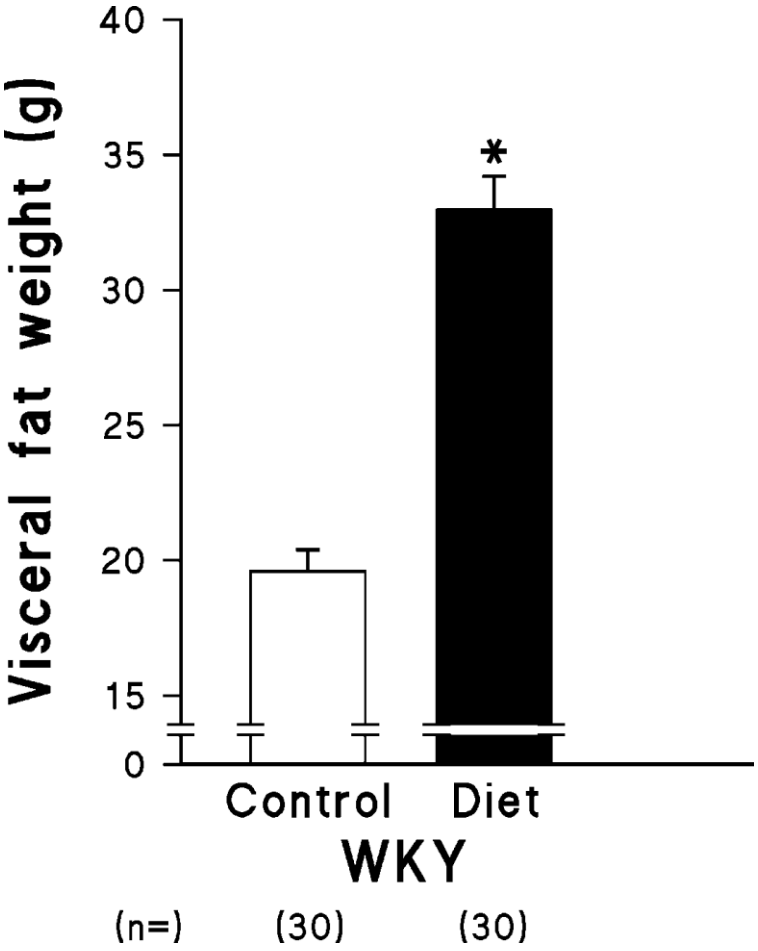


Figure 3.2 Visceral (retroperitoneal and mesenteric) fat weight in young Wistar Kyoto (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.0001$ vs Control group.

Table 3.1 Comparison of body morphometry in Wistar Kyoto (WKY) rats fed different diets for 5 months and subsequently receiving either isoproterenol (ISO) or the saline vehicle (vehicle) for 5 days.

	Final body weight (BW)(g)	Visceral fat (g)
WKY con-vehicle (n=16)	480±5	19.3±0.9
WKY con-ISO (n=14)	482±8	20.0±1.3
WKY diet-vehicle (n=16)	542±10*	32.1±1.6*
WKY diet-ISO (n=14)	560±12*	34.0±1.9*

Con, control diet; diet, experimental diet. *$p<0.0001$ versus respective Control diet-fed group.

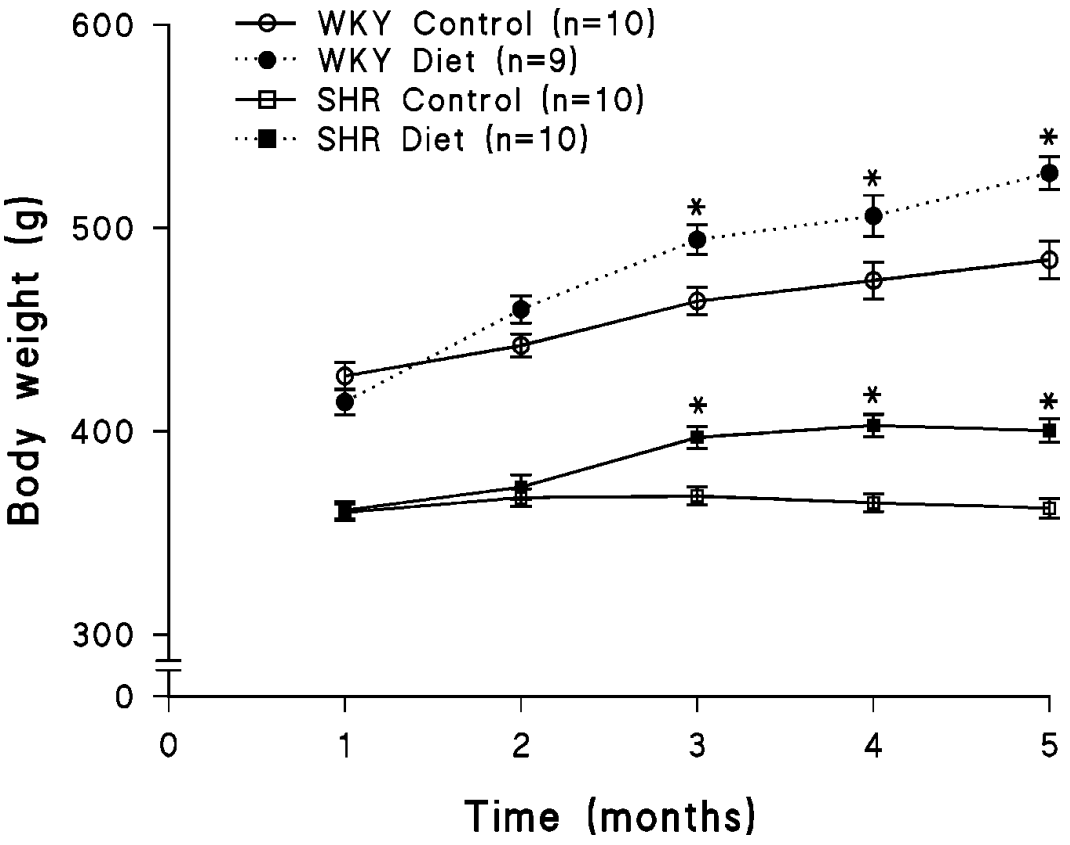


Figure 3.3 Changes in body weight over time in spontaneously hypertensive (SHR) and Wistar Kyoto Control (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). Body weights in diet-fed SHR and WKY rats were significantly greater than Control rats from 3 months on the experimental diet. *$p<0.0001$ vs respective Control diet-fed rats.

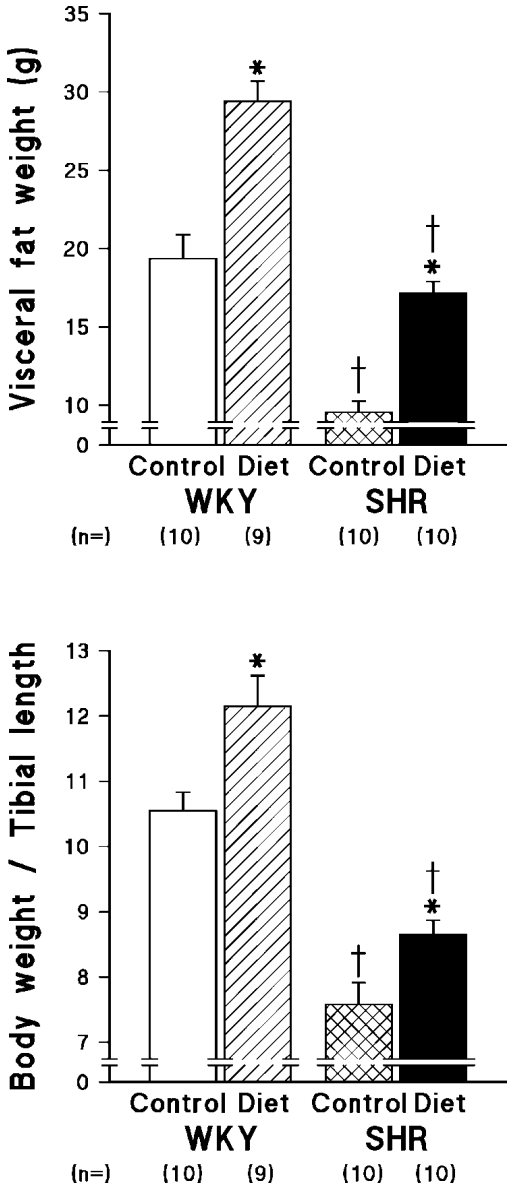


Figure 3.4 Visceral (retroperitoneal and mesenteric) fat weight and body weight normalized to tibial length (as an index of growth) in spontaneously hypertensive (SHR) and Wistar Kyoto Control (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.005$ vs respective Control diet-fed rats, † $p<0.0001$ vs respective WKY group.

index of body weight changes expressed as a proportion of growth) at the end of the study in elderly (8-9 months of age at the beginning of the study) WKY rats and SHR receiving either the experimental diet or the control diet. Although SHR weighed considerably less than WKY rats throughout the study period, after 5 months of feeding in both WKY rats and SHR the experimental diet resulted in an increase in body weight as compared to control diet fed rats in both groups (Figure 3.3). The body weight in both SHR and WKY rats receiving the experimental diet was significantly greater than the weight in rats receiving the control diet, from the third month of the study (Figure 3.3). SHR developed a significant increase in body weight normalized to tibial length (Figure 3.4), and a similar increase in visceral fat content in response to the experimental diet as WKY rats (Figure 3.4).

3.4 Systolic blood pressure in young Wistar Kyoto Rats.

Dietary-induced obesity had no effect on tail-cuff systolic BP in young (1 month of age at the beginning of the study) WKY rats (Experimental diet; Baseline SBP= 116±3 mm Hg, After 5 months of the study SBP= 118±4 mm Hg; Control diet; Baseline SBP= 112±4 mm Hg, After 5 months of the study SBP= 119±4 mm Hg).

3.5 Systolic blood pressure in elderly Wistar Kyoto and Spontaneously Hypertensive rats.

Figure 3.5 shows the tail-cuff systolic blood pressures measured in elderly (8-9 months of age at the beginning of the study) WKY and SHR before and after 5 months of feeding rats either a control or an experimental diet. Both at 1 and at 5 months of dietary intervention, systolic BP was markedly increased in SHR as compared to WKY rats, but the experimental diet failed to modify BP in either group.

3.6 Impact of obesity-inducing diet on percentage glycosylated haemoglobin (HbA_{1c}) in either young or elderly groups of rats.

The obesity-inducing diet failed to influence the percentage glycosylated haemoglobin (HbA_{1c}) in young WKY rats (Experimental diet=4.69±0.13%; Control diet= 4.68±0.05%). Similarly, the obesity-inducing diet failed to influence the HbA_{1c} in either elderly SHR or WKY rats (WKY rats; Experimental diet=4.67±0.08%; Control diet= 4.70±0.13%, SHR; Experimental diet=4.63±0.15%; Control diet=4.7±0.18%).

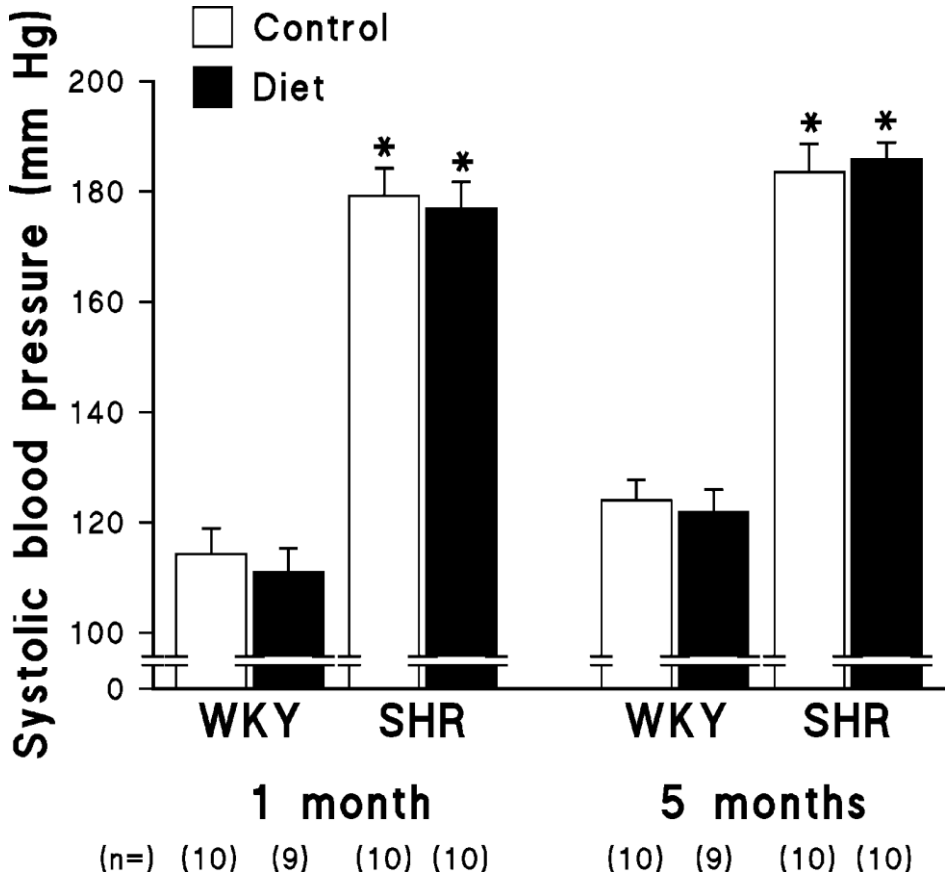


Figure 3.5 Tail-cuff systolic blood pressures in spontaneously hypertensive (SHR) and Wistar Kyoto Control (WKY) rats receiving either a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.0001$ vs respective WKY groups.

3.7 Impact of obesity and isoproterenol administration on heart weight and left ventricular weight in young WKY rats

Table 3.2 shows heart and left ventricular weights at the end of the study in young (1 month of age at the beginning of the study) WKY rats receiving either the experimental diet or the control diet for 5 months and either daily isoproterenol or the vehicle for 5 days prior to termination. Isoproterenol did not modify heart weight in either the control or the experimental diet groups, but increased left ventricular weight in the experimental diet group (Table 3.2). Feeding rats the experimental diet resulted in an increase in heart weight [Experimental diet (n=30)=1.23±0.02g; Control diet (n=30)= 1.14g±0.02g, $p<0.005$] and left ventricular weight [Experimental diet (n=30)=1.01±0.02g; Control diet (n=30)=0.94±0.02g, $p<0.005$]. As the experimental diet resulted in a greater increase in body weight (~15%), compared to heart (~8%) or left ventricular weight (~7%), a decrease in the heart and left ventricular weight-to-body weight ratios was noted in the experimental diet fed groups.

3.8 Impact of obesity and hypertension on heart weight and left ventricular weight in elderly WKY and Spontaneously Hypertensive rats

Table 3.3 shows heart and left ventricular weights at the end of the study in elderly (8-9 months of age at the beginning of the study) WKY rats and SHR receiving either the experimental diet or the control diet for 5 months. Despite having markedly

Table 3.2 Cardiac morphometry in Wistar Kyoto (WKY) rats fed different diets for 5 months and subsequently receiving either isoproterenol (ISO) or the saline vehicle (vehicle) for 5 days.

	HW (g)	LVW (g)	HW/BW	LVW/BW
WKY con-vehicle (n=16)	1.13±0.02	0.94±0.02	2.35±0.05	1.95±0.04
WKY con-ISO (n=14)	1.15±0.03	0.95±0.03	2.39±0.06	1.96±0.05
WKY diet-vehicle (n=16)	1.19±0.03	0.98±0.02	2.20±0.05	1.82±0.04**
WKY diet-ISO (n=14)	1.26±0.03	1.04±0.02*	2.26±0.03	1.85±0.02**

HW, heart weight; LVW, left ventricular weight; BW, body weight; Con, control diet; diet, experimental diet. *$p<0.05$, **$p<0.01$ versus respective Control diet-fed groups.

Table 3.3 Cardiac morphometry in elderly Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats fed different diets for 5 months.

	HW (g)	LVW (g)	HW/BW	LVW/BW
WKY con (n=10)	1.41±0.15	1.17±0.15	2.91±0.004	2.40±0.003
SHR con (n=10)	1.52±0.12	1.22±0.08	4.24±0.004*	3.41±0.003*
WKY diet (n=9)	1.52±0.07	1.23±0.05	2.88±0.005	2.33±0.004
SHR diet (n=10)	1.70±0.23	1.34±0.14	3.55±0.002*	3.55±0.002*

HW, heart weight; LVW, left ventricular weight; BW, body weight; Con, control diet; diet, experimental diet. *$p<0.0001$ versus respective WKY group.

lower body weights as compared to WKY rats throughout the study (Figure 3.3), SHR had similar heart and LV weights as compared to WKY rats (Table 3.3). Thus, when heart and left ventricular weight were normalized per 100g body weight, consistent with the effects of hypertension, SHR had striking increases in cardiac weight. Feeding rats the experimental diet resulted in a trend for an increase in heart and left ventricular weight in WKY rats and SHR (Table 3.3). Importantly, when LV weight was normalized for tibial length, SHRs having received the experimental diet exhibited striking LV hypertrophy as compared to the other 3 groups (Figure 3.6) ($p<0.05$ for an interaction between diet and hypertension).

3.9 Impact of obesity on LV dimensions and systolic function in young WKY rats receiving isoproterenol.

The experimental diet tended to increase left ventricular wall thickness [Experimental diet (n=18)=1.96±0.06mm; Control diet (n=18)=1.83±0.04mm, p=0.07] but did not modify left ventricular internal dimensions [Experimental diet (n=18)=8.54±0.12mm; Control diet (n=18)=8.44±0.17mm, p=0.65]. Table 3.4 shows LV internal dimensions and wall thickness at the end of the study in young (1 month of age at the beginning of the study) WKY rats receiving either the experimental diet or the control diet for 5 months and either daily isoproterenol or the vehicle for 5 days prior to termination. Neither the experimental diet given for 5 months, nor 5 days of isoproterenol administration modified either LV internal dimensions or wall thickness in young WKY rats.

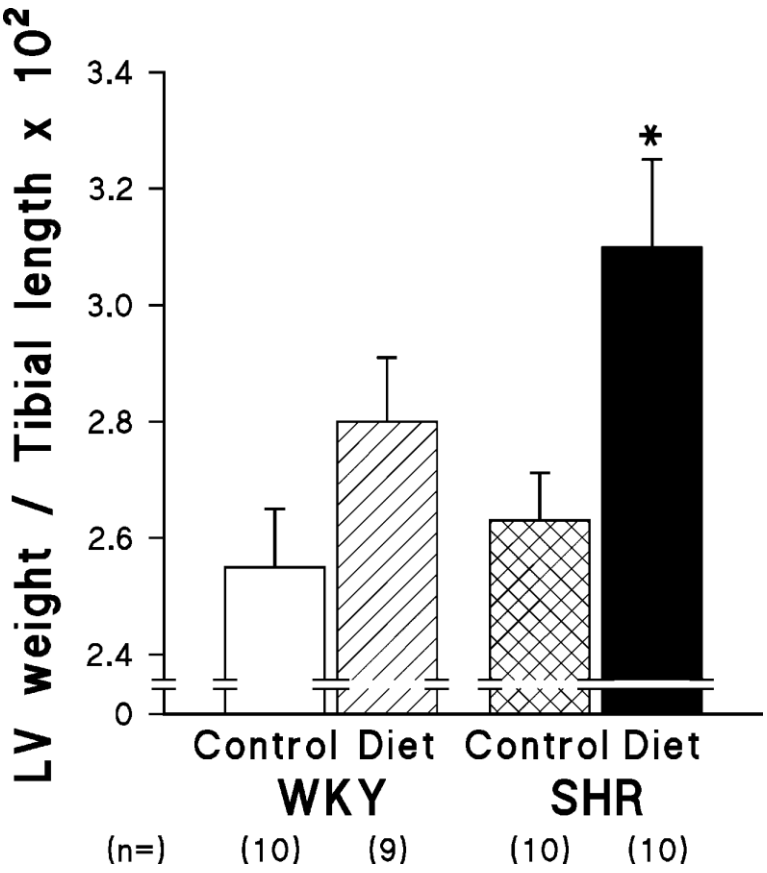


Figure 3.6 Left ventricular weight normalized to tibial length in spontaneously hypertensive (SHR) and Wistar Kyoto Control (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.05$ vs all other groups.

Table 3.4 Comparison of left ventricular diameters and wall thickness values in Wistar Kyoto (WKY) rats fed different diets for 5 months and subsequently receiving either isoproterenol (ISO) or the saline vehicle (vehicle) for 5 days.

	LVEDD (mm)	LVESD (mm)	ED PWT (mm)	ES PWT (mm)
WKY con-vehicle (n=8)	8.46±0.18	4.96±0.19	1.87±0.06	2.73±0.13
WKY con-ISO (n=10)	8.43±0.28	4.99±0.23	1.79±0.06	2.68±0.09
WKY diet-vehicle (n=8)	8.45±0.22	4.59±0.24	1.98±0.13	3.02±0.14
WKY diet-ISO (n=10)	8.61±0.12	5.28±0.18	1.96±0.06	2.81±0.10

LVEDD, LV end diastolic diameter; LVESD, LV end systolic diameter; ED PWT, LV end diastolic posterior wall thickness; ES PWT, LV end systolic posterior wall thickness Con, control diet; diet, experimental diet.

Figure 3.7 shows LV systolic chamber and myocardial function and heart rate at the end of the study in young (1 month of age at the beginning of the study) WKY rats receiving either the experimental diet or the control diet for 5 months and either daily isoproterenol or the vehicle for 5 days prior to termination. Moreover, Figure 3.7 shows changes in LV systolic chamber and myocardial function and heart rate in response to acute administration of isoproterenol in the different groups of rats. Neither the experimental diet given for 5 months, nor 5 days of isoproterenol administration modified baseline LV chamber or myocardial systolic function or heart rate. Moreover, neither the experimental diet given for 5 months, nor 5 days of isoproterenol administration modified the LV chamber or myocardial systolic function or heart rate response to acute isoproterenol administration.

3.10 Impact of obesity on LV dimensions and systolic function in elderly WKY and Spontaneously Hypertensive rats

Figure 3.8 shows LV end diastolic diameters and LV systolic chamber (FS_{end}) function and Table 3.5 shows LV internal dimensions at end systole, wall thickness, LV systolic myocardial function (FS_{mid}) and end diastolic and systolic relative wall thickness (h/r) at the end of the study in elderly (8-9 months of age at the beginning of the study) WKY rats and SHR receiving either the experimental diet or the control diet for 5 months. Consistent with a concentric LV geometry, SHR had reduced LV internal diameters

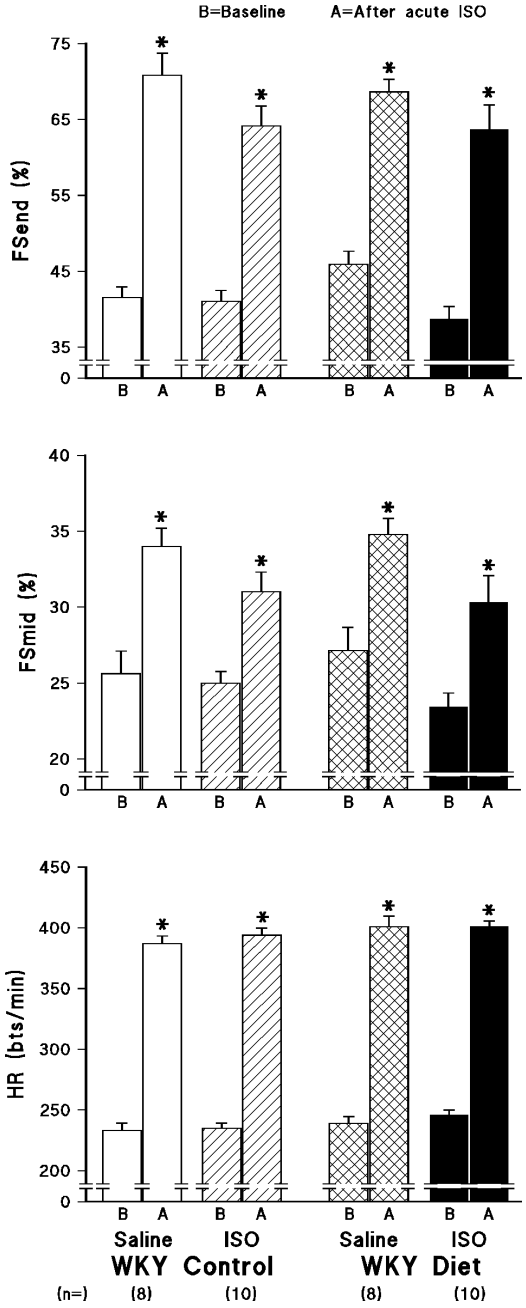


Figure 3.7 Effect of acute isoproterenol administration on left ventricular systolic chamber (endoocardial fractional shortening, FS_{end}) and myocardial (midwall fractional shortening, FS_{mid}) function as well as heart rate (HR) in young Wistar Kyoto control (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.0001$ vs Baseline data.

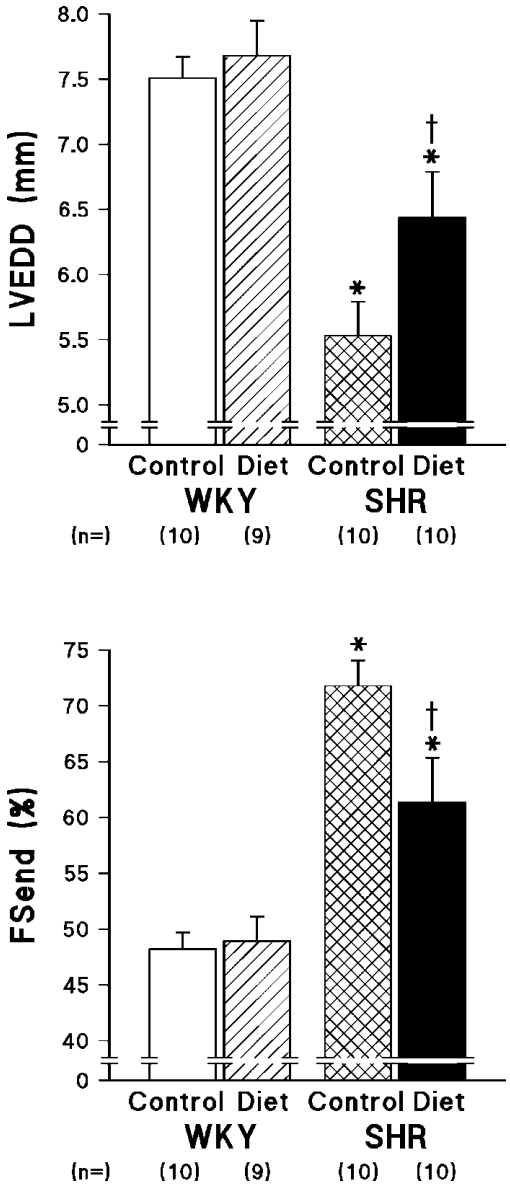


Figure 3.8 Left ventricular end diastolic diameter (LVEDD) and systolic chamber function (endocardial fractional shortening-FS_{end}) in Spontaneously hypertensive (SHR) and Wistar Kyoto Control (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.0001$ vs respective WKY groups,

† $p<0.05$ vs SHR Control.

Table 3.5 Left ventricular dimensions and myocardial function in elderly Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats fed different diets for 5 months.

	LVESD (mm)	ED PWT (mm)	ES PWT (mm)	ED h/r	ES h/r	FS_{mid} (%)
WKY con (n=10)	3.89±0.16	2.00±0.10	3.07±0.05	0.54±0.04	1.61±0.09	26.6±1.2
SHR con (n=10)	1.68±0.18*	2.98±0.19*	4.00±0.13*	1.13±0.12*	5.27±0.56*	33.3±1.7
WKY diet (n=9)	3.94±0.24	2.16±0.13	3.19±0.09	0.57±0.05	1.68±0.13	27.6±1.3
SHR diet (n=10)	2.69±0.38*†	2.56±0.10*†	3.68±0.14*†	0.83±0.08*†	3.37±0.55*†	29.5±2.6

LVESD, left ventricular end systolic diameter; ED PWT, LV end diastolic posterior wall thickness; ES PWT, LV end systolic posterior wall thickness; h/r, wall thickness to radius ratio. FS_{mid}, midwall fractional shortening; Con, control diet; diet, experimental diet. *$p<0.001$ versus respective WKY group, † $p<0.05$ vs SHR Control-diet fed group.

(Figure 3.8 and Table 3.5), a thicker posterior wall (Table 3.5), an increased LV relative wall thickness (h/r) (Table 3.5) and an increased systolic chamber function (FS_{end}) as compared to WKY rats (Figure 3.8). No differences were noted in systolic myocardial function (FS_{mid}) between the SHR and WKY rats (Table 3.5).

The experimental diet produced an increase in LV internal diameters at end diastole (Figure 3.8) and at end systole (Table 3.5) in SHR, but not in WKY rats. Moreover, the experimental diet produced a decrease in LV posterior wall thickness at end diastole and at end systole in SHR, but not in WKY rats (Table 3.5). The consequence of the dietary-induced changes in internal diameters and wall thickness in SHR was a reduced LV relative wall thickness (h/r) (Table 3.5). Although the dietary-induced changes in LV geometry in SHR failed to translate into a decreased myocardial function (FS_{mid}) (Table 3.5), SHR receiving the experimental diet developed a marked reduction in LV systolic chamber function (FS_{end}), whilst the experimental diet failed to modify FS_{end} in WKY rats (Figure 3.8).

3.11 Impact of obesity on isoproterenol-induced cardiomyocyte apoptosis and fibrosis

Figure 3.9 shows the percentage TUNEL positive stained cardiomyocytes (apoptosis) and the pathological score (fibrosis) at the end of the study in young (1 month of age at the beginning of the study) WKY rats receiving either the experimental diet or the control diet for 5 months and either daily isoproterenol or the vehicle for 5 days prior to termination. The experimental diet did not alter the degree of cardiomyocyte apoptosis

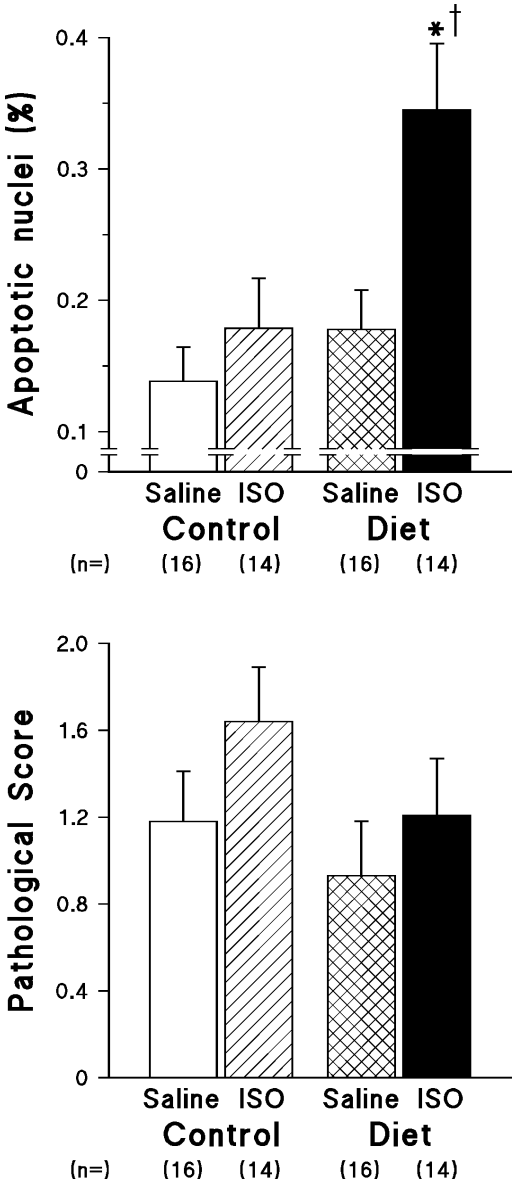


Figure 3.9 Percentage cardiomyocyte apoptotic nuclei and pathological score after 5 days of daily isoproterenol (ISO) or saline vehicle (vehicle) administration in young Wistar Kyoto control (WKY) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.05$ versus Control-ISO, †$p<0.01$ vs Control-saline and Diet-saline groups.

in rats receiving the vehicle for 5 days. However 5 days of daily isoproterenol administration resulted in an increased cardiomyocyte apoptosis in the experimental diet-fed but not the control diet-fed group. Neither isoproterenol nor the experimental diet influenced the degree of fibrosis.

3.12 Impact of obesity on cardiomyocyte apoptosis and fibrosis in elderly Wistar Kyoto and Spontaneously Hypertensive rats.

Figure 3.10 shows the percentage TUNEL positive stained cardiomyocytes (apoptosis) and the pathological score (fibrosis) at the end of the study in elderly (8-9 months of age at the beginning of the study) WKY rats and SHR receiving either the experimental diet or the control diet for 5 months. The presence of hypertension (SHR) alone was insufficient to promote excessive cardiomyocyte apoptosis or fibrosis. However, SHR fed the experimental diet developed a marked increase in cardiomyocyte apoptosis as compared to the group of rats fed the control diet. However, the diet failed to promote an increase in the pathological score in SHR.

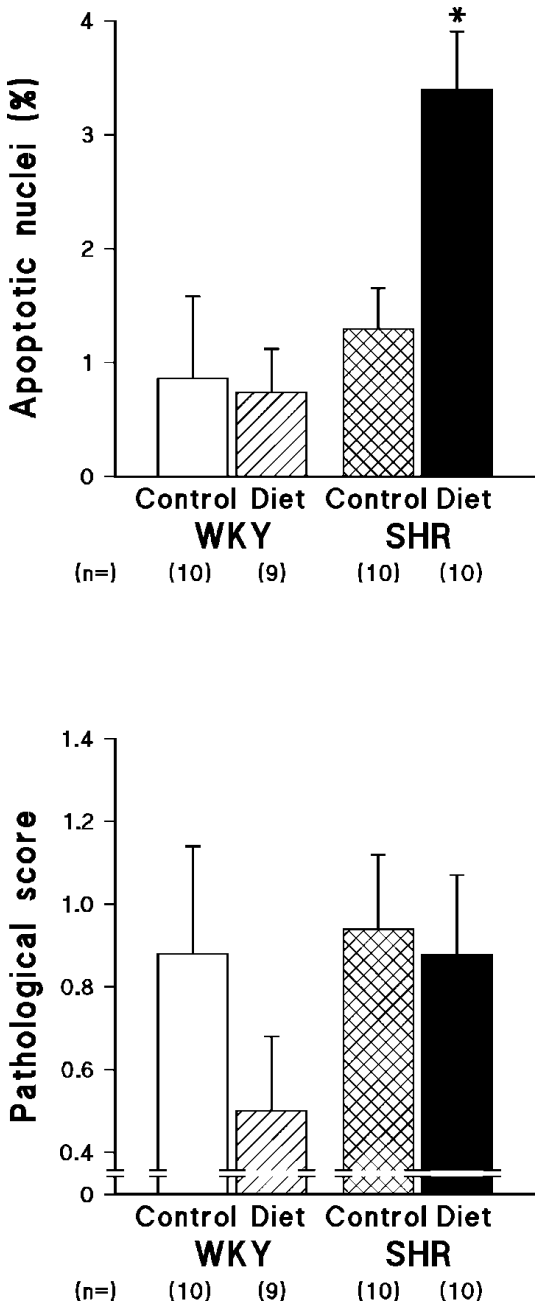


Figure 3.10 Percentage cardiomyocyte apoptotic nuclei and pathological score in elderly Wistar Kyoto Control (WKY) or Spontaneously Hypertensive (SHR) rats receiving a normal diet (Control) or a diet designed to increase caloric intake (Diet). *$p<0.005$ vs all other groups.

Chapter 4

Discussion.

4.1 Summary of main findings

The main findings of the present studies are as follows. An obesity-promoting diet given for 5 months to normotensive rats was associated with an enhanced adrenergic-induced cardiomyocyte apoptosis. Moreover, despite producing similar degrees of visceral obesity, an obesity-promoting diet given for 5 months resulted in the development of cardiomyocyte apoptosis in SHR, but not in normotensive WKY rats. Further, in SHR, but not in normotensive WKY control rats, the obesity-inducing diet mediated the development of an exaggerated degree of LV hypertrophy, and the development of LV dilatation (increased internal diameters and wall thinning) and a reduced LV pump function.

4.2 Comparison with previous studies reporting on obesity-associated cardiomyocyte apoptosis

A few studies have provided evidence to indicate that excessive cardiomyocyte apoptosis occurs in animal models of obesity. In this regard, cardiomyocyte apoptosis has previously been reported to occur in obese Zucker Diabetic Fatty rats (ZDF, *fa/fa*) (Zhou et al 2000) and in *ob/ob* mice (obese due to leptin deficiency) (Barouch et al 2006). Importantly however, in comparison to these previous studies (Zhou et al 2000, Barouch et al 2006) the outcome of the present study is unique in a number of respects. First, the present study is the first to show excessive cardiomyocyte apoptosis in association with dietary-induced obesity, rather than in association with genetic models of excess

adiposity. In this regard it is uncertain whether an increased cardiomyocyte apoptosis in these previous studies (Zhou et al 2000, Barouch et al 2006) represents an associated genetic effect, or an impact of obesity. The present study provides clear evidence that dietary-induced obesity is sufficient to produce excessive cardiomyocyte apoptosis. Second, the excessive cardiomyocyte apoptosis noted in the present study occurred in an animal model of modest obesity (body weights -9 to 15% greater than non-obese control animals) as opposed to previous models where body weights were ~ 45 to 84% greater than lean control animals (Mark et al 1999b, Zhou et al 2000). In this regard therefore, the present study is more likely to reflect the effects of mild-to-moderate obesity, the predominant form of obesity that occurs at a population level, in contrast to previous animal studies that are more likely to reflect the impact of more severe levels of obesity (Zhou et al 2000, Barouch et al 2006). Third, the present study clearly shows that interactions between dietary-induced obesity and either hypertension or excessive sympathetic activation are required to mediate the dietary induced effects noted. In this regard, previous studies reporting on obesity associated apoptosis have not addressed the potential role of an interaction between either blood pressure or LV hypertrophy and obesity in mediating the development of increases in cardiomyocyte apoptosis (Zhou et al 2000, Barouch et al 2006). Last, as shall be discussed in more detail in the subsequent discussion, the present study provides clear data to suggest that obesity-associated excessive cardiomyocyte apoptosis is not mediated through obesity-induced effects on blood pressure or an abnormal blood glucose control, but rather through interactions with blood pressure or sympathetic effects.

4.3 Is obesity-associated cardiomyocyte apoptosis an effect mediated through hypertension or diabetes mellitus?

As indicated in the aforementioned discussion, whether the excessive cardiomyocyte apoptosis noted in prior studies of obesity (Zhou et al 2000, Barouch et al 2006) can be attributed to the effects of conventional cardiovascular risk factors such as increases in blood pressure or the presence of an abnormal glucose control mediated by diabetes mellitus, is presently unclear. Indeed, obesity is well recognized as mediating the development of hypertension (Wilson et al 2002, Must et al 1999, Doll et al 2002, Niskanen et al 2004) and type II diabetes mellitus (Must et al 1999, Hu et al 2001, Schienkiewitz et al 2006). What is the evidence to suggest that obesity-associated cardiomyocyte apoptosis may or may not be caused by increases in blood pressure or the development of diabetes mellitus?

There is presently substantial evidence to indicate that hypertension or the associated cardiac hypertrophy may cause excessive cardiomyocyte apoptosis (Díez et al 1997, Li et al 1997, Liu et al 2000, Hamet et al 1995, Fortuño et al 1998, Gonzalez et al 2002, Teiger et al 1996). With respect to potential blood pressure effects explaining the relationship between obesity and excessive cardiomyocyte apoptosis in previous and the present study, obesity in the ZDF rat has indeed been shown to be associated with hypertension (Carlson et al 2000, Nagao et al 2003). Consequently it is uncertain whether excessive cardiomyocyte apoptosis in the ZDF rat can be attributed to blood pressure effects. In contrast however, *ob/ob* mice have a reduced blood pressure as compared to their lean counter-parts (Mark et al 1999b, Christoffersen et al 2003) and hence the

increase in cardiomyocyte apoptosis that occurs in this model of obesity cannot be attributed to increases in blood pressure. In support of the latter finding (Barouch et al 2006), in the present study, dietary-induced obesity was associated with excessive cardiomyocyte apoptosis independent of further blood pressure changes. However, in the present study obesity only promoted excessive apoptosis in the presence of hypertension and hypertensive LV hypertrophy or in the presence of an adrenergic stimulus. Consequently, the present study suggests that conventional cardiovascular risk factors, such as hypertension, or alternative risk factors, such as an increased sympathetic stimulation are indeed required to mediate obesity-induced adverse effects on myocardial apoptotic damage.

There is presently substantial evidence to indicate that diabetes mellitus may cause excessive cardiomyocyte apoptosis (Bäcklund et al 2004, Ghosh et al 2005, Cai et al 2006). With respect to potential blood glucose effects explaining the relationship between obesity and excessive cardiomyocyte apoptosis in previous models, obesity in the ZDF rat and the *ob/ob* mice has indeed been shown to be associated with diabetes mellitus (Coleman 1978, Corsetti et al 2000). Consequently it is uncertain whether excessive cardiomyocyte apoptosis in these animal models of obesity can be attributed to blood glucose effects. However, in contrast to these previous studies (Barouch et al 2006, Zhou et al 2000), in the present study obesity was not associated with abnormalities in blood glucose control as indexed by HbA_{1c} measurements. Hence, at least in the present study, obesity-associated increases in cardiomyocyte apoptosis cannot be attributed to abnormalities in blood glucose control.

4.4 Obesity-associated cardiac hypertrophy and potential relationships with cardiomyocyte apoptosis and dysfunction

In contrast to a number of studies that have demonstrated an increased heart and LV weight in obesity (du Toit et al 2008, Carroll et al 1997, Carroll et al 2002), in the present study, obesity produced a significant increase in LV weight in SHR, a modest increase in left ventricular weight in young WKY but no increase in elderly normotensive WKY rats. The most likely explanation for this apparent discrepancy in the elderly groups of rats studied is that in previous studies the obese animals had body weights that were ~18 to 48% greater than the non-obese control animals (du Toit et al 2008, Carroll et al 1997, Carroll et al 2002), whilst in the present study, the obese animals had body weights that were only ~9 % greater than the non-obese control animals.

Importantly, with respect to the potential implications of obesity-induced LV hypertrophy, it is indeed noteworthy in the present study that the group of rats in which obesity did promote a significant increase in LV weight (the SHR group), was also the group which exhibited an excessive cardiomyocyte apoptosis, LV dilatation and a decline in LV pump function. Whether this relationship between obesity-induced LV hypertrophy and obesity-associated excessive cardiomyocyte apoptosis, LV dilatation and decline in LV pump function is a cause-effect relationship cannot be determined from the present study design. However, as highlighted in chapter 1, section 4.0, cardiomyocyte apoptosis is an important mechanism involved in the myocardial response to pressure-overload in cardiac hypertrophy (Teiger et al 1996, Li et al 2007). Left ventricular hypertrophy is accompanied by cardiomyocyte upregulation of the

proapoptotic gene *bax* in chronic pressure-overload in rats (Condorelli et al 1999) and is associated with Gq activation (a class of GTP-binding proteins) which promotes hypertrophic growth and has been shown to induce cardiomyocyte apoptosis (Hirotani et al 2002, Adams et al 1998, Adams et al 2000).

4.5 Obesity-associated cardiac dysfunction and the relevance of associated cardiomyocyte apoptosis.

Although a number of pre-clinical studies have provided evidence to suggest that cardiomyocyte dysfunction occurs in insulin-resistant or obese states (Wold et al 2005, Caroll et al 1997, Relling et al 2006, Dong et al 2006, Ren et al 2000), when assessed *in vivo*, animal models of obesity have a preserved pump function (Caroll et al 2006, du Toit et al 2008) even if the same model of obesity produces an impaired pump function *ex vivo*. The present study provides clear evidence to support the notion that dietary-induced obesity is not necessarily associated with a reduced myocardial or pump function in normotensive rats. However, noteworthy is the finding that dietary-induced obesity did indeed promote pump dysfunction as assessed *in vivo* in the presence of hypertension, but not in the absence of hypertension. Thus, the present study provides the first evidence to indicate that obesity may indeed induce pump dysfunction even as assessed *in vivo*, but that this effect depends on the co-existence of hypertension.

The mechanisms of the dietary-induced pump dysfunction in SHR could be through LV dilatation and hence an increased LV wall stress. Indeed, end systolic relative wall thickness was markedly reduced in obese as compared to lean SHR, an effect that

would increase LV end systolic wall stress for a given LV systolic pressure. The mechanism of the LV dilatation could in turn be attributed to excessive cardiomyocyte apoptosis. Alternatively, the dietary-induced cardiomyocyte apoptosis could be attributed to an excessive myocardial wall stress mediated by the LV dilatation. In this respect, the present study design did not allow me to distinguish between cause-effect relationships.

4.6 Potential cellular mechanisms of obesity-associated cardiomyocyte apoptosis

Irrespective of whether the dietary-induced effect on cardiomyocyte apoptosis in SHR noted in the present study was mediated through LV hypertrophy, or an increased LV wall stress (produced by cardiac dilatation), the changes that occur at a cellular level that are responsible for this effect, deserve consideration. In this regard, the cellular mechanisms of stress-induced cardiomyocyte apoptosis have been summarized in chapter 1, (section 5.1), but in the present study these mechanisms were not explored. Further studies are therefore required to explore this possibility. In addition, the excessive cardiomyocyte aptoptosis that occurs in *ob/ob* mice may occur as a consequence of disruption of leptin signaling, as leptin treatment attenuates this effect on the myocardium (Barouch et al 2006). Again, this mechanism requires investigation in future studies.

An additional cellular mechanism that may explain the obesity-associated cardiomyocyte apoptosis reported on in the present study is through adrenergic over-activition. Indeed, as highlighted in Chapter 1 (Section 5.2), β-adrenergic activation results in an increased cardiomyocyte apoptosis (Osadchii et al 2007) and obesity may be associated with an enhanced sympathetic nervous system activity (Walgren et al 1987,

Schwartz et al 1983, Morgan et al 1995). Importantly, the mechanisms of adrenergic-induced cardiomyocyte apoptosis may also be through cellular oxidation and the generation of ROS. Indeed, isoproterenol administration is associated with the downregulation of antioxidants in the myocardium resulting in an increase in cardiac oxidative stress and the generation of ROS (Zhang et al 2005, Srivastava et al 2007).

With respect to a potential role of obesity-induced sympathetic activation explaining the excessive cardiomyocyte apoptosis noted in the present study, although I was unable to measure sympathetic nervous system activity in obese WKY or SHR, I was able to show that adrenergic-induced cardiomyocyte apoptosis is enhanced in obesity. Whether this effect can be attributed to an additional (additive) action of obesity-induced sympathetic activation, or whether obesity stimulates the same cellular targets as β-adrenoreceptors, could not be determined from the present study design. Importantly, however, contractile (FS_{end} and FS_{mid}) and chronotropic responses to β-adrenoreceptor activation in obese rats was similar to responses noted in lean rats, suggesting that β-adrenoreceptor-cAMP system responsiveness remained essentially unaltered in obese rats.

A further potential mechanism that could explain obesity-associated cardiomyocyte apoptosis in SHR in the present study is through activation of the renin-angiotensin-aldosterone system (RAAS), a system that is well recognized as being stimulated in obesity (Boustany et al 2004, Engeli et al 2005, Barton et al 2000, Tochikubo et al 1994, Sowers et al 1983). What is the evidence to support a notion that the RAAS may mediate excessive cardiomyocyte apoptosis in obese SHR? First, angiotensin II has been shown to cause cardiomyocyte apoptosis *in vitro* (Kajstura et al

1997) and hence any situation which is associated with RAAS activation, such as obesity, may promote increases in cardiomyocyte apoptosis. Second, cardiomyocytes isolated from SHR have an increased susceptibility to angiotensin II-induced apoptosis (Ravassa et al 2000) and cardiomyocyte apoptosis in SHR is accompanied by an increase in angiotensin-converting enzyme (ACE) activity in the left ventricle (Díez et al 1997). If these effects are related to the degree of LV hypertrophy, and obesity promotes LV hypertrophy as in the present study in SHR, obesity may mediate cardiomyocyte apoptosis in SHR by increasing the susceptibility of the myocardium in SHR to RAAS-induced cardiomyocyte apoptotic effects. As I did not explore the possibility that RAAS activation may mediate obesity-induced cardiomyocyte apoptosis in SHR in the present study, further studies are therefore required to explore the possibility that the susceptibility of SHR to obesity-induced cardiomyocyte apoptosis occurs through RAAS activation.

Obesity-induced cardiomyocyte apoptosis may also be mediated by the accumulation of triglycerides in the heart as reported to occur in obese ZDF rats (Zhou et al 2000). In this regard, a combination of lipolysis induced by catecholamines (Imura et al 1971) and a decrease in the expression of myocardial enzymes responsible for fatty acid oxidation results in the accumulation of myocardial fatty acids (Zhou et al 2000). The accumulation of myocardial fatty acids may result in a lipotoxic effect with increased levels of ceramides promoting cardiomyocyte apoptosis (Zhou et al 2000, Young et al 2002) through caspase-3 activation (Wang et al 2000). However, whether LV hypertrophy in hypertension increases the susceptibility of the myocardium to obesity-induced lipotoxic effects has not been assessed. Further studies are therefore required to

assess whether obesity-associated cardiomyocyte apoptosis noted to occur in SHR in the present study can be attributed to an increased susceptibility of the pressure-overloaded hypertrophic heart to myocardial lipotoxicity.

4.7 Cardiomyocyte apoptosis in previous studies in SHR

Excessive cardiomyocyte apoptosis has been reported to occur in SHR from 4 (younger) to 24 months (aged) of age (Díez et al 1997, Li et al 1997). In the present study the percentage cardiomyocyte apoptosis in SHR not receiving the experimental diet was no different to age-matched, normotensive WKY rats. These findings are not inconsistent with previous findings from our group in SHR (Veliotes et al 2005). Differences between studies in cardiomyocyte apoptosis in SHR may be attributed to the age of rats and the stage of the transition to heart failure when cardiomyocyte apoptosis is assessed. In this regard, Li et al (1997) noted excessive cardiomyocyte apoptosis in SHR with, but not without heart failure. Moreover, differences in the sensitivity of the techniques employed to detect cardiomyocyte apoptosis may account for apparent discrepancies in data between groups.

4.8 Obesity-associated hypertension and diabetes mellitus

In the present study, 5 months of feeding WKY rats and SHR a diet that promoted an increase in body weight did not affect systolic blood pressure or blood HbA_{1c}. Other rodent models of obesity produced by diets either high in fat or moderately high in fat

have been associated with increases in systolic blood pressure and blood glucose concentrations (Boustany et al 2004, Smith et al 2006, Dobrian et al 2001, Dobrian et al 2000, Boustany et al 2005). Moreover, obesity induced in SHR fed a sucrose-rich diet has been reported to result in an increased systolic blood pressure after 3 weeks on the diet (Preuss et al 2006). However, not all studies support the notion that dietary-induced obesity is associated with increases in blood pressure (du Toit et al 2008). Moreover, obesity produced by feeding rats a high fructose diet may either increase or have no effect on systolic blood pressure (Hwang et al 1987, D'Angelo et al 2005). It is possible that blood pressure changes in rats with obesity could be a result of a heightened stress response associated with restraint and tail-cuff inflation involved in the technique (D'Angelo et al 2005). Importantly, in the present study, the lack of blood pressure effects associated with dietary-induced obesity were reproduced in young and elderly normotensive rats and in rats with pre-existing hypertension (SHR). Whether more severe forms of obesity may have produced a blood pressure effect remain to be determined. Furthermore, whether 24 hour blood pressure monitoring using telemetric techniques could unveil a blood pressure effect also requires further study.

4.9 Clinical implications

The prevalence of obesity is increasing in both developed and developing countries (Bourne et al 2002, Flegal et al 2002, Ogden et al 2006). Although body weight is independently related to the development of heart failure (Contaldo et al 2002, Kenchaiah et al 2002, Ingelsson et al 2005), the mechanisms of these effects are

uncertain. Although reductions in cardiac systolic and diastolic function occur in overweight people, which persist after adjustment for blood pressure, age, gender and left ventricular mass (Peterson et al 2004, Wong et al 2004), more recent studies suggest that a reversal of abnormalities in systolic function may not be associated with weight loss in obese people (Skilton et al 2007, Wong et al 2006). Furthermore, although a number of studies suggest that myocardial contractile disturbances may occur in animal models of obesity (Caroll et al 1997, Relling et al 2006, Dong et al 2006, Ren et al 2000), no study has been able to show an obesity-associated reduction in pump function *in vivo*. In addition, although a number of animal studies have demonstrated that obesity is associated with excessive cardiomyocyte apoptosis (Zhou et al 2000, Barouch et al 2006), these studies have not excluded the possibility that hypertension and an abnormal blood glucose control mediate these effects. In the present dissertation I have been able to show that obesity interacts with pressure-overload hypertrophy and with adrenergic activation to promote excessive cardiomyocyte apoptosis through effects that cannot be attributed to changes in blood pressure or blood glucose control. As cardiomyocyte apoptosis may have a causal role in the pathophysiology of ventricular dysfunction and its progression to cardiac failure (Cheng et al 1996, Abbate et al 2003, Olivetti et al 1997, Frustaci et al 1999, Kocher et al 2001, Li et al 1997), the data reported on in the present dissertation provide direct evidence to support the notion that obesity may indeed contribute toward cardiac dysfunction, albeit in synergy with alternative factors such as hypertensive hypertrophy or sympathetic activation.

4.10 Limitations

The limitations of the present study are as follows. First, the modest degree of obesity induced in elderly WKY and SHR may have limited the outcomes of the study. However, as indicated in the aforementioned discussion employing a model of modest as opposed to severe obesity is nevertheless entirely consistent with the high prevalence of mild-to-moderate obesity which presently exists world-wide, whilst more severe forms of obesity are less common (McTigue et al 2006, Davenport et al 2009, Neovius & Rasmussen 2008, Abdulla et al 2008, López-García et al 2008, Wattie et al 2008). Second the technique used to detect cardiomyocyte apoptosis (TUNEL technique) may overestimate the number of apoptotic nuclei, as it labels both DNA fragmentation and cells undergoing DNA repair. Alternative techniques such as the assessment of myocardial caspase-3 activity or other methods could have been employed to support the present outcomes. Indeed, active caspase-3 may have also been a more sensitive technique as it is activated during the early phases of apoptosis (Nicholson et al 1995). However, the assessment of myocardial caspase-3 activity requires freeze-clamped tissue, the collection of which would have precluded the assessment of heart and ventricular weights. Third, as indicated in the aforementioned discussion I have not identified a potential mechanism responsible for the obesity-associated increase in percentage cardiomyocyte apoptosis in SHR or in normotensive rats receiving a daily adrenergic stimulus. However, as previously outlined, there are a considerable number of potential mechanisms that could play a role, all of which require further study. Hence evaluating all of these mechanisms goes beyond the boundaries of the present dissertation. Fourth,

with regard to the signalling mechanisms that may induce the observed changes in cardiomyocyte apoptosis in the present model, Western blots would have provided insight. However Western blots require freeze-clamped heart tissue. To achieve this, the sample size would have to be increased for additional heart tissue samples. In addition, the isolated perfused heart experiments do not allow for the collection of fresh tissue samples required for Western blots. With regard to the renin-angiotensin aldosterone system (RAAS), as with western blots myocardial Ang II requires freeze-clamped heart tissue. To achieve this, the sample size would have to be increased for additional heart tissue samples. In addition, the isolated perfused heart experiments do not allow for the collection of fresh tissue samples required to determine myocardial Ang II activity. Furthermore no clear blood pressure differences were observed in our study and hence it is unlikely that serum Ang II would be changed.

4.11 Conclusion

In conclusion, the results of the present study indicate that mild-to-moderate obesity is indeed associated with an enhanced cardiomyocyte apoptosis but through an interaction with pressure-overload hypertrophy and an excessive sympathetic activation. These findings therefore suggest possible mechanisms through which obesity may play a role in mediating the development of heart failure.

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