

CARDIOVASCULAR FUNCTION IN ANIMAL MODELS OF METABOLIC SYNDROME  
AND TYPE 2 DIABETES  
-THE ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS)

by

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## ABSTRACT

Activation of inducible nitric oxide synthase (iNOS) and oxidative stress have been shown to be associated with compromised cardiovascular function in streptozotocin (STZ)-induced type 1 diabetes. The aim of the project is to investigate cardiovascular abnormalities in a rat model of type 2 diabetes (Zucker diabetes fatty or ZDF rats) and two models of metabolic syndrome (fructose-fed rats and Zucker obese rats), and to provide direct evidence linking iNOS and oxidative stress to abnormal cardiovascular function in these disorders. Blood pressure, cardiac contractility, cardiac index, regional flow, vascular resistance and venous tone were measured in diseased as well as normal rats. Biochemical analyses such as activities of iNOS, immunostaining of iNOS and western-blot analysis of iNOS in the heart tissue were carried out. The results showed that cardiac contractile response to dobutamine was compromised in the ZDF rats, and this was associated with increased myocardial protein expression as well as activity of iNOS. The formation of peroxynitrite was increased in the heart tissue of the ZDF rats. Selective inhibition of iNOS by 1400W (N-3-aminomethyl-benzyl-acetamidine) did not alter responses to dobutamine in the control rats, but augmented the contractile effects of dobutamine in the diabetic rats. The regional blood flow was altered in the ZDF rats, and iNOS played a negligible role in regulating regional flow in the ZDF rats. Although venous response to noradrenaline was also altered in the Zucker obese rats, NOS may not be involved in venous tone regulation. Anti-oxidative treatment with N-acetylcysteine inhibited the development of insulin resistance, blood pressure elevation and the increase of 8-isoprostane formation in the fructose-fed rats. We conclude that heart function is compromised and regional blood flow is altered in the ZDF rats. Activation of iNOS plays an important role in suppressing heart dysfunction but does not affect regional blood flow. In Zucker obese rats with metabolic syndrome, iNOS may not be involved in changes of venous function. Oxidative stress is associated with both abnormality of heart dysfunction in type 2 diabetes (by formation of peroxynitrite due to iNOS activation) and development of hypertension and insulin resistance in metabolic syndrome.

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## LIST OF ABBREVIATIONS

ATP III	Adult Treatment Panel III
ANOVA	analysis of variance
apo B	apolipoprotein B
AUC	area under the curve
BF	blood flow
BP	blood pressure
CI	cardiac index
cNOS	constitutive NOS
cGMP	cyclic 3',5'-guanosine monophosphate
CO	cardiac output
DOCA-salt	deoxycorticosterone-sodium salt
+dP/dt	maximal rate of increase of left ventricular pressure
EDRF	endothelial-derived relaxing factor
eNOS	endothelial NOS
FAP	final arterial pressure
GC	guanylate cyclase
GK rats	Goto-Kakizaki rats
GTP	guanosine triphosphate
HDL	high-density lipoprotein
HR	heart rate
IDDM	insulin-dependent diabetes mellitus, type 1 diabetes
IDF	International Diabetes Federation
iNOS	inducible nitric oxide synthase
ISI	insulin sensitivity index
LPS	lipopolysaccharide
LV	left ventricular

LVEDP	LV end diastolic pressure
LVP	LV systolic pressure
LVDP	left ventricular developed pressure
MAP	mean arterial pressure
MCFP	mean circulatory filling pressure
NT	nitrotyrosine
L-NAME	N <sup>G</sup> -monomethyl-L-arginine
NIDDM	non-insulin-dependent diabetes mellitus, type 2 diabetes
NCEP	National Cholesterol Education Program
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
$\cdot\text{NO}_2$	nitrogen dioxide
N <sub>2</sub> O <sub>3</sub>	dinitrogen trioxide
OLETF rats	Otsuka-Long-Evans-Tokushima Fatty rats
O <sub>2</sub> <sup>-</sup>	superoxide anion
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
RPP	rate pressure product
STZ	streptozotocin
TPR	total peripheral resistance
VPP	venous plateau pressure
WHO	World Health Organization
ZDF rats	Zucker diabetic fatty rats
XOR	xanthine-oxidoreductase
1400W	N-3-aminomethyl-benzyl-acetamidine

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# **1. INTRODUCTION**

## **1.1 Overview of diabetes mellitus**

### **1.1.1 Definition and prevalence of diabetes mellitus**

Diabetes mellitus is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

Diabetes has become a major public health problem and social burden in North America and around the world (King et al., 1998). According to a report of the World Health Organization (WHO) in 2000, over 171 million people were affected by diabetes around the world and this number will increase to more than 300 million by the year 2025 (WHO, 2000). In Canada, more than two million people have diabetes and it has been estimated that over three million Canadians will suffer from the disease by the end of the decade. It has been estimated that diabetes is a contributing factor in the deaths of approximately 41,500 Canadians each year (Canadian Diabetes Association., 2007). In the U.S., an estimated 7% of the population or 20.8 million people had diabetes according to a report released in 2005 (Centers for Disease Control and Prevention, 2005). Because of the increasing prevalence, diabetes exerts a substantial burden on the direct health care costs in many countries. In 1998, the total economic burden of diabetes and its chronic complications in Canada was estimated to be between 4.76 and 5.32 billion U.S. dollars. The direct medical costs associated with diabetes care, before considering any complications, were \$573 million. If the costs associated with the complications of diabetes were considered, the amount spent was around \$637 million (Dawson et al., 2002). In the United States, direct medical and indirect yearly expenditures attributable to diabetes were estimated at \$132 billion in 2002 (Hogan et al., 2003; Centers for Disease Control and Prevention, 2005). Diabetes has become a worldwide concern, and its associated morbidity and mortality will continue to cast an enormous burden upon personal, public and economic costs.

### **1.1.2 Classification of diabetes mellitus**

Uniform diagnostic criteria for diabetes were first recommended by the American Diabetes Association and WHO in 1979 and 1980. Originally, diabetes mellitus was mainly classified as two types: insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). The classification and diagnostic criteria were later updated and revised in the late 1990s (Alberti and Zimmet, 1998). The Canadian Diabetes Association (CAD) adopted the updated diagnostic criteria in 1998.

According to the current revised criteria, diabetes mellitus is classified into the following categories: (1) Type 1 diabetes (formerly called type I, IDDM or juvenile diabetes) is caused by  $\beta$ -cell destruction, usually leading to absolute insulin deficiency. It occurs in comparatively young subjects and accounts for approximately 10% of all cases of diabetes; (2) Type 2 diabetes (formerly called NIDDM, type II or adult-onset diabetes) is characterized by insulin resistance in peripheral tissue with relative insulin deficiency or an insulin secretory defect. It accounts for approximately 90% of all diabetic cases; (3) Other specific types of diabetes include those of known etiologies, such as diabetes caused by genetic defects of beta-cell function or insulin action. This group of diabetes also includes those caused by drug- or chemical-induced pancreatic dysfunction; (4) Gestational diabetes refers to diabetes that occurs during pregnancy.

### **1.1.3 Diagnostic criteria of diabetes mellitus**

Under the most recent guidelines of diagnostic criteria, diabetes mellitus is diagnosed when two fasting plasma glucose readings are 126 mg/dl (7.0 mmol/L) or higher. Other options for diagnosis include two-hour postprandial plasma glucose (2hrPPG) readings of 200 mg/dL (11.1 mmol/L) or higher after a glucose load of 75 g or two casual glucose reading of 200 mg/dL (11.1 mmol/L) or higher (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

#### **1.1.4 Cardiovascular complications in diabetes**

Consistent hyperglycemia in chronic diabetes can cause metabolic abnormalities which affect functions of various organs and eventually lead to complications. Both type 1 and type 2 diabetes are associated with long-term complications that threaten life and the quality of life. Specific long-term complications of diabetes include: (1) Retinopathy with potential loss of vision; (2) Nephropathy leading to end-stage renal disease and failure; (3) Neuropathy with risk of foot ulcers, amputation, sexual dysfunction, and potentially disabling dysfunction of the stomach, bowel, and bladder; (4) Cardiovascular complications such as arteriosclerosis, congestive heart failure, diabetic cardiomyopathy etc.

Diabetic patients are very susceptible to cardiovascular complications. It has been shown that the incidence of cardiovascular diseases is 2-3 fold higher in diabetic patients than nondiabetic patients (Kannel and McGee, 1979). The death rate from cardiovascular diseases was also significantly higher in diabetic patients and up to 80% of mortality in diabetic patients is related to cardiovascular events (Kannel and McGee, 1979). Diabetes can cause both macrovascular and microvascular pathological changes. Macrovascular pathological changes may contribute to the development of atherosclerosis, coronary heart disease, stroke, peripheral vascular disease, hypertension and myocardial infarction. Microvascular pathological changes of diabetes can result in diabetic retinopathy, nephropathy, cardiomyopathy and neuropathy (Lorenzi and Cagliero, 1991; Tooke 2000; Uccella et al., 1991). Diabetic patients have an increased risk of congestive heart failure (Kannel et al., 1974; Kannel and McGee, 1979). Both systolic and diastolic function may be impaired in diabetic patients. Defective ventricular relaxation, increased left ventricular end-systolic volume and decreased left ventricular ejection fractions have been reported in diabetic patients (Ahmed et al., 1975; Fein, 1990; Shapiro, 1982). Diabetic cardiomyopathy is a common heart complication that affects heart function in patients with diabetes. The pathological condition of diabetic cardiomyopathy was originally described by Rubler et al. in 1972 on the basis of observations in four diabetic patients who presented with heart failure without evidence of hypertension, coronary arterial disease, valvular or congenital heart disease (Rubler et al., 1972). Later,

diabetic cardiomyopathy was defined as cardiac dysfunction caused by diabetes independent of dominant pathophysiological changes such as coronary atherosclerosis, macroangiopathy and autonomic neuropathy (Davies, 2000; Richardson et al., 1996). Cardiac dysfunction in diabetic cardiomyopathy may be caused by defects in myocardial cellular organelles such as myofibrils, mitochondria, sarcoplasmic reticulum and sarcolemma. Impairment of calcium handling and lipid accumulation in the cardiomyocytes may also play a role in depressing heart function in patients with diabetic cardiomyopathy (Lagadic-Gossman et al., 1996; Yokoyama et al., 2004; McNeill, 1996). The exact mechanisms that lead to cardiac dysfunction in diabetic cardiomyopathy are still not very clear.

## **1.2 Metabolic syndrome**

### **1.2.1 Definition of metabolic syndrome**

Metabolic syndrome was identified first in adults by Reaven (Reaven, 1988) as a series of abnormalities that tended to coexist in patients who have insulin resistance. The cluster of symptoms has been variously labeled as syndrome X, insulin resistance syndrome, or dysmetabolic syndrome. Slightly differing definitions of metabolic syndrome are being used by different health organizations. In the concept proposed by WHO, insulin resistance is the underlying cause of metabolic syndrome, and so insulin resistance or glucose intolerance is required for its diagnosis (Alberti et al., 1998 and 2006). In the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) definition, however, insulin resistance is not required for the diagnosis of metabolic syndrome. A diagnosis of metabolic syndrome is established when at least 3 of the following criteria are present: abdominal obesity, high levels of triglycerides, low levels of high-density lipoprotein (HDL) cholesterol, hypertension, and increased fasting glucose. Building on the NCEP and WHO definitions, the International Diabetes Federation (IDF) has recently proposed a new definition for metabolic syndrome, which requires a diagnosis of central obesity, and two of the following: raised triglycerides, reduced HDL-cholesterol, raised blood pressure (BP), or raised fasting plasma glucose (International Diabetes Federation. 2006). Currently, the NCEP ATP III definition is

the most widely used criterion for diagnosing metabolic syndrome (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003; Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). It is applicable to routine clinical practice and quantifies variables that are easily measurable.

### **1.2.2 Prevalence of metabolic syndrome**

Metabolic syndrome is a common health problem around the world. According to a new report from the third national health and nutrition examination survey, 25% of the adult American population meets the diagnostic criteria of metabolic syndrome (Park et al., 2003). The percentage increases to 44% if only Americans over fifty years old are considered (Alexander et al., 2003b; Alberti and Zimmet, 1998). The DECODE (Diabetes Epidemiology: Collaborative analysis of Diagnostic criteria in Europe) study found that 31% of men and 34% of women have metabolic syndrome in the European population (Qiao, 2006). Currently, the incidence of metabolic syndrome in youth is also increasing. An investigation that analyzed data from 1988-1992 and from 1999-2000 found that the prevalence of metabolic syndrome in youth had increased from 4.2% to 6.4% in these short time periods (Duncan et al., 2004). Another investigation which analyzed data from the third National Health and Nutrition Examination Survey (1988-1994) using the NCEP (ATP III) diagnostic criteria showed that the overall prevalence of the metabolic syndrome among adolescents aged 12 to 19 years was 4.2% and the syndrome was present in 28.7% of overweight adolescents (body mass index  $\geq$  95th percentile) (Cook et al., 2003). All these studies indicate that metabolic syndrome is becoming a common health problem that affects both adolescents and older people of our society.

### **1.2.3 Metabolic syndrome and cardiovascular diseases**

Patients with metabolic syndrome are at increased risk for developing cardiovascular disease (Alexander, 2003a and 2003b; Wilson et al., 2005; Kendall and Harmel, 2002; Isomaa et al., 2001). Epidemiological studies have shown that metabolic syndrome predicts the



development of cardiovascular morbidity and mortality (Lakka et al., 2002; Hu et al., 2004) and patients with metabolic syndrome have a three-times greater risk of coronary heart disease and stroke than those without the syndrome (Isomaa et al., 2001). Metabolic syndrome has been found to be an important predictor of subclinical heart dysfunction in patients without overt cardiovascular disease (Wong et al., 2005; Isomaa et al., 2001). Among individuals with no previous cardiovascular disease, cardiovascular mortality increased much faster in those with metabolic syndrome than in those without, and increased prevalence of coronary heart disease was found among adults over 50 years of age with metabolic syndrome (Alexander, 2003b). In the peripheral vessels, components of metabolic syndrome such as hypertension, hyperlipidemia and insulin resistance are associated with the development of endothelial dysfunction (Panza et al., 1990; Balletshofer et al., 2000; Kamata and Yamashita, 1999; Katakam et al., 1998).

### **1.3 Insulin resistance: the link between metabolic syndrome and type 2 diabetes**

Metabolic syndrome and type 2 diabetes are closely linked. Data from the San Antonio Heart Study show that metabolic syndrome is a stronger risk factor for diabetes than it is for cardiovascular diseases. Patients with metabolic syndrome are four times more likely to develop diabetes than those without the syndrome but twice as likely to develop cardiovascular disease (Lorenzo et al., 2003). In patients with type 2 diabetes, the prevalence of the metabolic syndrome is as high as 75.6% (Bruno et al., 2004). Currently, there is sufficient evidence that insulin resistance is an important link to the development of metabolic syndrome and type 2 diabetes.

Insulin resistance refers to a decrease in the biological and physiological response to insulin, resulting in over-secretion of insulin to compensate for the impairment of glucose transport in skeletal muscle and fat cells (Sowers and Frohlich, 2004; Sowers, 2004). Insulin resistance is a common abnormal condition present in both metabolic syndrome and type 2 diabetes. Studies have demonstrated that hyperinsulinaemia, which develops in response to insulin resistance, precedes the development of type 2 diabetes (Vuguin et al., 2004; Hanley et

al., 2005). There is evidence which shows that abnormalities of metabolic syndrome, namely, obesity, hyperlipidemia and hypertension are all associated with the development of hyperinsulinemia and insulin resistance. For example, it has been found that basal and total 24-hour rates of insulin secretion are 3-4 times higher in obese subjects compared with non-obese subjects (Polonsky et al., 1988) and visceral/subcutaneous adiposity is associated with the development of insulin resistance (Wagenknecht et al., 2003). Hypertension is prevalent in obesity and in type 2 diabetes, both of which are associated with insulin resistance. Various prospective and cross-sectional studies have documented a correlation between insulin resistance, hyperinsulinaemia and elevated BP (Frontoni et al., 2005; Sarafidis et al., 2006; Zarich et al., 2005). Abnormality in vascular activity in the presence of insulin resistance is one of the main mechanisms that lead to elevated BP in these conditions. It has been shown that impaired brachial artery reactivity and endothelial dysfunction are associated with increased severity of insulin resistance (Caballero et al., 1999). Lipoprotein abnormalities in diabetes and metabolic syndrome consist of increases in plasma levels of triglycerides, apolipoprotein B (apo B), and small dense LDL particles, with marked reductions in plasma levels of HDL-C (including HDL<sub>2</sub>-C) and apo A-I (Brunzell, 2002). The presence of these characteristics have been associated with insulin resistance (Fujimoto et al., 1994; Tchernof et al., 1996). Studies also showed that healthy subjects with increased visceral adiposity and insulin resistance have elevated plasma levels of triglycerides, sdLDL, and apo B. In addition, subjects with an elevated total cholesterol/HDL cholesterol ratio were found to have higher systolic and diastolic BPs, in addition to being insulin-resistant, glucose-intolerant and hypertriglyceridemic (Nieves et al., 2003). All these findings suggest that insulin resistance is associated with type 2 diabetes, obesity, primary hypertension and dyslipidemia. In the state of insulin resistance, hyperinsulinemia is the result of increased amounts of insulin secreted by  $\beta$  cells in the pancreas. Progressive deterioration of the metabolic state results in eventual failure of endogenous hyperinsulinemia to compensate fully for the insulin resistance, thereby causing impaired glucose tolerance, and ultimately diabetes (DeFronzo, 1992; DeFronzo et al., 1992).

## **1.4 Haemodynamic changes in diabetes**

### **1.4.1 Changes of cardiac contractility in type 1 and type 2 diabetes**

Cardiac contractile dysfunction is very common in patients with type 1 and type 2 diabetes (Bell, 2003). It has been shown that the incidence of congestive heart failure is increased by 2.5-5 fold in male and female diabetic patients, and this is independent of age, hypertension and coronary artery disease (Kannel et al., 1979). Depression of left ventricular (LV) contractility has been shown in rats with streptozotocin (STZ)-induced type 1 diabetes (Dai and McNeill, 1992; Dowell et al., 1986; Litwin et al., 1990; Okayama et al., 1994). However, there is currently a paucity of information on cardiac function of rats with type 2 diabetes and published research results are not consistent. *In vivo* echocardiographic measurements have shown depressed (Zhou et al., 2000) or increased (Fredersdorf et al., 2004) baseline cardiac contractile function of 19-20 week old Zucker diabetic fatty (ZDF) rats, an animal model of type 2 diabetes. *In vitro* studies have shown unchanged baseline contractile function of isolated perfused hearts from 11-week-old ZDF rats (Chatham and Seymour, 2002). Inconsistent results regarding cardiac contractile function in these rats may be related to variability of the age of the animals or severity of diabetes and the use of different methods for the evaluation of cardiac function. It remains to be determined if ZDF rats have reduced cardiac contractile response to adrenoceptor agonists in the chronic phase of diabetes.

### **1.4.2 Changes in cardiac output (CO) and blood flow in rats and humans with type 1 diabetes**

Varying results have been reported regarding CO and regional blood flow in type 1 diabetes. At an early stage (six days) of STZ-induced diabetes, CO and hindquarter arterial flow were decreased in rats (Brands et al., 2000). When diabetes was induced by withdrawing intravenous insulin-replacement, a progressive decrease in CO was observed in STZ-treated rats and this was associated with significant increases in glomerular filtration rate and renal blood flow (Brands et al., 2000). CO as well as flows to the skeletal muscle and skin beds was

found to be reduced, but flows to the kidneys and intestine were increased at eight weeks after the onset of STZ-induced diabetes (Hill and Larkins, 1989). However, unchanged CO has also been reported at four weeks after injection of STZ (Litwin et al., 1990). In type 1 diabetic patients, Doppler echocardiograms also detected higher CO compared with controls, whereas total peripheral resistance (TPR) was found to be lower in diabetic patients (de Simone et al., 2000). A previous study in our lab found that baseline CO and cardiac index (CI) were similar between the control and STZ-induced diabetic rats (CO,  $100 \pm 22$  and  $99 \pm 13$  ml/min; CI,  $232 \pm 58$  and  $280 \pm 36$  ml/min kg, respectively). TPR was also similar between the control and diabetic rats ( $0.99 \pm 0.05$  and  $0.89 \pm 0.05$  mmHg min/ml). Blood flow in the diabetic rats was decreased in the kidneys, increased in the intestine and colon/caecum, but similar in other organs and tissues (Cheng et al., 2004b).

### **1.4.3 Changes in CO and blood flow in rats and humans with type 2 diabetes**

Blood flow measurements have been made in only limited beds of animals with type 2 diabetes. CO was higher but CI was lower in 12 and 24 week old db/db mice (Van den Bergh et al., 2006). Relative to control rats, pancreatic blood flow (BF) was shown to be increased in 3-4 month old non-obese diabetic Goto-Kakizaki rats (GK rats; Svensson et al., 2007) and in the prediabetic (non-obese) phase, but not in the diabetic phase, of Otsuka-Long-Evans-Tokushima Fatty rats (OLETF rats; Iwase et al., 2002). The GK rats also had increased duodenal but similar colonic BF relative to the controls (Svensson et al. 2007). *Psammomys abesus* (sand rat) in the early stage of type 2 diabetes were found to have reduced renal BF (Hilzenrat et al., 1996). There is a need to determine if type 2 diabetes differentially affects the distribution of BF to various organs and tissues.

## **1.5 Nitric oxide (NO), nitric oxide synthase (NOS) and cardiovascular function**

### **1.5.1 Overview of NO and NOS**

In 1980, Furchgott and Zawadzki first described their observations that acetylcholine-mediated vascular relaxation occurred only when endothelium was present in the

aorta, inferring the existence of a substance that caused the relaxation of the vessels. The substance released was termed endothelial-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Later on, it was shown that endothelial cells produce NO and NO release accounts for the biological activity of EDRF (Palmer et al., 1987; Palmer et al., 1988). NO is synthesized by the oxygen-dependent enzyme NOS, using the semi-essential amino acid, L-arginine, as the natural precursor which is oxidized to NO and citrulline. The cofactors of the enzyme involved in this processes are NADPH, flavin-adenine-dinucleotide, flavinmononucleotide and tetrahydrobiopterin (BH<sub>4</sub>). NOS exists in three major isoforms: the neuronal nitric oxide synthase (nNOS or NOS 1), the inducible nitric oxide synthase (iNOS, or NOS 2) and the endothelial nitric oxide synthase (eNOS or NOS 3). There is evidence that eNOS mainly exists in platelets and endothelial cells, nNOS in neurons and iNOS in vascular endothelial cells, smooth muscle cells, cardiomyocytes, macrophages and mast cells (Mayer and Andrew, 1998; Colasanti and Suzuki, 2000). Both eNOS and nNOS are constitutive and calcium-calmodulin-dependent. The constitutively produced NO is able to diffuse lumenally and ablumenally. It is a highly reactive molecule with a half-life of only a few seconds. Constitutively produced NO is involved in a variety of physiological regulatory process such as regulation of vascular tone, neurotransmission, immune defense, inhibition of smooth muscle cell proliferation and platelet adherence (Cooke and Dzau, 1997; Munzel et al., 1997; Murad, 1998). In contrast, the activation of iNOS is non-calcium-calmodulin-dependent, and it requires an inducing factor such as inflammation, endotoxin, ischemia or other disorders. When iNOS is activated and induced in pathophysiological conditions, a large quantity of NO is produced, and this may cause injuries to tissues and organs. In physiological conditions, NO functions as an endogenous activator at concentrations of 100-500 nM to activate guanylate cyclase (GC) which converts guanosine triphosphate (GTP) to cyclic 3',5'-guanosine monophosphate (cGMP), a second messenger in cells. However, at higher concentrations (1-3  $\mu$ M) such as when iNOS is induced and activated, NO can react with superoxide anion (O<sub>2</sub><sup>-</sup>) and result in the production of peroxynitrite, which is toxic to cells and tissues. In this process, reactive nitrogen species (RNS) such as nitrogen dioxide (NO<sub>2</sub>) and dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>)

are also produced, which cause oxidative stress and cytotoxicity as a result of the oxidation of deoxyribonucleic acid (DNA), thiols, amino acids and metals in cells (Beckman and Koppenol, 1996).

### **1.5.2 NO and cardiovascular function**

NO plays a key role as a mediator in multiple physiological as well as pathological processes affecting cardiovascular function. Under physiological conditions, production of NO in the endothelium is primarily catalyzed by constitutively-produced eNOS. Synthesis and release of NO in the endothelium can be stimulated by neurohormonal substances, including acetylcholine, substance P, bradykinin, adenosine diphosphate, or by mechanical stimuli, such as shear stress and cyclic strain (Cannon, 1998). The produced NO diffuses from the endothelium to the underlying smooth muscle cells to activate soluble guanylyl cyclase (GC) and results in the formation of cGMP. The latter activates protein kinase G which in turn decreases the cytosolic concentration of calcium thereby causing the relaxation of vascular smooth muscle cells and vasodilation (Cannon, 1998; Behrendt and Ganz, 2002). In addition to the effect of vasodilation, NO also influences vascular tone and homeostasis by inhibiting the vasoconstrictive, thrombotic, inflammatory, and fibrotic actions of angiotensin II (Ang II) and endothelin-1 by down-regulating the syntheses of angiotensin-converting enzyme, angiotensin II type 1 receptors, and endothelin-1 in the endothelium (Raij, 2001). Besides dilating peripheral arteries, NO also mediates dilation of large coronary arteries and cardiac microvasculature (Quyyumi et al., 1995a and 1995b).

In the heart, NO goes well beyond its vasodilator and antithrombotic actions. All three isoforms, namely, iNOS, nNOS and eNOS are expressed in cardiomyocytes (Massion et al., 2003; Kelly et al., 1996). The neuronal isoform nNOS is expressed in cholinergic and nonadrenergic, noncholinergic nerve terminals, in specialized conduction tissue in the heart (Schmidt et al., 1992; Tanaka et al., 1993) and in sympathetic nerve terminals, where it has been postulated to play a role in catecholamine release and reuptake (Schwarz et al., 1995; Kaye et al., 1995). NO produced by eNOS can inhibit cell growth, platelet aggregation and monocyte

adhesion. It can also decrease oxygen consumption and oppose the inotropic action of catecholamines (Balligand and Cannon, 1997). Studies in mice have demonstrated varying inotropic effects following eNOS or nNOS gene deletion, with some mice having greater myocardial contractility (Massion et al., 2003; Brunner et al., 2001; Cotton et al., 2001). In pathophysiological conditions, iNOS has been found to be activated in vascular endothelial cells, smooth muscle cells, cardiomyocytes, macrophages and mast cells (Mayer and Andrew, 1998; Colasanti and Suzuki, 2000), and this results in the production of large amounts of NO in the cells and tissues. The large amounts of NO produced by iNOS is a two edged weapon that can be both beneficial and harmful. On one hand, large amount of NO is necessary in the process of antibacterial and anti-inflammatory and other protective or compensatory actions in response to pathological changes. On the other hand, however, too much NO production can impair contractile function of cardiomyocytes (Balligand et al., 1993; Brady et al., 1992; Schulz et al., 1992) and vascular smooth muscle cells (Fleming et al., 1991; Rees, et al., 1990; Knowles and Moncada, 1992).

The role of iNOS in cardiovascular diseases has been widely investigated in both humans and animals. For example, over-expression and induction of iNOS were detected in hearts from animals with heart failure, such as rats with experimental autoimmune myocarditis, rats with volume and pressure-overload heart failure and mice with myocardial infarction (Zhang et al., 2007; Feng et al., 2001; Gealekman et al., 2002; Ishiyama et al., 1997). Rats with volume-overload heart failure had activation of iNOS as well as reduced contractility to isoproterenol in the papillary muscle (Gealekman et al., 2002). In humans, it has been reported that NO produced in the heart was associated with the attenuation of positive inotropic response to dobutamine ( $\beta$ -adrenoceptor agonist) in patients with left ventricular dysfunction (Hare et al., 1995). The expression of iNOS was induced in the heart of an animal model of congestive heart failure induced by chronic transverse aortic constriction. Selective iNOS inhibition with 1400W (N-3-aminomethyl-benzyl-acetamidine) significantly attenuated systolic overload-induced myocardial hypertrophy and pulmonary congestion (Zhang et al., 2007). However, inconsistent results regarding the

effect of iNOS induction has also been observed. For example, in patients with dilated cardiomyopathy, increased gene expression of endomyocardial iNOS or eNOS was found to be correlated with enhanced LV stroke volume and work, with a beneficial rightward shift in the diastolic LV pressure-volume relation and increased LV preload reserve (Heymes et al., 1999). More studies are needed to clarify the role of iNOS activation on cardiovascular function in conditions of contractile abnormality.

## **1.6 NO and NOS in diabetes**

### **1.6.1 Hyperglycemia and iNOS**

There is evidence that iNOS is activated at the acute phase of diabetes through the influence of hyperglycemia. Perfusion of rat hearts for 2 h with a solution high in glucose increases the expression of the iNOS (but not eNOS) gene, the generation of NO and superoxide, and apoptosis of cardiac cells (Ceriello et al., 2002a). The exposure of aortic endothelial cells to high glucose for 7-10 days increases the release of peroxynitrite, a highly reactive and cytotoxic oxidant derived from the reaction of NO with superoxide (Ceriello et al., 2002b). Diabetes is associated with increased production of cytokines in addition to hyperglycemia. There is evidence that hyperglycemia, in the presence of cytokines, stimulates cellular production of iNOS. For example, high glucose enhances stimulated iNOS promoter activity and iNOS mRNA expression upon challenge with lipopolysaccharide (LPS) and interferon-gamma in murine macrophage cells (cell line RAW 264.7) and murine glomerular mesangial cells (Sharma et al., 1995). As well, iNOS is undetectable in rat aortic endothelial cells; but is activated in the presence of high glucose and interleukin-1 beta.

### **1.6.2 Diabetes and eNOS**

The vascular endothelium is damaged in both types of diabetes, and this alters the bioavailability of endothelium-derived dilators, e.g., NO and prostacyclin. The formation or action of eNOS is reported to be increased, unchanged or decreased in clinical as well as experimental type 1 and 2 diabetes (Farkas et al., 2000; Chan et al., 2000). Discrepancies may



be due to variations in the model, duration/severity of diabetes, method of measuring the concentration or effect of NO, presence or absence of inactivators of NO, e.g.,  $O_2^-$  that reacts with NO or glycation end-products that quench NO, and the type of vessels studied (conduit or resistance artery). For example, relaxation to acetylcholine is reduced in the perfused mesenteric bed but unchanged in the aorta of the same rat with STZ-induced diabetes (Taylor et al., 1994). Response to noradrenaline is reduced in the aorta but normal in mesenteric microvessels of rats with alloxan-induced diabetes (Fortes et al., 1983).

### **1.6.3 Type 1 diabetes and iNOS**

NO is one of the most important endogenous vasodilators. Functional and biochemical studies have shown that iNOS is activated in type 1 diabetes, and that iNOS can be detected in the mesenteric artery at week 12-14 (Bardell and MacLeod, 2001) and in cardiac myocytes from diabetic rats at week 8 (Smith et al., 1997) after injection of STZ, and in platelets of patients with type 1 diabetes (Tannous et al., 1999). The expression of iNOS protein was found to be increased in the myocardium, small mesenteric arteries and aorta as early as 3 weeks after the induction of diabetes by STZ, but the expression of eNOS was decreased at week 9 (Nagareddy et al., 2005) in STZ diabetic rats. NO production and iNOS mRNA expression in macrophages were increased in rats or mice with autoimmune-prone (type I) and STZ-induced diabetes and these increases were abolished by treatment with insulin (Stevens et al., 1997). Results from our previous studies showed that relative to control rats, those with STZ-induced diabetes had decreased *in vivo* cardiac contractility and arterial and venous constriction to noradrenaline, as well as increased myocardial iNOS activity, iNOS mRNA and immunostaining of iNOS. Furthermore, selective inhibition of iNOS by administration of 1400W augmented vascular and left ventricular contractile response to noradrenaline in the diabetic rats but not the controls (Cheng et al., 2004b; Cheng and Pang, 2004; Cheng et al., 2003a). Nitrotyrosine, an *in vivo* biomarker for oxidative damage induced by peroxynitrite and other reactive nitrogen species, was identified in the hearts of rats with type 1 diabetes but not those of control rats (Cheng et al., 2004b). These results suggest that iNOS plays a

pathophysiological role on cardiac contractile function in rats with type 1 diabetes and the mechanism may involve the formation of peroxynitrite from overproduction of NO by iNOS.

#### **1.6.4 Role of iNOS and eNOS in cardiovascular dysfunction in type 2 diabetes**

The cardiovascular role of iNOS and eNOS in type 2 diabetes remains unclear. There is evidence that iNOS is activated in type 2 diabetes. The expression of eNOS was shown to be decreased, but that of iNOS was increased in the internal mammary artery from patients with type 2 diabetes (Okon et al., 2005). Expression of iNOS was higher in the hearts of 7 and 14 weeks old ZDF rats with type 2 diabetes (Zhou et al., 2000). The expression of eNOS was increased in the cerebral arteries of 3-4 month old ZDF rats (Schwaninger et al., 2003) and aorta of 12 and 36 week old GK rats (Kobayashi et al., 2004), but decreased in the aorta of 11 month old ZDF rats (Erdely et al., 2004). The functional cardiovascular influence of eNOS and iNOS in type 2 diabetes remains unexplored. Nitrotyrosine and iNOS-dependent peroxynitrite are detected in the blood (Ceriello et al., 2001, 2002a, 2002b; Zou et al., 2002) and platelets (Tannous et al., 1999) of patients with type 2 diabetes. The '+' allele of the iNOS promoter variant or allele 210bp of the iNOS gene is present in patients with type 2 diabetes and are associated with increased frequency of nephropathy, neuropathy and retinopathy (Kumaramanickavel et al., 2002; Morris et al., 2002). Furthermore, iNOS is identified in the penile cavernosal smooth muscle and endothelium of diabetic patients (Seftel et al., 1997). Immunoactivity of iNOS, iNOS expression and/or nitrotyrosine are increased in the retina of rats with type 2 diabetes (Carmo et al., 2000; Ellis et al., 2002). At 7 and 14 week old, myocardial expressions of iNOS mRNA in the ZDF rats are 2-4 fold those of lean rats (Zhou et al., 2000). There is also evidence that iNOS activation causes apoptosis of pancreatic  $\beta$ -cells and insulin resistance (Wang et al., 1998; Sugita et al., 2002; Perreault and Marette, 2001). As well, iNOS is over-expressed in the muscle tissue of ZDF rats (Perreault and Marette, 2001). These indicate that iNOS plays an important role in cardiovascular dysfunction in type 2 diabetes.

### **1.7 Involvement of NOS in changes of vascular reactivity and distribution of regional blood flow in type 2 diabetes**

Microvascular and macrovascular complications are common in diabetic patients (McMillan, 1997; Yamagishi and Imaizumi, 2005). Diabetes-induced microvascular changes can alter the diameter of resistance arteries thereby affecting CO and the distribution of BF. The cardiovascular role of NOS in type 2 diabetes remains unclear. The expression of eNOS was shown to be decreased, but that of iNOS was increased in the internal mammary artery from patients with type 2 diabetes (Okon et al., 2005). The expression of eNOS was increased in the cerebral arteries of 3-4 month old ZDF rats (Schwaninger et al., 2003) and aorta of 12 and 36 week old GK rats (Kobayashi et al., 2004), but decreased in the aorta of 11 month old ZDF rats (Erdely et al., 2004). The functional vascular influence by eNOS and iNOS in type 2 diabetes remains unexplored. Currently, there is no information on how regional BF is differentially altered in organs and tissues in animal models of type 2 diabetes and how iNOS and constitutive NOS (cNOS) influence regional haemodynamics in type 2 diabetes.

### **1.8 Obesity/ metabolic syndrome and iNOS**

Animal models of obesity and metabolic syndrome are found to be associated with the activation of iNOS. Perreault and Marette (2001) have shown that the expression of iNOS mRNA is increased in the skeletal muscle and adipose tissue of mice fed a high-fat diet (relative to the chow-fed mice). In addition, mice fed a high-fat diet develop insulin resistance in the skeletal muscle. Targeted disruption of the gene encoding iNOS protects against the development of insulin resistance. Mice fed with an obesogenic high-fat diet were found to have increased expression of iNOS in aorta with evidence of increased vascular NO production. Knockout of iNOS gene in obese mice was shown to protect against the development of resistance to the glucoregulatory and vascular effects of insulin (Noronha et al., 2005). These results suggest a pathological role of iNOS in the development of insulin resistance and other abnormalities in metabolic syndrome.

## **1.9 Venous function in the presence of obesity in metabolic syndrome**

Obesity is at present a worldwide epidemic, and is associated with the development of hypertension, diabetes and coronary artery disease (Kopelman, 2000). Although the relationship between obesity and hypertension in metabolic syndrome is recognized, the mechanism by which obesity increases BP remains poorly understood. BP is a product of CO (or venous return) and peripheral vascular resistance, the latter being increased in obese patients with hypertension (Kopelman, 2000). There is currently a paucity of information on how metabolic syndrome affects venous function.

There is evidence that hypertension is associated with altered venous function as determined by measurements of mean circulatory filling pressure (MCFP) or venous compliance. MCFP is the pressure that would occur throughout the circulation if all vascular pressures were brought into equilibrium, and is an index of body venous tone (Pang, 2001; Pang, 2000). Venous compliance is the ratio of a change in volume to a concomitant change in the transmural distending pressure (Pang, 2001; Pang, 2000). Increased MCFP has been reported in various animal models of hypertension, including deoxycorticosterone-sodium salt (DOCA-salt) hypertensive rats (Fink et al., 2000), spontaneous hypertensive rats (Martin et al., 1998), two-kidney 1-clip hypertensive rats (Edmunds et al., 1989) as well as angiotensin II- (Ang II, Young et al., 1980) or aldosterone-induced hypertensive dogs (Pan and Young, 1982). However, unchanged MCFP has also been shown in DOCA-salt hypertensive rats (Yamamoto et al., 1983). Studies have also demonstrated reduced venous compliance in the large limb veins, but not jugular veins, of dogs with perinephritic hypertension (Overbeck, 1972), and reduced mesenteric compliance in dogs with one-kidney but not two-kidney perinephritic hypertension (Simon et al., 1975). Rats with two-kidney 1-clip or renal medullectomy-induced hypertension, though, did not show a change in venous compliance (Edmunds et al., 1989; Edmunds et al., 1990). Due to technical limitations, venous function in humans can only be assessed in regional beds; however, studies in hypertensive patients also show alterations in venous function. Venous occlusion plethysmography studies have shown reduced total vascular compliance in the forearms of hypertensive haemodialysis patients

relative to those of normotensive patients (Kooman et al., 1992), as well as in the forearms of patients with either hypertension, obesity, or both (Stepniakowski and Egan, 1995). Vascular compliance is also reduced in the lower limbs of hypertensive patients (Milio et al., 1997). It is well known that the sympathetic nervous system plays a key role in regulating both arterial and venous pressure. However, studies on the changes in sympathetic nerve activity in obesity-related hypertension have been inconclusive (Pamidimukkala and Jandhyala, 1996; Morgan et al., 1995; Alonso-Galicia et al., 1996; Pan and Young, 1982). Results from different laboratories regarding vascular response to  $\alpha$ -adrenoceptor agonists have been inconsistent (Subramanian and MacLeod, 2003; et al., 2002; Ouchi et al., 1996). Studies need to be done to characterize whether venous function is altered in the presence of characteristics of metabolic syndrome such as obesity and insulin resistance. Hence, it is also of interest to see if NO/NOS plays a role in the regulation of venous tone in animals with metabolic syndrome.

### **1.10 Inhibitors of the action of iNOS**

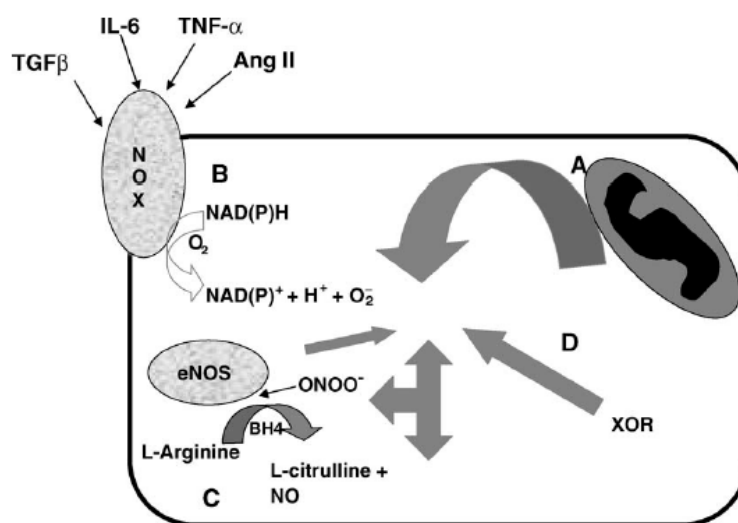
Investigation of the *in vivo* role of iNOS requires the use of an inhibitor of iNOS. 1400W (N-3-aminomethyl-benzyl-acetamidine) has been found to be the most selective, potent and efficacious iNOS inhibitor and its action is virtually irreversible (Garvey et al., 1997; Alderton et al., 2001). The *in vivo* toxicity of this compound is low. In one study, rats tolerated i.v. infusion of 120 mg/day of 1400W for 7 days (Garvey et al., 1997). We have previously found that 1400W (i.v. 3 mg/kg) prevented the late phase fall in mean arterial pressure (MAP) without worsening CO and tissue BF<sub>s</sub> in rats with LPS-induced septicemia, and it did not alter MAP, CO or flow in control rats (Cheng et al., 2003b). In contrast,  $N^{\omega}$ -nitro-L-arginine (L-NNA, non-selective NOS inhibitor) does not prevent the decline in MAP in LPS-treated rats, and drastically reduced CO through an increase in vascular resistance. Therefore 1400W, through selective inhibition of iNOS, may be beneficial in the management of conditions associated with an overproduction of NO. In 3 wk STZ diabetic rats, acute administration of 1400W increased MAP and cardiac contractile responses to noradrenaline

(Cheng et al., 2004b).

## **1.11 Oxidative stress**

### **1.11.1 Overview of oxidative stress**

Oxidative stress refers to the situation of a serious imbalance between production of free radicals and antioxidant defence which leads to potential tissue damage (Halliwell 1995). Free radical species includes a variety of highly reactive molecules such as  $O_2^{\cdot -}$ , hydroxyl radicals ( $OH^{\cdot}$ ), peroxy radical  $ROO^{\cdot}$  in the reactive oxygen species (ROS) group and  $NO^{\cdot}$ ,  $NO_2^{\cdot}$ , peroxynitrite in reactive nitrogen species (RNS) group. Free radical reactions play a key role in defence mechanisms as with neutrophils, macrophages and other cells of the immune system. However, if too many free radicals are produced, they may cause tissue injury and cell death (Halliwell 1992, 1996). Sources of free radicals and oxidative stress include: (A) uncoupling of mitochondrial oxidative phosphorylation; (B) activation of the NAD(P)H oxidase system by inflammatory mediators such as IL-6 and TNF- $\alpha$  and growth factors such as TNF- $\beta$  and Ang II; (C) uncoupling of NO synthesis and its augmentation by positive feedback through peroxynitrite formation; and (D) the xanthine-oxidoreductase (XOR) system (Fig 1.11.1; Mehta et al. 2006). To avoid free radical overproduction, antioxidants are synthesized to neutralize free radicals. Antioxidants include a manifold of enzymes such as superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, glutathione, and compounds such as vitamin C. In pathological conditions, both increase in the formation of ROS/RNS and a decrease in the ability to inactivate ROS/RNS by antioxidants may result in tissue damage (Halliwell, 1995).



**Figure 1.11.1** Sources of oxidative stress. TGFβ, Transforming growth factor beta; IL-6, Interleukin-6; TNF-α, tumor necrosis factor-alpha; NO<sub>x</sub>, a type of nitrogen oxide; NAD(P)<sup>+</sup>, Nicotinamide adenine dinucleotide phosphate; BH<sub>4</sub>, Tetrahydrobiopterin; XOR, xanthine oxidoreductase; NO, Nitric oxide.

### 1.11.2 Oxidative stress in metabolic syndrome and diabetes

There is experimental and clinical evidence that the generation of ROS, such as O<sub>2</sub><sup>-</sup> and hydrogen peroxide, are increased in type 1 and type 2 diabetes as well as in metabolic syndrome (Mehta et al., 2006). Hyperinsulinemia (increased plasma insulin) and insulin resistance (suppressed insulin-stimulated glucose uptake) are associated with several metabolic and cardiovascular disorders that include hypertension and diabetes mellitus (DeFronzo and Prato, 1996; Bressler et al., 1996; Ginsberg, 2000; DeFronzo, 1997). There is evidence that ROS, such as O<sub>2</sub><sup>-</sup> and hydrogen peroxide, are involved in the pathogenesis of endothelial dysfunction (Lum and Roebuck, 2001), insulin resistance and hypertension (Evans et al., 2003). This may involve reduced production of vasodilators such as endothelial-derived NO, increased inactivation of NO (Lum and Roebuck, 2001) and/or generation of vasoconstrictors such as isoprostanes through peroxidation of arachidonic acid (Lum and Roebuck, 2001). There is also evidence that free radical scavengers, such as vitamin E (Jain and Wise, 1995), glutathione (Faure et al., 1997) and SOD (Nakazono et al., 1991) are depressed in patients and experimental

animals with hypertension and/or insulin resistance. Furthermore, treatments with antioxidants have been shown to reduce BP in the spontaneously hypertensive rats (Noguchi et al., 2001; Vaziri et al., 2000; Vasdev et al., 2002; Vasdev et al., 2001). Moreover, treatment of diabetic animals with probucol (lipid-lowering and antioxidant) (Kaul et al., 1995), or with vitamin E (Faure et al., 1997; Lacy et al., 1998; Paolisso et al., 1993) has been shown to reduce insulin resistance. It is plausible to speculate that antioxidants have a protective role against the development of hyperinsulinemia, insulin resistance and hypertension. N-acetylcysteine is a free radical scavenger which increases intracellular levels of glutathione and acts as an antioxidant (Kelly, 1998). In a previous study, it was found that supplementation with N-acetylcysteine improved cardiac contractile function in STZ-induced type 1 diabetes (Cheng et al., 2005). The effects of chronic N-acetylcysteine treatment on hypertension, insulin resistance and other metabolic abnormalities in animal models of metabolic syndrome and type 2 diabetes remain to be studied.

### **1.11.3 Biological markers of oxidative stress**

Biological markers of oxidative stress currently used in research include 3-nitrotyrosine for protein damage, F<sub>2</sub>-isoprostanes, malondialdehyde and/or thiobarbituric acid reactive substances for lipid peroxidation damage, and 8-hydroxydeoxyguanosine for DNA damage (Rosen et al., 2001). Nitrotyrosine is derived from the attack of tyrosine residues in proteins by various RNS (such as peroxynitrite, NO<sup>•</sup>) and therefore is a biomarker for RNS-induced oxidative damage (Kajstura et al., 2001; Frustaci et al., 2000; Halliwell, 1997). F<sub>2</sub>-isoprostanes are generated by the non-enzymatic free radical oxidation of arachidonic acid in membrane phospholipids and lipoproteins when there is increased oxidative stress in the body and they are reliable biomarkers of lipid peroxidation and oxidative stress (Davi et al., 1999; Morrow et al., 1999; Barden et al., 1996).

## **1.12 Animal models**

### **1.12.1 Obese Zucker rats (ZF/fa/fa; 14-16 wk) versus aged-matched lean Zucker**



### **(Gmi/fa/+) control rats**

The obese Zucker rat is an established model of obesity (Argiles JM. 1989). In addition to being obese, these rats have metabolic syndrome characterized by hyperglycemia, insulin resistance and a modest increase in BP (Pamidimukkala and Jandhyala, 1996; Alonso-Galicia et al., 1996; He and MacLeod, 2002; Schreihöfer, Hair et al., 2005; Alonso-Galicia et al., 1996).

### **1.12.2 Fructose-fed rats and normal-chow-fed control rats**

The fructose-fed rat is a model of acquired hypertension that exhibits insulin resistance, hyperinsulinemia and hypertriglyceridemia (Galipeau et al., 2002) as well as endothelial dysfunction (Kamata and Yamashita, 1999; Katakam et al., 1998). These abnormalities are associated with the human condition of metabolic syndrome X, and are important risk factors of coronary heart disease (Reaven, 2003; Timar et al., 2000).

### **1.12.3 Zucker diabetic fatty rats (homozygous recessive; ZDF/fa/fa) versus aged-matched Zucker lean rats (homozygous dominant; +/+)**

The ZDF is an inbred rat model that closely mimics human adult onset diabetes (type 2) and some of the major diabetes-related complications. Homozygous recessive males (fa/fa) develop obesity, hyperlipidemia, fasting hyperglycemia and type 2 diabetes. Homozygous dominant (+/+) and heterozygous (fa/+) lean genotypes remain normoglycemic. The ZDF rat is an excellent model for type 2 diabetes based on impaired glucose tolerance caused by the inherited obesity gene mutation which leads to insulin resistance. The mutation, which occurs in both the ZDF and Zucker rat, results in shortened leptin receptor protein which does not effectively interact with leptin. This mutation is phenotypically expressed as obesity with high levels of normal leptin in the blood (Considine, 2005). In the ZDF rat, hyperglycemia is initially manifested at about 7 weeks of age, and all obese male rats are fully diabetic by 12 weeks. Between 7 and 10 weeks, blood insulin levels are high, but these subsequently decrease as the pancreatic beta cells cease to respond to the glucose stimulus. This loss of

response to glucose is associated with the disappearance of glucose type 2 transporters (GLUT2) on the beta cells in the islets.

### **1.13 Rationale and objectives of the study**

It is known that small amounts of NO, as synthesized by eNOS and nNOS, are important for physiological function, but large amounts of NO, as produced by iNOS in pathophysiological conditions can be detrimental (Colasanti and Suzuki, 2000). Previous experiments in our laboratory have shown that at 3 weeks after induction of type 1 diabetes by injection of STZ, conscious rats have diminished cardiac and vascular contractile responses to adrenergic agents due to the induction of iNOS, and that the suppression of iNOS activity ameliorates cardiovascular dysfunction. However, the functional role of iNOS is unclear in type 2 diabetes. There is evidence that iNOS is activated in the hearts of rats with type 2 diabetes and metabolic syndrome. The degree of influence of iNOS in these metabolic disorders is likely complex and dependent on the duration of diabetes (acute or chronic) or metabolic disorder (e.g., degree of hyperinsulinemia, obesity or hyperglycemia), and organs/tissues where iNOS is induced (heart and/or specific vasculature). The activation of iNOS in the heart may lead to depressed left ventricular contractility. Alterations of NOS activity in particular vasculatures may cause abnormality in arterial as well as venous function in diabetes. Constrictive functional changes of resistance and capacitance vessels may influence BP, venous return and CO. It is not known how these haemodynamic parameters are altered in metabolic syndrome and type 2 diabetes. In type 1 diabetes, it has been found that the depressed cardiovascular function is associated with oxidative stress and decreased antioxidative capacity due to the activation of iNOS. There is evidence to show that the gene expression of iNOS as well as the generation of ROS is increased in metabolic syndrome and type 2 diabetes. In addition, it has been found that free radical scavengers, such as glutathione (Faure et al., 1997) and SOD (Nakazono et al., 1991) are depressed in patients and experimental animals with hypertension and/or insulin resistance. Hence we speculate that cardiovascular function is depressed in type 2 diabetes and changes of NOS activity caused by the induction of

iNOS play an important role in the suppressed cardiovascular function in metabolic syndrome and type 2 diabetes. Treatments with selective iNOS inhibitor (1400W) or antioxidant (N-acetylcysteine, a glutathione donor) may protect against the development of metabolic as well as haemodynamic abnormalities in metabolic syndrome and diabetes.

In particular, we would like to find out:

- 1) If hyperglycemia in type 2 diabetes is associated with the over-expression and activation of iNOS and if activation of iNOS is associated with compromised cardiovascular function at the diabetic (20 wk) phase of ZDF rats (type 2 diabetes) relative to changes in the control rats.
- 2) If acute treatment of an iNOS inhibitor (1400W) restores cardiovascular function in ZDF rats.
- 3) If the regional distribution of BF and CO are altered in ZDF (20 wk of age) rats relative to the lean controls and if NOS is involved in altered vascular tone and BF in type 2 diabetes in the presence of obesity.
- 4) If obesity in metabolic syndrome is associated with altered venous function (obese Zucker rats) and if NOS is involved in the alteration of vascular function (arterial and venous) in metabolic syndrome.
- 5) If oxidative stress is associated with the development of hypertension and insulin resistance in metabolic syndrome in the absence of hyperglycemia. If chronic treatment of fructose-fed rats (an animal model of metabolic syndrome) with N-acetylcysteine (a free radical scavenger and glutathione donor) has a protective action against the progression of hypertension, hyperinsulinemia and insulin resistance.

#### **1.14 Hypothesis of the study**

We hypothesize that iNOS is activated at the established phases of type 2 diabetes. The activation of iNOS is associated with the suppressed heart function and alteration of regional BF in type 2 diabetes. The pathophysiological process involves oxidative stress via

the formation of peroxynitrite due to the excessive amounts of NO produced by iNOS. In metabolic syndrome, changes of NOS activity are not only associated with alteration of arterial contractility but also are involved in changes of venous function. Oxidative stress is involved in the development of hypertension and insulin resistance in metabolic syndrome and is a common occurrence in both type 2 diabetes and metabolic syndrome.

In particular, we hypothesize:

- 1) In ZDF rats (animal model of type 2 diabetes), iNOS is activated in the heart as well as blood vessels and this is associated with compromised cardiac contractility, cardiac output and the alteration of vascular activity as well as the regional distribution of BF.
- 2) The acute administration of a selective inhibitor of iNOS (1400W) improves cardiac contractility and vascular function in ZDF rats.
- 3) CO and regional flow are changed in ZDF rats which is associated with altered NOS function.
- 4) MCFP (index of body venous tone) and pressor responses to noradrenaline and N<sup>G</sup>-nitro-arginine methyl ester (L-NAME, inhibitor of NOS) are increased in Zucker obese rats relative to Zucker lean rats.
- 5) Chronic N-acetylcysteine treatment increases insulin sensitivity, preventing the development of hypertension and hyperinsulinemia in fructose-fed rats.

## **2. MATERIALS AND METHODS**

### **2.1 Animals**

Animals used in the experiments (Zucker diabetic fatty rats, Zucker obese rats and Sprague-Dawley rats) were ordered from Charles River Lab., Québec, Canada. All the rats were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

### **2.2 Methods**

#### **2.2.1 Anaesthesia of animals**

In experiments conducted in conscious animals, the rats were anaesthetized with halothane (1.5% in air) while surgery was performed. All inserted cannulae were filled with heparinized normal saline (heparin 25 IU/ml in 0.9% NaCl) and tunneled subcutaneously to the back of the neck, exteriorized and secured. After surgery, rats were allowed 6 or 24 hours to recover from the effects of surgery and anaesthesia before use. At the time of the experiments, the rats were given 1 h to equilibrate before the commencement of studies.

In experiments conducted in anaesthetized animals, the rats were anaesthetized with thiobutabarbital (100 mg/kg, i.p.), tracheotomised, and allowed to breath spontaneously in room air. Body temperature was maintained at 37°C by a rectal thermometer and a heat lamp connected to a Thermistemp Temperature Controller (Model 71, Yellow Springs Instrument Co., Ohio, USA).

#### **2.2.2 Blood pressure and heart rate (HR) measurements**

Under anaesthesia, polyethylene cannulae (PE50) filled with heparinized saline (0.9% NaCl, 25 I.U./ml) were inserted into the left or right femoral artery for the measurement of BP via a pressure transducer (p23DB, Gould Statham, CA, USA). BP and HR were displayed and recorded by a BIOPAC computerized data acquisition system (MP 150). In experiments conducted in conscious animals, cannulae were filled with heparinized normal saline (heparin 25 IU/ml in 0.9% NaCl) and tunneled subcutaneously to the back of the neck, exteriorized and

secured.

### **2.2.3 Measurement of left ventricular function**

Cannulae were inserted into the left ventricle via the right carotid artery for the recording of left ventricular peak systolic pressure (LVP), left ventricular end-diastolic pressure (LVEDP) and maximum rate of rise of LV pressure (+dP/dt). Left ventricular developed pressure (LVDP) was derived by subtracting LVEDP from LVP, and rate pressure product (RPP =  $HR \times LVDP$ ) was calculated as an index of cardiac performance. After 6 h of recovery from anaesthesia and surgery, each rat was placed in a small cage and allowed to wander freely. Pressure transducers (P23DB, Gould Statham, Oxnard, CA) were connected to the implanted cannulae, and all pressure recordings were stored in a computer using AcqKnowledge software (Biopac System Inc., Goleta, CA, USA).

### **2.2.4 Measurement of mean circulating filling pressure (MCFP)**

MCFP was measured as previously described (Pang 2000). The rats were anaesthetized with halothane and a polyethylene cannula (PE50) was inserted into the left iliac artery to record MAP. Cannulae were inserted into the right iliac vein for the administration of drugs, and into the inferior vena cava through the left iliac vein for the measurement of central venous pressure. All pressures were measured by P23DB pressure transducers (Gould Statham, CA, USA) and displayed on a polygraph (Grass 7D, Grass Instruments, Quincy, MA, USA). A saline-filled, balloon-tipped catheter was advanced into the right atrium through the right external jugular vein. All cannulae were filled with heparinized saline (0.9% NaCl, 25 I.U./ml), tunneled subcutaneously to the back of the neck, exteriorized and secured. Central venous pressure was measured after transiently stopping the circulation through injection of a small volume of fluid into the right atrial balloon. Within 5 secs following inflation of the balloon, MAP decreased to a plateau value (final arterial pressure, FAP), while central venous pressure increased to a plateau value (venous plateau pressure, VPP). In order to correct for incomplete equilibration of arterial and venous pressures during circulatory arrest, MCFP was calculated as follows:  $MCFP = VPP + 1/60 (FAP - VPP)$ , where 1/60 represents the ratio of

arterial to venous compliance. The experiments were conducted in conscious animals after 24 hours of recovery from the effect of surgery and anaesthesia.

### **2.2.5 Measurement of cardiac output and regional blood flow**

Under thiobutabarbital anaesthesia, surgery includes cannulation of the left ventricle via the right carotid artery for the injection of radioactively labeled microspheres, and cannulation of the right femoral artery for blood withdrawal (Pang, 1983). The left femoral artery and vein were also cannulated for the recording of MAP and the administration of drugs, respectively. The experiments were conducted in unconscious animals given 1 hour of stabilization after surgery. At the time of experiments, a well-stirred suspension (100-200  $\mu$ l) containing 20,000-40,000 microspheres (15  $\mu$ m diameter) labeled with either  $^{57}\text{Co}$ ,  $^{51}\text{Cr}$  or  $^{103}\text{Ru}$  (Perkin-Elmer Life Sciences, Boston, MA, USA) was injected sequentially into each rat in a random order and flushed over 10 s into the left ventricle. Each determination involves injection into the left ventricle over 10 s of a well-stirred suspension (250  $\mu$ l) containing 20,000-25,000 microspheres. Beginning at 10 s before the injection of each set of microspheres, blood was withdrawn (Harvard pump) from the right femoral arterial cannula into a heparinized saline-filled syringe at 0.35 ml/min for 45 s. The blood was returned to the rats after the counting of radioactivity by a gamma counter with a 3-inch NaI crystal (1185 series, Nuclear-Chicago, IL, USA). At the end of the experiments, both kidneys were removed, weighed and counted for radioactivity. If the counts of any sets of microspheres between the two kidneys differed by 15%, the experiment was rejected. Afterwards, the remaining whole organs, adipose tissue, as well as 30 g each of skeletal muscle (from areas of the chest, abdomen, back, hindlimb, and forelimb) and skin (from areas of the chest, abdomen and back) were also removed for counting. Corrections were made in each experiment for the spillover of radioactivity from  $^{51}\text{Cr}$  into the  $^{57}\text{Co}$  channel, and for spillover of  $^{103}\text{Ru}$  into the  $^{57}\text{Co}$  and  $^{51}\text{Cr}$  channels.

Calculations were done as follows:

Cardiac Output (CO, ml/min)

$$= \frac{\text{Blood withdrawal rate (ml/min)} \times \text{tissue cpm}}{\text{cpm in withdrawn blood}}$$

Total peripheral resistance (TPR, mm Hg min/ml)

$$= \frac{\text{MAP (mm Hg)}}{\text{CO (ml/min)}}$$

Blood flow (BF, ml/min)

$$\text{BF} = \frac{\text{rate of withdrawal of blood} \times \text{organ c.p.m.}}{\text{c.p.m. in withdrawn blood}}$$

Arterial conductance (ml/mm Hg min)

$$= \frac{\text{Blood flow (ml/min)}}{\text{MAP (mm Hg)}}$$

Values for BF and arterial conductance were normalized for tissue weight.

### **2.2.6 Drug administration**

Cannulae were inserted into the right and/or left iliac veins for the administration of drugs. In experiments conducted in conscious animals, cannulae were filled with heparinized normal saline (heparin 25 IU/ml in 0.9% NaCl) and tunneled subcutaneously to the back of the neck, exteriorized and secured. The cannulae were re-connected and flushed at the time of experiments.

### **2.2.7 Evaluation of insulin sensitivity by oral glucose tolerance test and insulin sensitivity index**

Rats were fasted for 12 h and given an oral glucose tolerance test. Each rat was given, by oral gavage, a solution containing 40% glucose (1 g/kg body wt). Blood samples were taken at 0, 10, 20, 30, 60 and 90 min after gavage for the measurements of plasma glucose and insulin via a Glucose Reagent Kit (Infinity<sup>TM</sup>, Sigma) and radioimmunoassay kit (Linco Research Inc, MO), respectively. Total area under the curves for insulin and glucose were calculated using the trapezoidal method. The insulin sensitivity index was calculated from the



data of plasma glucose and insulin according to the formula of Matsuda and DeFronzo (1999). It has been shown that results derived from the insulin sensitivity index correlate well with those from the euglycemic hyperinsulinemic clamp technique (Matsuda and DeFronzo, 1999).

#### **2.2.8 15-F<sub>2t</sub>-isoprostane assay**

Enzyme-linked immunoassay was used to measure free 15-F<sub>2t</sub>-isoprostane concentration (pg/ml) in the plasma according to the instructions of the manufacturer (EIA Kit, Cayman Chemical, Ann Arbor, MI) with slight modifications. Briefly, the ventricular tissues (0.4 g) were homogenized in phosphate-buffered saline (PBS, 1.0 ml). Afterwards, 15-F<sub>2t</sub>-isoprostane in the homogenate was extracted with ethanol (2.0 ml) containing butylated hydroxytoluene (0.005%) to prevent oxidation, and centrifuged (2,000 g) for 10 min. The supernatant was mixed with potassium hydroxide (15%) for the determination of total 15-F<sub>2t</sub>-isoprostane by base hydrolysis. The pH was adjusted to 7.0-7.5 with HCl. The samples were then incubated at 40 °C for 1 h, and diluted 10-fold with ultra pure water. Each sample was passed through a 4 ml 15-F<sub>2t</sub>-isoprostane (8-isoprostane) affinity column (Cayman Chemical) for purification. The affinity column was first washed with column buffer (2 ml), UltraPure water (2 ml), then elution solution (2 ml) to elute 15-F<sub>2t</sub>-isoprostane from the columns. The elution solution in test tubes was evaporated and dried by a stream of nitrogen. Then, enzyme immunoassay buffer (0.5 ml) was added to dissolve 15-F<sub>2t</sub>-isoprostane. The concentration of 15-F<sub>2t</sub>-isoprostane was quantified by enzyme immunoassay (Xia, Godin et al., 2003). Plasma samples (50 µl) were added in triplicate into a 96-well plate followed by the addition of 15-F<sub>2t</sub>-isoprostane acetylcholinesterase tracer and the antibody. The prepared plates were incubated overnight at room temperature. The next day, the plates were washed five times with the washing buffer, followed by the addition of Ellman's reagent. After 80 min, the plates were read at 405 nm with the operator blinded. Tissue 15-F<sub>2t</sub>-isoprostane content was expressed as pg/g tissue.

### **2.3.9 Plasma glucose, insulin and triglyceride assays**

Plasma glucose concentration was measured with a glucose reagent kit (Infinity™, Thermo DMA, Inc., OH, USA), and plasma triglyceride concentration was determined with an enzymatic colorimetric kit (Infinity™, Thermo DMA, Inc., OH, USA). Enzyme-linked immunoassay was used to measure the concentration of plasma insulin according to the instructions of the manufacturer (EIA Kit, Crystal Chem Inc., IL, USA).

### **2.2.10 Assay of NOS activity**

Frozen myocardial tissues from experiments were homogenized with a Polytron homogenizer in 5 volumes (W/V) of cold (4°C) homogenization buffer containing sucrose (250 mM), ethylenediaminetetraacetic acid (EDTA, 0.1 mM), phenylmethane sulphonyl fluoride (1 mM), dithiothreitol (1 mM) and potassium phosphate (5 mM) at pH 7.4. The homogenate was then centrifuged at 10,000g at (4°C) for 20 min. Activity of iNOS in the supernatant was quantified by measuring the formation of radiolabeled L-[<sup>3</sup>H]-citrulline from L-[<sup>3</sup>H]-arginine as described by Wu et al. (2001) but with slight modifications. The supernatant (50 µl) was incubated in a reaction mixture containing Tris-HCl (25 mM, pH 7.4), flavin adenine dinucleotide (10 µM), tetrahydrobiopterin (10 µM), L-arginine (0.2 µCi), L-[<sup>3</sup>H]-arginine (Amersham), NADPH (1 mM), CaCl<sub>2</sub> (2 mM) and calmodulin (10 µg/ml, Roche Diagnostics) in a total volume of 100 µl for 1 hour at 37°C. The reaction was terminated by the addition of cold, stop buffer (100 µl, 50/6 mM of HEPES-EDTA, pH < 5.5). Dowex 50W (200-400, 1 ml 8% cross-linked, Na<sup>+</sup> form; 1:1 w/v in water) was added to the solution to remove excess L-[<sup>3</sup>H]arginine. Afterwards, the samples were centrifuged at 5,000 g for 10 min. An aliquot (500 µl) of the supernatant was removed to a vial, and scintillation liquid (4 ml) was added for the counting of radioactivity using a standard scintillation counting procedure (Beckman Coulter, USA, LS 6000TA). The non-specific radioactivity of the enzymatic reaction was determined in the presence of L-NNA (5 mM, nonselective inhibitor of NOS) in the reaction mixture. Ca<sup>2+</sup>-independent activity (iNOS) was measured by the addition of 1 mM EDTA and EGTA (instead of Ca<sup>2+</sup> and calmodulin). Protein content in the supernatant was determined by

the DC Protein Assay using a standard curve prepared with bovine serum albumin (DC protein assay reagents package, Bio-Rad Laboratories Inc., USA). NOS activity was expressed as fmol/min/mg protein. L-(<sup>3</sup>H)-arginine was obtained from Amersham Biosciences (Baie D'Urfé, Québec, Canada), and calmodulin (10 µg/ml) was obtained from Calbiochem (San Diego, CA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA). The activity of iNOS was expressed as fmol/min/mg protein.

#### **2.2.11 Immunostaining of iNOS and Nitrotyrosine**

Heart tissues were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), processed and embedded in paraffin wax. The tissues were then cut into 5-µm sections. All sections were deparaffinized and rehydrated in clearing solution and a graded ethanol series. Endogenous peroxidase was blocked by incubating the sections in methanol/hydrogen peroxide (methanol, 47.2 ml; H<sub>2</sub>O<sub>2</sub> 30%, 0.8 ml) for 30 min at room temperature. The sections were then labeled with primary antibodies against iNOS (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) or nitrotyrosine (Cayman Chemical Company, Ann Arbor, MI USA). Some sections were treated with mouse nonspecific immunoglobulin G (1:200, Vector Laboratories, Burlingame, CA, USA) instead of the primary antibody, and they served as negative controls. After incubation with the primary antibody, the sections were washed three times in PBS and stained with peroxidase-conjugated secondary antibodies for 1 h at room temperature. Sections were then incubated with diaminobenzidine tetrahydrochloride /H<sub>2</sub>O<sub>2</sub> (25 mg tetrahydrochloride and 2 drops of 30% H<sub>2</sub>O<sub>2</sub>) for 5 min.

#### **2.2.12 Western blot analysis of cardiac iNOS and nitrotyrosine protein expression**

Samples of frozen heart tissue were powdered in liquid nitrogen and then homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonider P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1% PMSF, 1% sodium orthovanadate and 1% protease inhibitor cocktail at 4°C. The homogenate was centrifuged at 10,000 g at 4°C for 30 min to remove the

insoluble material. Supernatants were collected, and the protein concentration was then measured with the Bradford protein assay reagent (Bio-Rad Laboratories, Inc, Hercules, CA, USA), using BSA as a standard. Equal amounts of protein (60 µg) from each sample were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% skim milk and then incubated overnight at 4°C with primary antibody against iNOS (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and nitrotyrosine (Cayman Chemical Company, Ann Arbor, MI USA). Following a 30-min wash, the membranes were incubated with secondary antibody conjugated to HRP for 1 h at room temperature. The membranes were then washed for 30 min and exposed to enhanced chemiluminescence reagents (Amersham Biosciences Corp., Piscataway, NJ, USA) for 1 min and developed on film. Densitometric analysis was performed to quantify the signal intensity.

## **2.3 Experimental protocols**

### **2.3.1 Study 1: Role of iNOS on cardiac contractile function in Zucker diabetic fatty rats.**

Male Zucker diabetic fatty rats (ZDF/ fa/fa) and aged-matched Zucker lean rats (12 weeks old; n=7 each group) were maintained under 12:12-h light-dark cycle and given standard laboratory chow and water *ad libitum*. Body weight, food and fluid intake were monitored each week.

When the rats were at 20 weeks of age, blood samples (0.05 ml) were collected from the tail vein for the measurement of blood glucose via the use of a glucose meter (AccuSoft Advantage). The rats were anaesthetized with halothane (1.5% in air) when surgery was conducted. The surgery included insertion of polyethylene cannula (PE50) into the left iliac artery for monitoring arterial pressure and HR, cannulation of the right and left iliac veins for the administration of drugs, and of the left ventricle via the right carotid artery for the recording of parameters for evaluation of left ventricular function. All cannulae were filled with heparinized normal saline (heparin 25 IU/ml in 0.9% NaCl) and tunneled subcutaneously to the back of the neck, exteriorized and secured. After 6 h of recovery from anaesthesia and surgery, each rat was placed in a small cage and allowed to wander freely. Pressure transducers

(P23DB, Gould Statham, Oxnard, CA) were connected to the implanted cannulae, and all pressure recordings were stored in a computer using AcqKnowledge software (Biopac System Inc., Goleta, CA, USA). The rats were given 1 h to equilibrate before conducting the experiments. Dose-response curves of HR, LVP, LVEDP and  $+dP/dt$  to dobutamine (1, 3, 10, 30  $\mu\text{g/kg/min}$ ) were constructed before and after the administration of 1400W (3 mg/kg over 5 min followed by 3 mg/kg/h i.v. for 1 h). This dose of 1400W has previously been found to significantly suppress iNOS activity in rats (Cheng et al. 2003b and 2004a). Each dose of dobutamine was infused until a plateau response was attained (2-5 min after the start of infusion), and this was followed by a recovery period of 10-15 min. At the end of the experiments, the hearts from the 1400W-treated control rats and ZDF rats were excised, cleaned in cold saline and divided into two-halves. One-half was flash frozen and stored at  $-70\text{ }^{\circ}\text{C}$  for the assay of iNOS activity and protein expression of both iNOS and nitrotyrosine, and the other half was fixed in 4% paraformaldehyde for immunostaining of iNOS and nitrotyrosine. In addition, hearts from three controls and three ZDF rats were excised and frozen in liquid nitrogen without receiving 1400 W for the measurement of baseline iNOS activity. Each pair of heart tissues from control and ZDF rats was assayed concurrently within 1 month of harvest. All tissues were assayed within 1 month.

### **2.3.2 Study 2: Selective *versus* non-selective suppression of NOS on regional haemodynamics in Zucker diabetic fatty rats.**

Male Zucker diabetic fatty rats (ZDF/ fa/fa) and aged-matched Zucker lean rats (12 weeks old; Charles River Lab., Québec, Canada; n=7 per group) were maintained under 12:12-h light-dark cycle and given standard laboratory chow and water *ad libitum*. Body weight, food and fluid intake were monitored each week. All the rats were cared for in accordance with the guidelines of the Canadian Council on Animal Care. At 20 weeks of age, blood samples (0.05 ml) were collected from the tail vein to assess blood glucose levels (using a glucometer: AccuSoft Advantage). The rats were anaesthetized with thiobutabarbital (100 mg/kg i.p.), tracheotomized, and allowed to breathe spontaneously in room air. Cannulae

were inserted into the left ventricle via the right carotid artery for the injection of radioactively labeled microspheres, and into the right femoral artery for blood withdrawal, as required for the measurement of flow and CO (Pang, 1983). The left femoral artery and vein were also cannulated for the recording of MAP by a pressure transducer (PD23DB, Gould, Statham, CA, USA) and the administration of drugs, respectively. The rats were given 1h to equilibrate before the start of experiments. HR was derived electronically from the upstroke of the arterial pulse pressure by a Biopac MP150 data acquisition system (Biopac System Inc., Goleta, CA, USA). All cannulae were filled with heparinized saline (0.9% NaCl, 25 I. U. /ml). A well-stirred suspension (100–200  $\mu$ l) containing 20,000–40,000 microspheres (15  $\mu$ m diameter) labeled with either  $^{57}\text{Co}$ ,  $^{51}\text{Cr}$  or  $^{103}\text{Ru}$  (Perkin-Elmer Life Sciences, Boston, MA, USA) was injected and flushed over 10 s into the left ventricle. Beginning at 10 s before the injection of each set of microspheres, blood was withdrawn (Harvard pump) from the right femoral arterial cannula into a heparinized saline-filled syringe at 0.35 ml/min for 45 secs. The microspheres were sequentially injected into each rat in a random order. The blood was returned to the rats after the counting of radioactivity by a gamma counter with a 3-inch NaI crystal (1185 series, Nuclear-Chicago, IL, USA). A first set of microspheres was injected to determine baseline flow. Following baseline measurements, 1400W was administered (3 mg/kg, i.v. injected over 5 min, followed by 3 mg/kg/h for 1 hour). This dose of 1400W was found to significantly suppress iNOS activity in rats, Cheng et al., 2004). Following 1400W administration, L-NAME (8 mg/kg, i.v. bolus) was given. This dose of L-NAME was found to significantly suppress NOS activity in rats (Leung et al., 2003). Haemodynamic measurements were taken once the response to 1400W or L-NAME had reached a steady state and full impact. Tissue and organs were then collected, weighed and counted for radioactivity. Only visceral adipose tissue was collected for calculation of BF.

### **2.3.3 Study 3: Venous function and role of NOS in conscious obese Zucker rats with metabolic syndrome.**

Male obese Zucker ( $Zf/fa/fa$ ) and male lean Zucker ( $ZUC-lepr^{fa}$ ) rats (12 weeks old)

from Charles River Laboratories (Québec, Canada) were maintained under 12 h (light) : 12 h (dark) cycle and given standard laboratory chow and water *ad libitum*. Food and fluid intake were monitored each week. At 14 weeks of age, a blood sample (0.8 ml, after 12 h fasting) was collected from the tail for the measurement of plasma glucose, insulin and triglycerides.

At 16 weeks of age, the rats received surgery and were given 24 h to recover. On the day of the study, each rat was placed in a small cage and allowed to wander freely. The rats were equilibrated for 1 h prior to the construction of MCFP response curves to noradrenaline (2.5, 10 and  $30 \times 10^{-9}$  mol/kg/min, i.v.). Each dose of noradrenaline was infused until a plateau response was attained (2-10 min after the start of infusion), which was followed by a recovery period of 10–15 min. MAP and central venous pressure were measured at the baseline and at the plateau phase of response to noradrenaline. At 30 min after administration of the last dose of noradrenaline, the rats were given L-NAME (8 mg/kg, i.v.). This dose of L-NAME was found in a previous study to cause a maximum pressor response in rats (Wang et al., 1995).

#### **2.3.4 Study 4: Effects of chronic N-acetylcysteine treatment on fructose-induced insulin resistance and hypertension in rats**

Male rats (5 weeks of age, Sprague-Dawley, Charles River Laboratories, Québec, Canada) were randomly assigned into four groups as follows: normal chow (n = 6); normal chow plus N-acetylcysteine (n = 6); fructose-fed (n = 8); and fructose-fed rats plus N-acetylcysteine (n = 6). At seven weeks of age, the rats were fed fructose (60% of diet) or normal chow, and given tap water with or without added N-acetylcysteine (1-2 g/L). Food consumption was measured once weekly. Fluid consumption was monitored every two days, and the amount of N-acetylcysteine consumed by each rat during the study was adjusted ( $1.5 \pm 0.2$  g/day per kg body weight) according to the amount of fluid consumed. All the rats were maintained under a 12-12-h light-dark cycle, and cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. After 10 weeks of fructose and/or N-acetylcysteine treatment, an oral glucose tolerance test was performed to determine insulin sensitivity, and a blood sample (1.5 ml) was collected for the measurement of plasma

triglycerides and 15-F<sub>2t</sub>-isoprostane concentrations. After 12 weeks of treatment, intra-arterial pressure was measured and a dose-response curve to methoxamine ( $\alpha$ -adrenoceptor agonist, 15-60  $\mu$ g/kg min) was constructed in the four groups of conscious, unrestrained rats at dose-intervals of 3-5 min. BP was recorded prior to and at the plateau phase of response to each dose of methoxamine.

## **2.4 Drugs and chemicals**

All the reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

## **2.5 Statistical analyses**

All data are presented as mean  $\pm$  standard error of the mean (SEM). Student's non-paired *t*-tests were used to compare data of body weight, food and fluid intake, and plasma concentrations of insulin, glucose and triglycerides. Data with multiple time points or from different dose levels were analyzed by the linear model repeated-measures analysis of variance (ANOVA) followed by Newman-Keuls test for comparison of group means. Data among groups or within the same group of rats were analyzed by one-way or two-way ANOVA followed by Newman-Keuls test. Values within the same group for MCFP pre- and post-drug administration were analyzed using paired *t*-test. Statistical significant difference was set at  $P < 0.05$ .



### **3. RESULTS**

#### **3.1 Study 1: Role of iNOS on cardiac contractile function in Zucker diabetic fatty rats.**

##### **3.1.1 General characteristics and baseline values**

At 12 weeks of age, the body weight of the ZDF rats was significantly higher ( $P<0.05$ ) than that of the control rats ( $418 \pm 9$  versus  $346 \pm 10$  g, respectively). However, at 20 weeks of age, the body weight of the ZDF rats was slightly, but not significantly, higher than that of the control rats (Table 3.1-1). The ZDF rats had a higher intake of food and fluid relative to the lean rats. Blood glucose was markedly higher in the ZDF rats compared to the controls. Baseline values of MAP, LVDP and  $+dP/dt$  were not significantly different between the ZDF rats and controls at 20 weeks of age (Table 3.1-2). However, baseline HR and RPP were significantly lower in the ZDF rats relative to those of the control rats, while baseline LVEDP was slightly, but not significantly, higher in the ZDF rats than the controls. Administration of 1400W did not significantly alter baseline readings in either group of rats, and HR and RPP of the ZDF rats remained lower than those of the control rats following the administration of 1400W.

##### **3.1.2 Dose-HR, LVDP, $+dP/dt$ and RPP responses to dobutamine**

Dobutamine dose-dependently increased LVDP and  $+dP/dt$  in both the ZDF and control groups (Figure 3.1-1A and 3.1-1B); however, curve analysis show that the increase in the ZDF rats was significantly less than that in the control rats. Dobutamine dose-dependently increased HR in both the ZDF and control rats (Figure 3.1-2A). Increases in HR were also significantly less in the ZDF relative to the control rats. The increase in RPP in response to dobutamine was also markedly less in ZDF rats compared to control rats (Figure 3.1-2B).

##### **3.1.3 Effect of 1400W on cardiac responses to dobutamine**

The acute administration of 1400W did not affect the response of LV  $+dP/dt$  (Figure 3.1-1B) or HR (Figure 3.1-2A) to dobutamine in either the control or ZDF rats. Treatment

with 1400W, however, significantly increased the responses of LVDP as well as RPP to dobutamine in the ZDF rats, but not in the control rats (Figs. 3.1-1A and 3.1-2B).

#### **3.1.4 Activity of iNOS in the myocardium**

Relative to control rats, the activity of iNOS was significantly higher in the heart tissue of the ZDF rats. Treatment with 1400W reduced the activity of iNOS in ZDF rats (to the same level as that of the control rats), but did not affect iNOS activity in the control rats (Figure 3.1-3).

#### **3.1.5 Immunostaining and Western blot analysis of iNOS or nitrotyrosine**

In the negative controls, no immunoreactivity of iNOS nitrotyrosine was observed (Figure 3-4A). Both iNOS and nitrotyrosine immunostaining (indicated by arrows) was present in the hearts of the control and ZDF rats, but was markedly more prominent in the hearts of the diabetic rats (Fig 3.1-4C) than the control rats (Figure 3.1-4B). Western blot analysis showed increased expressions of both iNOS (Figure 3.1-5A and 3.1-5B) and nitrotyrosine (Figure 3.1-5C and 3.1-5D) in hearts of ZDF rats at 20 weeks of age than hearts of the age-matched lean control rats.

**Table 3.1-1** Body weight, blood glucose, food and fluid intake of 20-week old control and Zucker diabetic fatty (ZDF) rats

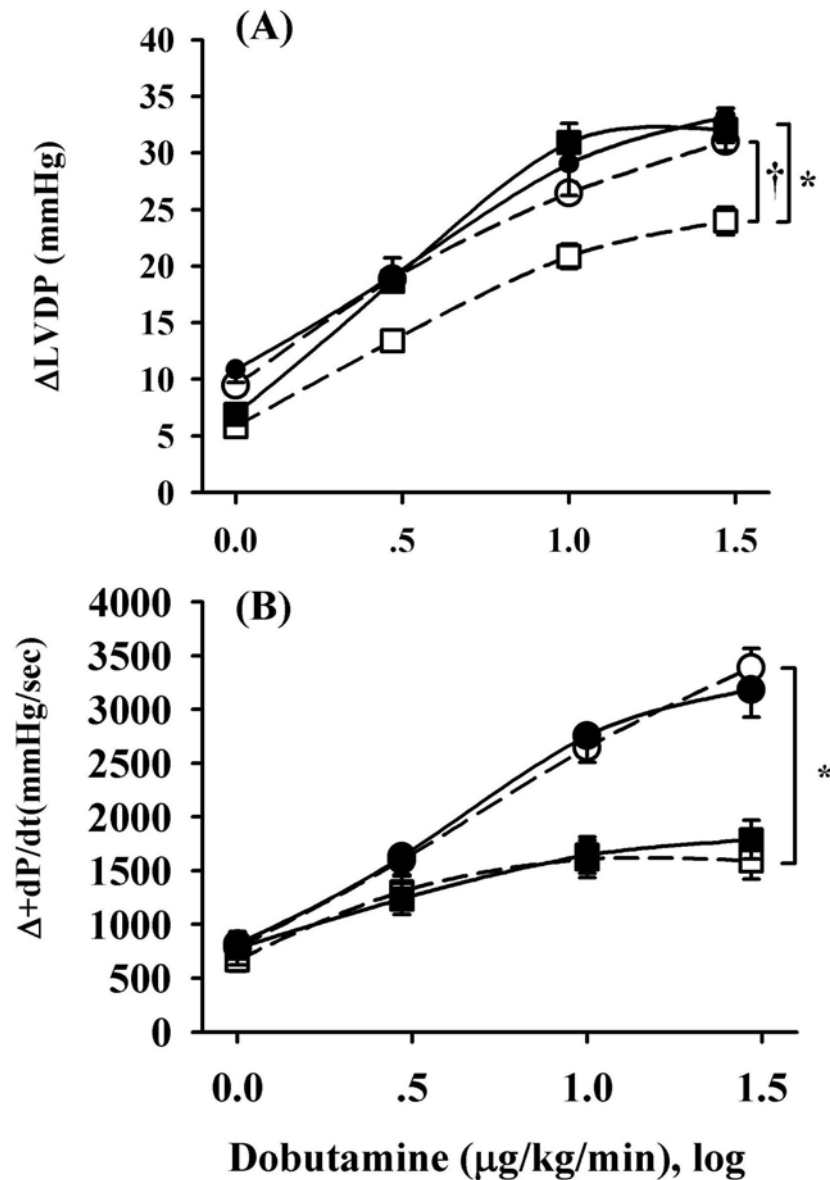
	Control rats	ZDF rats
Body weight (g)	434 ± 9	451 ± 11
Fluid intake (ml/ day)	46 ± 1	160 ± 3 <sup>*</sup>
Food intake (g/ day)	53 ± 3	80 ± 3 <sup>*</sup>
Blood glucose (mmol/L)	7.8 ± 0.3	26 ± 6 <sup>*</sup>

Data are mean ± SEM; n = 7 per group. <sup>\*</sup>Significantly different from control ( $P < 0.05$ ).

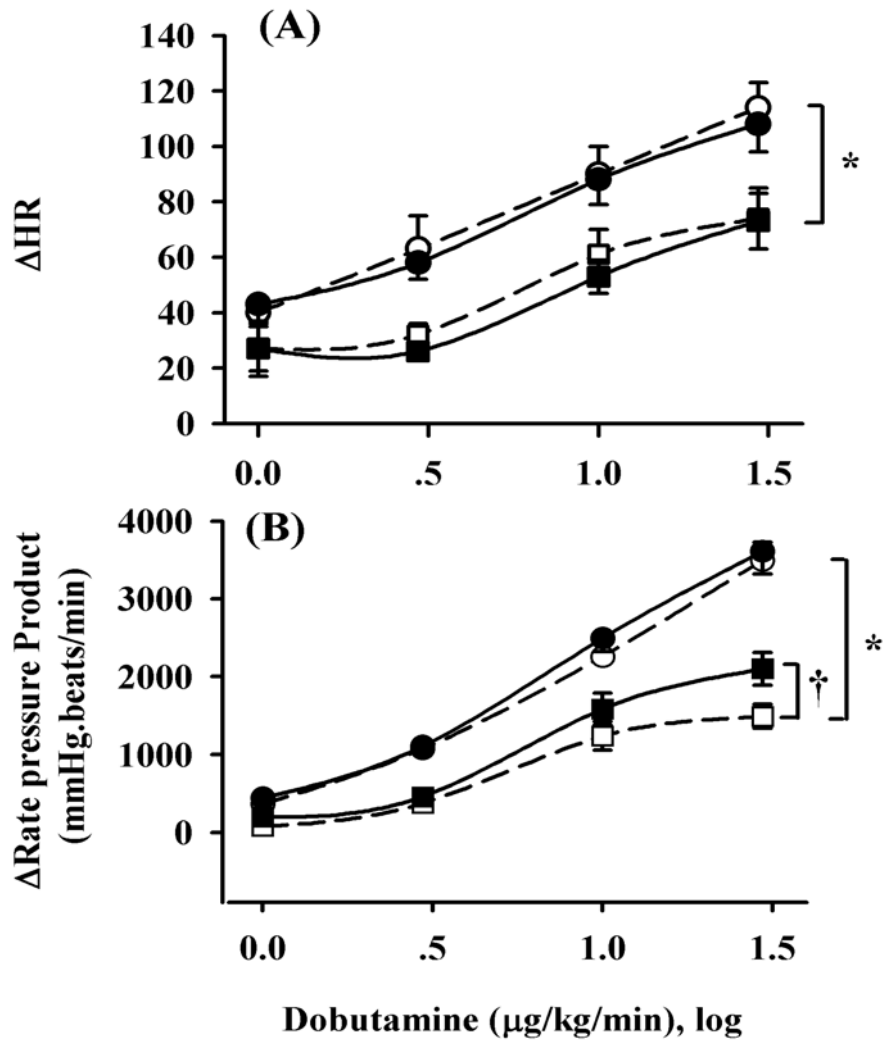
**Table 3.1-2** Baselines values of MAP, LVDP, LVEDP, HR and +dP/dt of LVP before and after administration of 1400W in conscious control and Zucker diabetic fatty (ZDF) rats at 20 weeks of age

	Control rats		ZDF rats	
	Before	1400W	Before	1400W
MAP (mmHg)	114 ± 1	115 ± 2	117 ± 1	116 ± 2
LVDP (mmHg)	178 ± 3	176 ± 3	177 ± 3	175 ± 4
LVEDP (mmHg)	7 ± 1	8 ± 1	11 ± 2	12 ± 2
HR (beats/min)	377 ± 12	376 ± 12	333 ± 11*	327 ± 11*
+dP/dt (mmHg/sec)	9994 ± 172	9998 ± 224	10108 ± 289	9696 ± 359
RPP (mmHg beats/min)	66968 ± 2426	65972 ± 2392	59021 ± 2421*	57259 ± 2776*

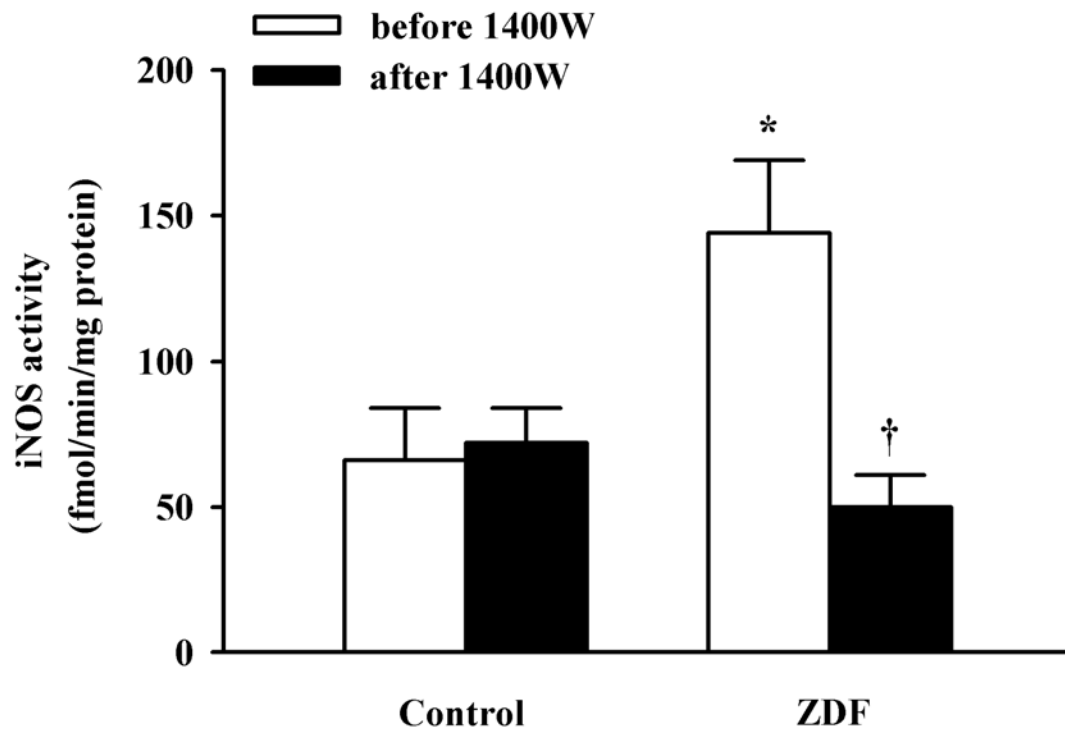
LVP, left ventricular pressure; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; +dP/dt, maximal rate of increase of LVP; RPP, rate pressure product. Data are mean ± SEM, n = 7 per group. \*Significantly different from controls ( $P < 0.05$ ).



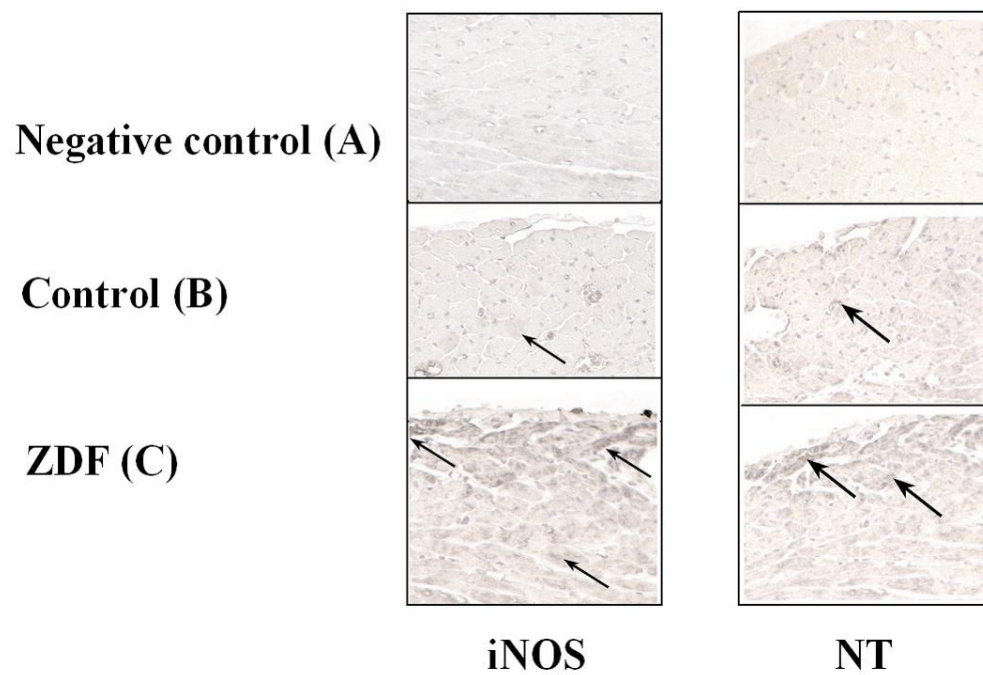
**Figure 3.1-1** Responses (mean  $\pm$  SEM,  $n = 7$  per group) to dobutamine (1, 3, 10, 30  $\mu\text{g/kg/min}$ ) on left ventricular developed pressure (LVDP, A) and  $+dP/dt$  (B) in conscious control and Zucker diabetic fatty (ZDF) rats before and after treatment with 1400W (3 mg/kg followed by 3 mg/kg h, i.v.). \*Significantly different ( $P < 0.05$ ) from responses in the control rats. †Significantly different ( $P < 0.05$ ) from responses prior to the administration of 1400W within the same group. Open circles = control rats; open squares = ZDF rats; black circles = control rats after 1400W; black squares = ZDF rats after 1400W.



**Figure 3.1-2** Responses (mean  $\pm$  SEM,  $n = 7$  per group) to dobutamine (1, 3, 10, 30  $\mu\text{g/kg/min}$ ) on heart rate (HR, A) and rate pressure product (B) in conscious control and Zucker diabetic fatty (ZDF) rats before and after treatment with 1400W (3 mg/kg followed by 3 mg/kg h, i.v.). \*Significantly different ( $P < 0.05$ ) from responses in the control rats. †Significantly different ( $P < 0.05$ ) from responses before the administration of 1400W within the same group. Open circles = control rats; open squares = ZDF rats; black circles = control rats after 1400W; black squares = ZDF rats after 1400W.

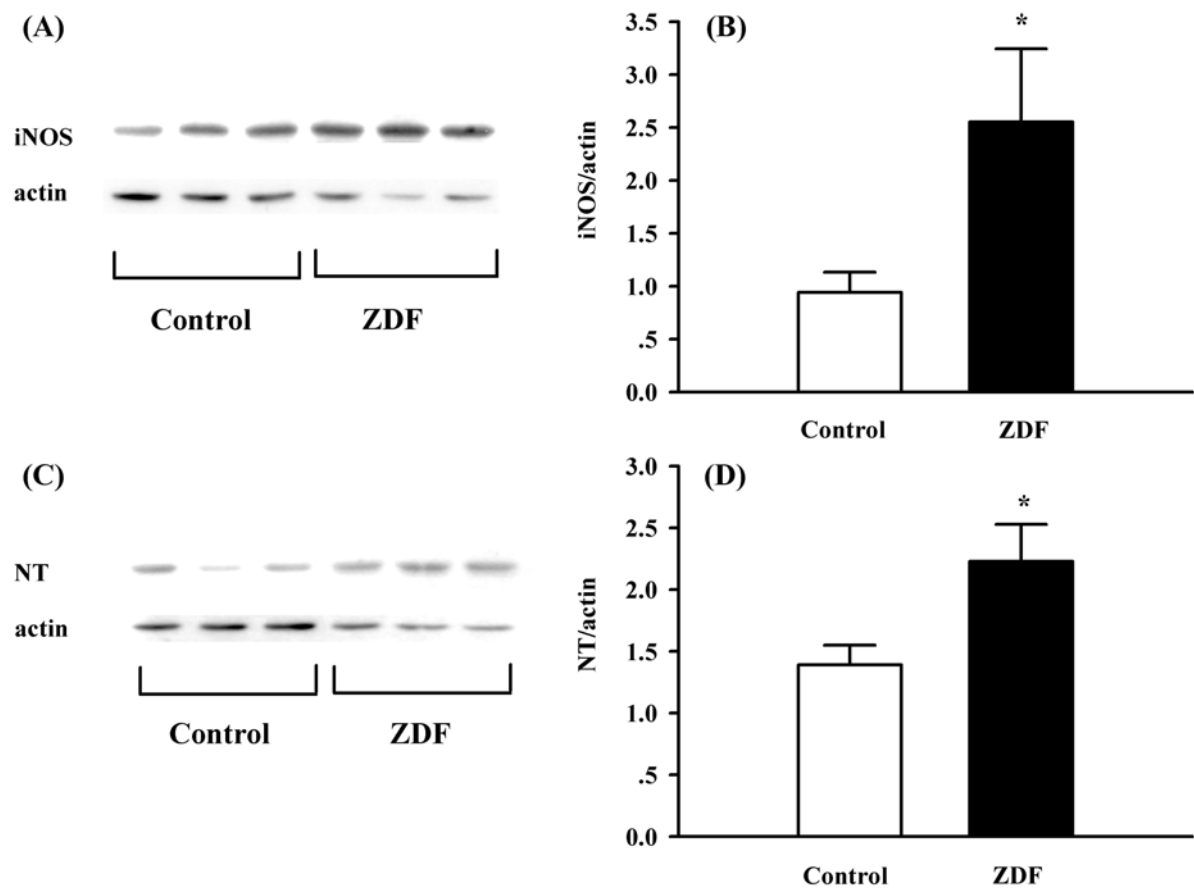


**Figure 3.1-3** Activity of inducible nitric oxide synthase (iNOS) in the heart tissue of control and Zucker diabetic fatty (ZDF) rats (n = 3 per group) before and after treatment with 1400W (3 mg/kg followed by 3 mg/kg h, i.v.). \*Significantly different ( $P < 0.05$ ) from the control. †Significantly different ( $P < 0.05$ ) from value before the administration of 1400W within the same group.



**Figure 3.1-4** Immunostaining of inducible nitric oxide synthase (iNOS) and nitrotyrosine (NT) in representative heart tissue of control (B) and Zucker diabetic fatty (ZDF) rats (C). The negative control (A) involved staining with mouse nonspecific immunoglobulin G instead of the primary iNOS antibody.





**Figure 3.1-5** Western blot and densitometric quantification analysis of inducible nitric oxide synthase (iNOS, A, B) and nitrotyrosine (NT, C, D) in heart tissues of control and Zucker diabetic fatty (ZDF) rats (n = 3 per group). \*Significantly different ( $P < 0.05$ ) from control group.

## **3.2 Study 2: Effect of selective *versus* non-selective suppression of NOS on regional haemodynamics in Zucker diabetic fatty rats.**

### **3.2.1 General characteristics**

At 20 weeks of age, the ZDF rats had slightly lower ( $P>0.05$ ) body weight and significantly higher blood glucose than the controls (Table 3.2-1). The normalized weights (organ weight/body weight) of the liver, intestine, caecum/colon, kidneys and adipose tissue of the ZDF rats were significantly higher than those of the controls (Table 3.2-1). The normalized weights of other organs and tissues were similar between the two rat groups.

### **3.2.2 Baseline values**

The two groups of rats had similar baseline MAP, CO and TPR. Baseline HR was, however, significantly lower in ZDF relative to the control rats (Figure 3.2-1). The ZDF rats had lower BF (normalized by weight) to the intestines, kidneys and adipose tissues (Figure 3.2-2B) relative to the control rats (Figure 3.2-2A). Arterial conductance values were significantly lower in the intestines, caecum/colon, kidneys and adipose tissue of the ZDF rats (Figure 3.2-3B) relative to those of the controls (Figure 3.2-3A). Baseline BF and conductance in other organs and tissues were similar between the two groups.

### **3.2.3 Effect of 1400W and L-NAME**

The administration of 1400W did not significantly alter baseline values of MAP, CO, HR, and TPR in either group of rats (Figure 3.2-2A, B). The drug also did not alter BF or conductance in any organs or tissues in the two groups (Figure 3.2-3A, B).

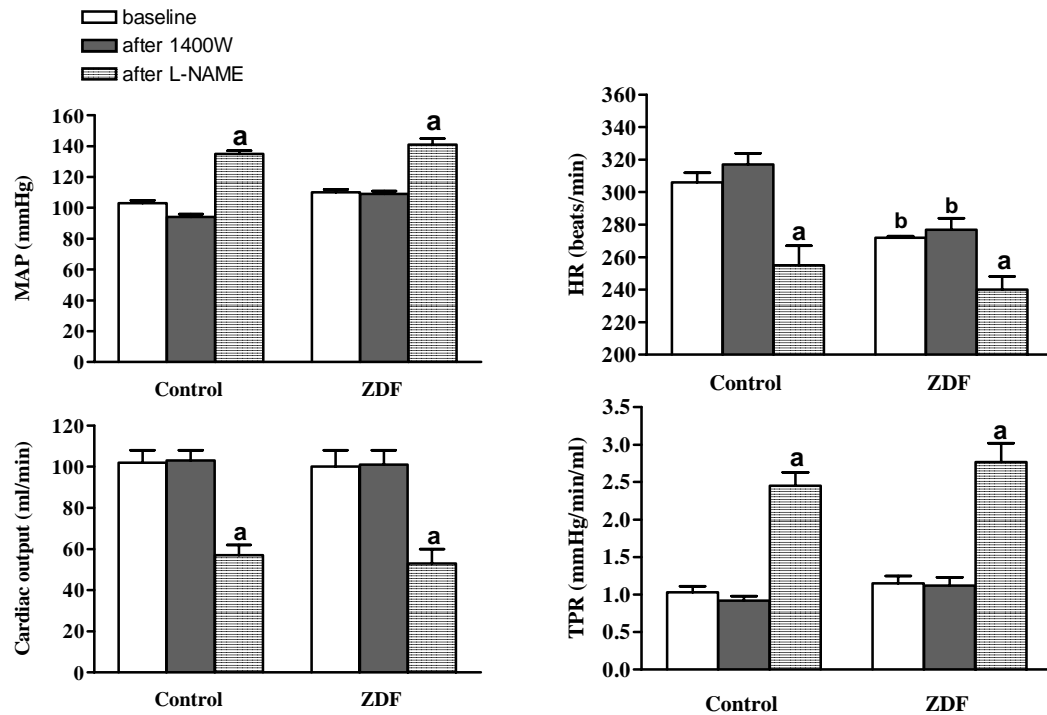
Treatment with L-NAME significantly increased MAP and TPR, and decreased CO and HR, in both the ZDF and control rats. Regional BF and arterial conductance were decreased by L-NAME in all organs and tissues in both the ZDF (Figure 3.2-2A and 3.2-3A) and control rats (Figure 3.2-2B and 3.2-3B); however, the reductions did not reach statistical significance in the testes and adipose tissue of the control rats, and in the testes and liver of the ZDF rats.

Relative to the control rats, L-NAME-induced decreases in flow and conductance in the ZDF rats were markedly less in the pancreas, and greater in the hearts and brains (Figure 3.2-4).

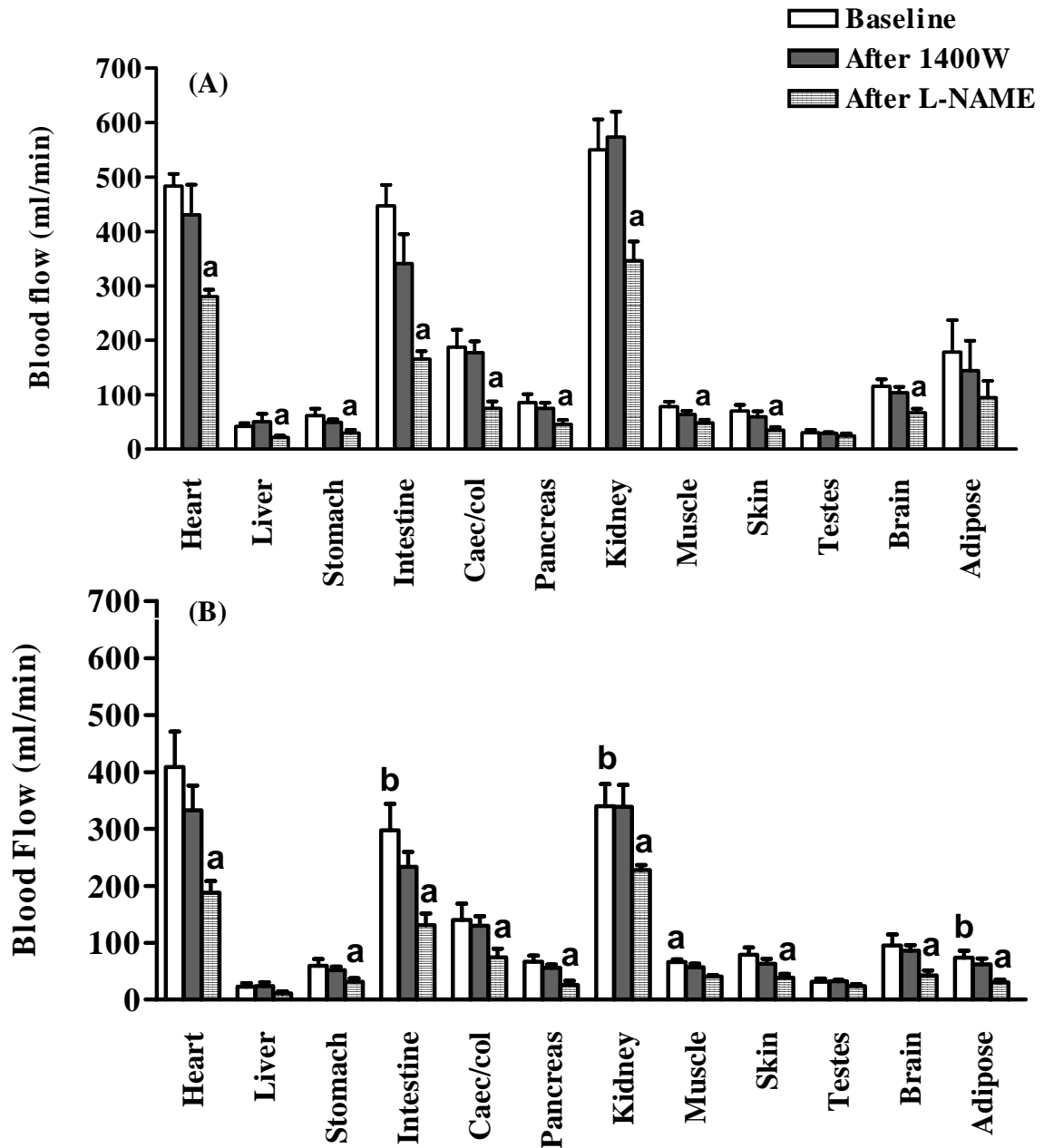
**Table 3.2-1** Blood glucose, body weight and normalized organ weight (lung, heart, liver, stomach, intestine, caecum and colon, pancreas, kidneys, spleen, testes, brain and adipose tissue) of control and ZDF rats at 20 weeks of age

	Control	ZDF
Body weight (g)	441 ± 11	421 ± 13
Plasma glucose (mM)	6.4 ± 0.3	28 ± 2*
<i>Tissue/body weights (g/kg)</i>		
Heart	3.3 ± 0.2	3.0 ± 0.1
Liver	33.4 ± 1.6	57.3 ± 4.2*
Stomach	3.9 ± 0.2	5.8 ± 0.3
Intestine	7.5 ± 0.4	17.2 ± 1.6*
Caecum and colon	3.8 ± 0.3	7.9 ± 0.9*
Pancreas	3.1 ± 0.3	3.6 ± 0.4
Kidneys	7.7 ± 0.1	13.0 ± 0.7*
Testes	6.0 ± 0.2	6.6 ± 0.4
Brain	4.5 ± 0.1	4.1 ± 0.3
Adipose tissue	25.6 ± 2.8	89.5 ± 17.1*

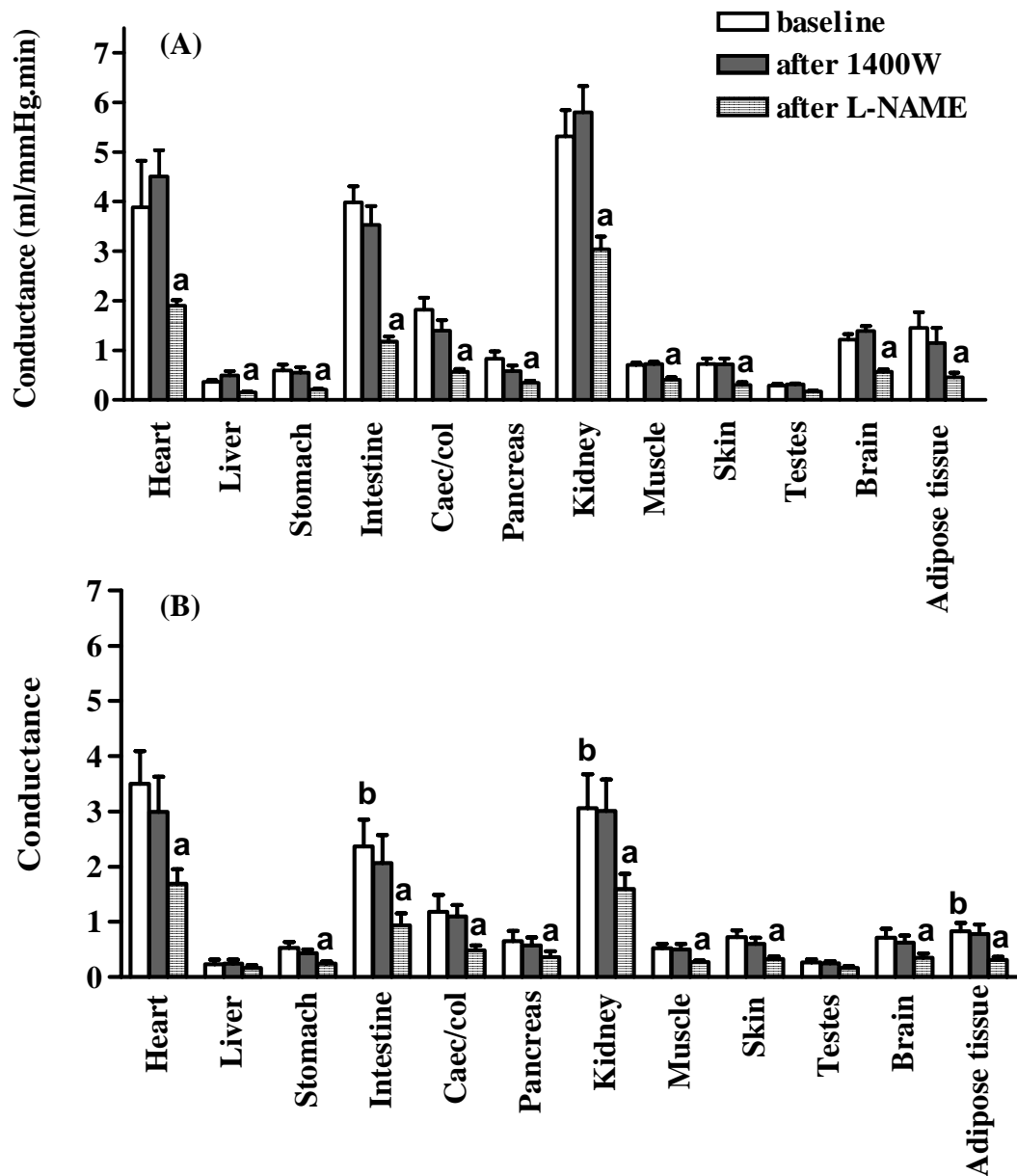
\*Significantly different from control (p<0.05).



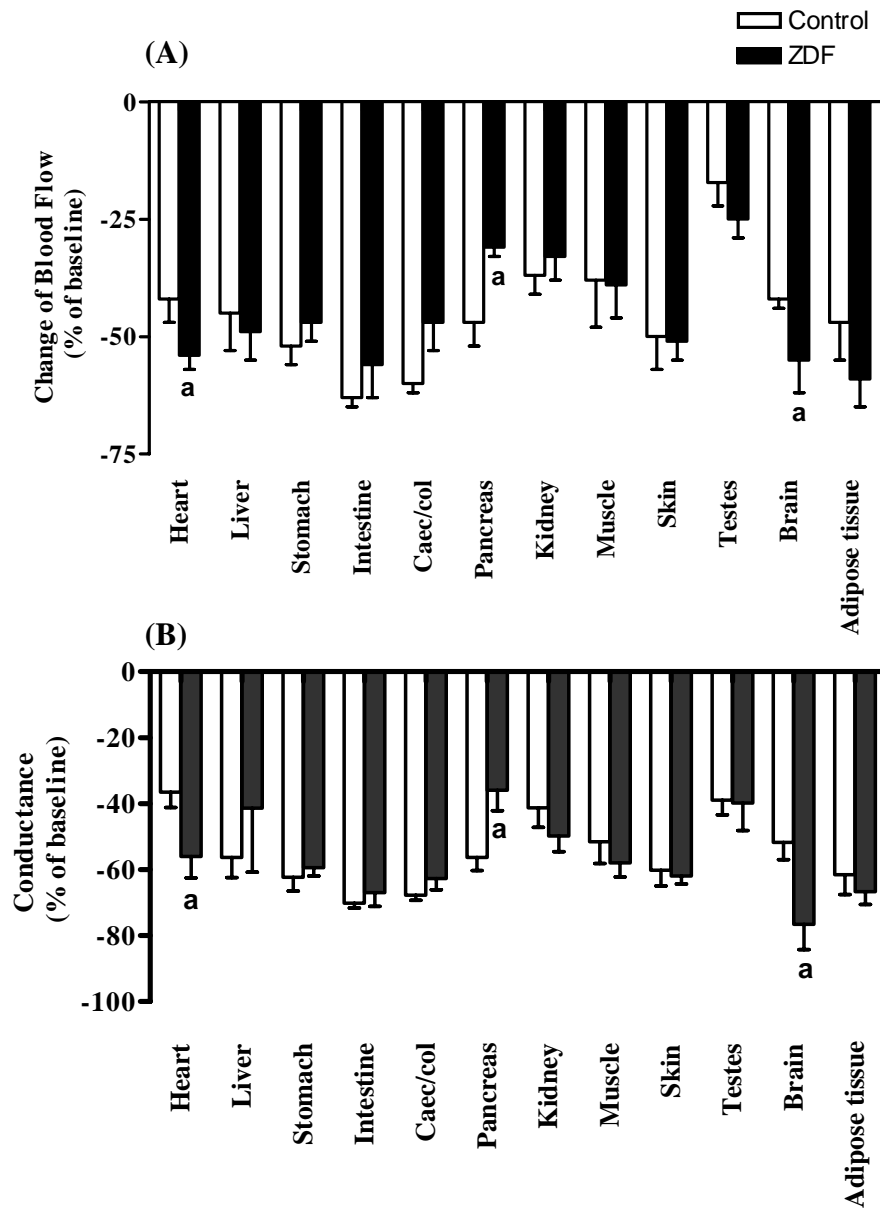
**Figure 3.2-1** Mean arterial pressure (MAP), heart rate (HR), cardiac output and total peripheral resistance (TPR) in anaesthetized control and Zucker diabetic fatty (ZDF) rats treated with vehicle (0.9% NaCl), 1400W (3 mg/kg, 3 mg/kg/h) or L-NAME (8 mg/kg). All values are mean  $\pm$  SEM (n = 7 per group). <sup>a</sup>Significantly different (p<0.05) from baseline and after 1400W; <sup>b</sup>Significantly different (p<0.05) from control group.



**Figure 3.2-2** Regional blood flow (mean  $\pm$  SEM,  $n = 7$ ) in control (A) and Zucker diabetic fatty (ZDF, B) rats treated with the vehicle (0.9% NaCl), 1400W (3 mg/kg followed by 3 mg/kg/h) and N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 8 mg/kg). Blood flow was normalized as per 100 g tissue in all organs and tissues, but per 1,000 g in the muscle, skin and adipose tissue. <sup>a</sup>Significantly different ( $P < 0.05$ ) from baseline values within the same group. <sup>b</sup>Significantly different from corresponding reading in the control rats.



**Figure 3.2-3** Regional conductance (mean  $\pm$  SEM,  $n = 7$ ) in control (A) and Zucker diabetic fatty (ZDF, B) rats treated with the vehicle (0.9% NaCl), 1400W (3 mg/kg followed by 3 mg/kg/h) and L-NAME (8 mg/kg). Blood flow was normalized as per 100 g tissue in heart, liver, stomach, intestine, caecum/colon, pancreas, kidneys, testes, brain, and as per 1,000 g in muscle, skin and adipose tissue. <sup>a</sup>Significantly different ( $P < 0.05$ ) from baseline values within the same group. <sup>b</sup>Significantly different from corresponding reading in the control rats.



**Figure 3.2-4** Effect of L-NAME (8 mg/kg, i.v. bolus) on percent change (mean  $\pm$  SEM,  $n = 7$  per group) in blood flow (A) and arterial conductance (B) in control and Zucker diabetic fatty (ZDF) rats. Percent changes in flow and conductance were calculated from the corresponding baseline values obtained immediately prior to the administration of L-NAME. <sup>a</sup>Significantly different ( $P < 0.05$ ) from values prior to the administration of L-NAME.



### **3.3 Study 3: Venous function and the role of NOS in conscious obese Zucker rats with metabolic syndrome**

#### **3.3.1 General characteristics**

By 14 weeks of age, the obese rats had markedly higher body weight, plasma insulin and plasma triglycerides levels than the lean rats (Table 3.3-1). The obese rats also had a higher intake of food and fluid, and slightly higher concentrations of plasma glucose. In addition, baseline systolic and diastolic BP was higher in the obese rats than in lean rats (Table 3.3-1).

#### **3.3.2 MAP, HR and MCFP responses to noradrenaline**

Baseline MAP was higher in the obese than lean Zucker rats, but baseline HR and MCFP were similar between the two groups of rats (Table 3.3-2).

Noradrenaline caused dose-dependent increases in MAP and MCFP, and dose-dependent reductions in HR in both groups of rats (Figure 3.3-1). The changes in MAP and MCFP were significantly less in the obese rats relative to the lean rats. However, the change in HR was similar between the two groups.

#### **3.3.3 MAP, HR and MCFP responses to L-NAME**

L-NAME increased MAP and decreased HR in both groups of rats (Table 3.3-2). The pressor and bradycardic effects to L-NAME were significantly greater in the lean rats than in the obese rats. L-NAME did not significantly alter MCFP in either group of rats, although the changes were slightly though insignificantly greater in the lean rats (11%) than obese rats (2%).

**Table 3.3-1** General parameters of obese Zucker and lean Zucker rats at 14 weeks of age

	Lean Zucker Rats	Obese Zucker rats
Baseline systolic blood pressure (mmHg)	122 ± 1	148 ± 6 <sup>a</sup>
Baseline diastolic blood pressure (mmHg)	92 ± 3	97 ± 1 <sup>a</sup>
Body weight (g)	374 ± 7	556 ± 11 <sup>a</sup>
Fluid intake (ml)	38 ± 1	50 ± 6 <sup>a</sup>
Food intake (g/day)	24 ± 1	39 ± 4 <sup>a</sup>
Plasma glucose (mM)	5.5 ± 0.1	6.2 ± 0.1 <sup>a</sup>
Plasma insulin (ng/ml)	3.5 ± 0.4	21.1 ± 2.4 <sup>a</sup>
Plasma triglyceride (mM)	1.29 ± 0.17	6.77 ± 0.51 <sup>a</sup>

Values are means ± SEM (n = 6 per group).

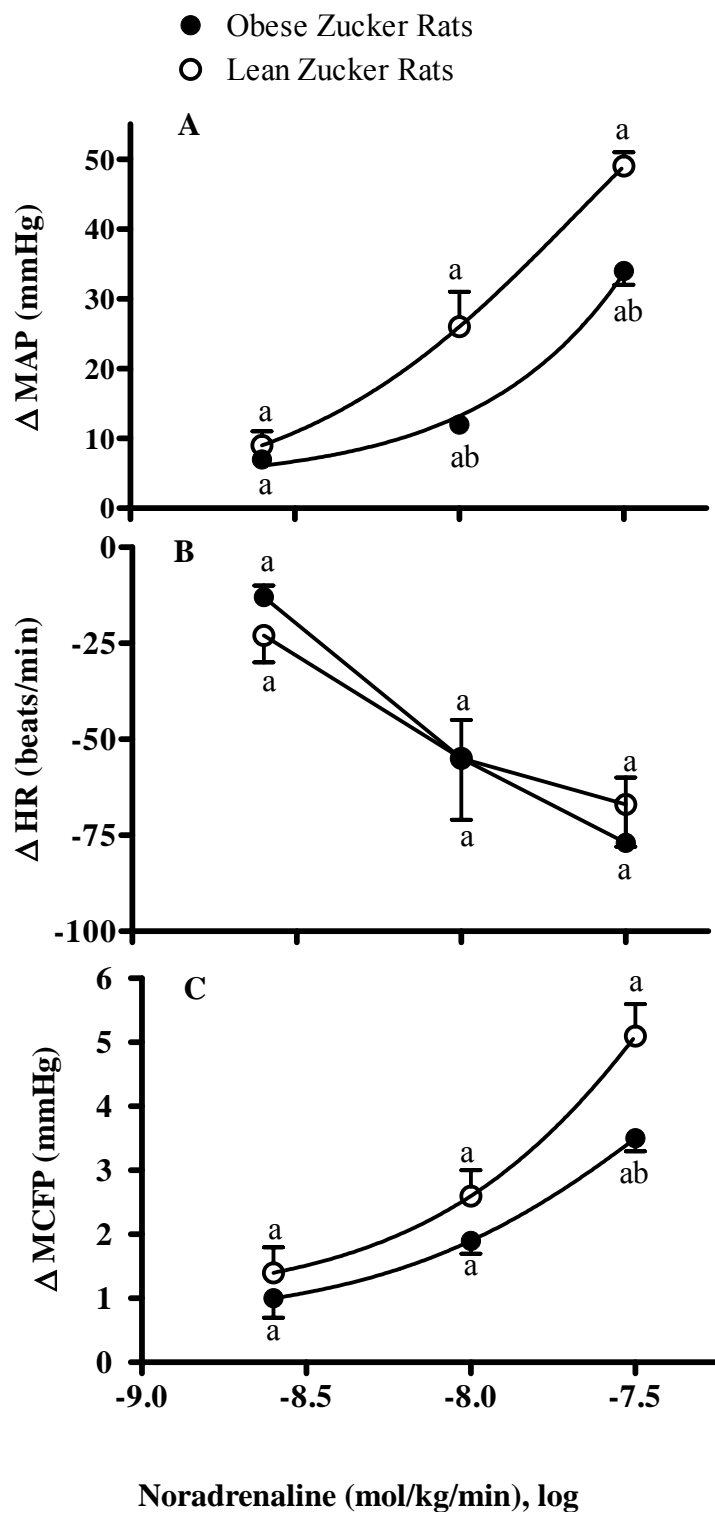
<sup>a</sup>Significantly different from the Lean Zucker rats ( $P < 0.05$ ).

**Table 3.3-2** Mean arterial pressure (MAP), heart rate (HR) and mean circulatory filling pressure (MCFP) before and after administration of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 8 mg/kg i.v.) in Lean Zucker and obese rats at 16 weeks of age

	Lean Zucker rats	Obese Zucker rats
MAP (mmHg)		
Baseline	105 ± 1	119 ± 1 <sup>a</sup>
Effect of L-NAME	+24 ± 7	+7 ± 1 <sup>a</sup>
HR (beats/per min)		
Baseline	402 ± 8	387 ± 2
Effect of L-NAME	-39 ± 8	-27 ± 3 <sup>a</sup>
MCFP (mmHg)		
Baseline	6.4 ± 0.5	6.3 ± 0.2
Effect of L-NAME	+0.7 ± 0.4	+0.1 ± 0.2

Values are means ± SEM (n = 6 per group).

<sup>a</sup>Significantly different from the lean Zucker rats ( $P < 0.05$ ).



**Figure 3.3-1** Dose-response curves for the effects (mean  $\pm$  SEM,  $n = 6$  per group) of noradrenaline (i.v.) on mean arterial pressure (MAP, A), heart rate (HR, B) and mean circulatory filling pressure (MCFP, C) in conscious obese Zucker rats and lean Zucker rats at 16 weeks of age. <sup>a</sup>Significantly different from baseline ( $P < 0.05$ ). <sup>b</sup>Significantly different from control ( $P < 0.05$ ).

### **3.4 Study 4: Effects of chronic N-acetylsysteine on fructose-induced insulin resistance and hypertension in rats**

#### **3.4.1 General characteristics**

There were no significant differences in body weight and consumption of food or fluid among the four groups at the beginning of the study (data not shown). At 10 weeks after treatment, body weights and fluid intake were similar between the control rats and fructose-fed rats; however, body weights and fluid intake were decreased in the groups treated chronically with N-acetylcysteine relative to the respective controls (Table. 3.4-1). There were no significant differences in the intakes of food among the groups.

#### **3.4.2 Oral glucose tolerance**

Upon oral glucose challenge, the plasma glucose profiles were similar among all the groups (Figure 3.4-1A). The fructose-fed rats, however, secreted more insulin than the rats given only normal chow, as indicated by the greater peak insulin response as well as the area under the curve (Figure 3.4-1B). Chronic treatment with N-acetylcysteine insignificantly decreased insulin secretion in the rats fed only chow, but significantly decreased insulin secretion in the fructose-fed rats which reached a similar level as that in the chow-fed control rats (Figure 3.4-1B).

#### **3.4.3 Insulin sensitivity index (ISI)**

A comparison of insulin sensitivity indices (calculated from the data of oral glucose tolerance test) shows that the fructose diet significantly decreased insulin sensitivity (Figure 3.4-2). Chronic treatment with N-acetylcysteine prevented significantly the decrease in insulin sensitivity in the fructose-fed rats, and increased insulin sensitivity insignificantly in the control chow-fed rats.

#### **3.4.4 Plasma 15-F2t-isoprostane assay**

Plasma 15-F2t-isoprostane and triglyceride concentrations in the fructose-fed group were markedly higher than the corresponding values in control group fed only chow. Chronic treatment of the fructose-fed rats with N-acetylcysteine significantly inhibited the fructose-induced increase in plasma triglycerides, and completely abolished the increase in plasma 15-F2t-isoprostane (Table 3.4-1).

#### **3.4.5 Blood pressure**

At 12 weeks after the initiation of treatment, systolic and diastolic blood pressures were significantly higher in the fructose-fed group than in the control group given rat chow. Treatment with N-acetylcysteine prevented the increases in systolic and diastolic blood pressures caused by the fructose diet (Figure 3.4-3).

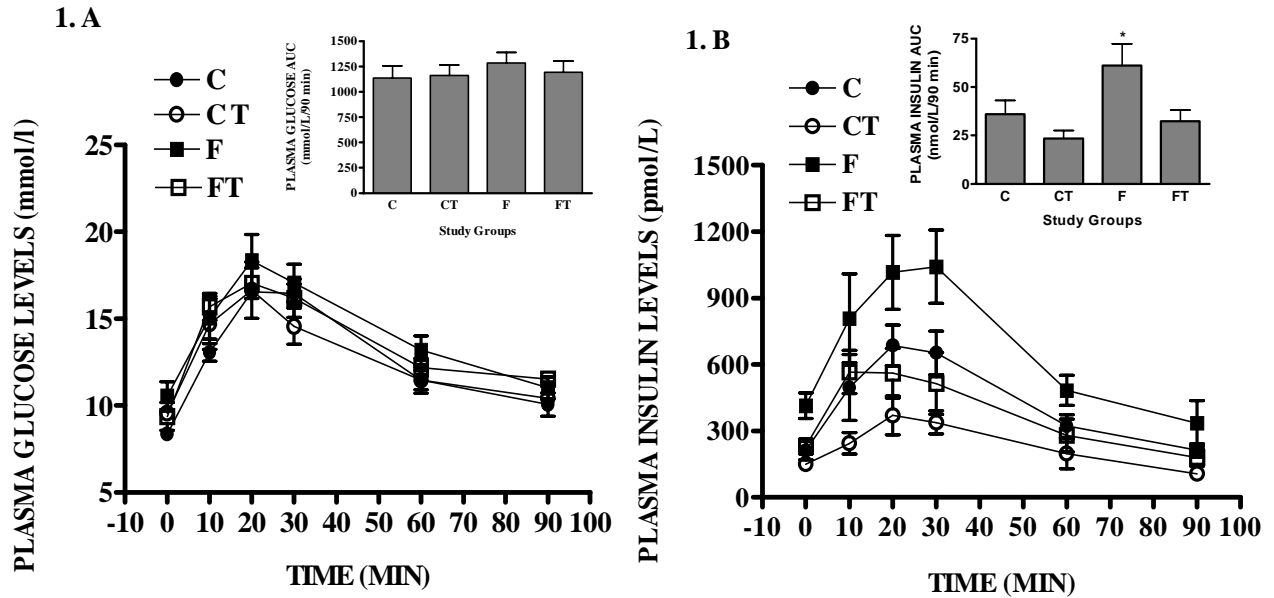
Low doses of methoxamine (15 and 30  $\mu\text{g/kg/min}$ ) caused similar increases in systolic BP in all groups of rats (Figure 3.4-4). Pressor response to the high dose of methoxamine (60  $\mu\text{g/kg/min}$ ) was significantly decreased in the fructose-fed rats treated with N-acetylcysteine relative to the other three groups.

**Table 3.4-1** Baseline values of body weight, food and fluid intake, and plasma glucose, insulin, triglycerides and 15-F<sub>2t</sub>-isoprostane in four groups of rats after treatment for 10 weeks with normal chow (C), normal chow plus N-acetylcysteine (1-2 g/L; CT), fructose (60% of diet, F), and fructose plus N-acetylcysteine (FT).

Parameter measured	C	CT	F	FT
	(n=6)	(n=6)	(n=8)	(n=6)
Body weight (g)	622 ± 16	570 ± 13	617 ± 11	567 ± 21
Food intake (g/day)	35 ± 2	43 ± 5	37 ± 3	40 ± 2
Fluid intake (g/kg/day)	58 ± 5	40 ± 3 <sup>a</sup>	55 ± 4	43 ± 4 <sup>a</sup>
Plasma glucose (mM)	8.3 ± 0.2	9.6 ± 0.3	10.5 ± 0.8	9.3 ± 0.8
Plasma insulin (ng/ml)	1.23 ± 0.25	0.92 ± 0.16	2.5 ± 0.48 <sup>a</sup>	1.38 ± 0.20 <sup>b</sup>
Triglycerides (mmol/L)	1.35 ± 0.32	1.15 ± 0.2	5.29 ± 0.86 <sup>a</sup>	3.56 ± 0.3 <sup>ab</sup>
15-F <sub>2t</sub> -Isoprostane (pg/ml)	62.3 ± 13	74.6±14.4	115.6 ± 11.8 <sup>a</sup>	52.4 ± 14.5 <sup>b</sup>

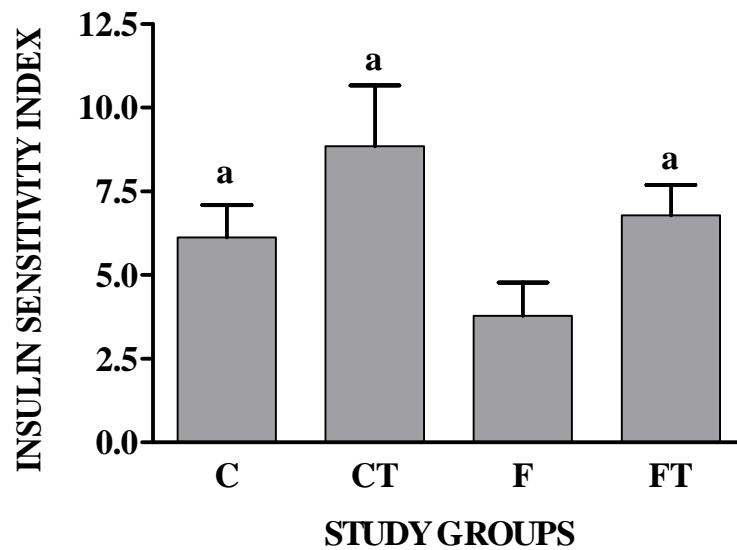
<sup>a</sup>Significantly different ( $p < 0.05$ ) from the control rats given normal chow ( C ).

<sup>b</sup>Significantly different ( $p < 0.05$ ) from the fructose-fed control rats (F).

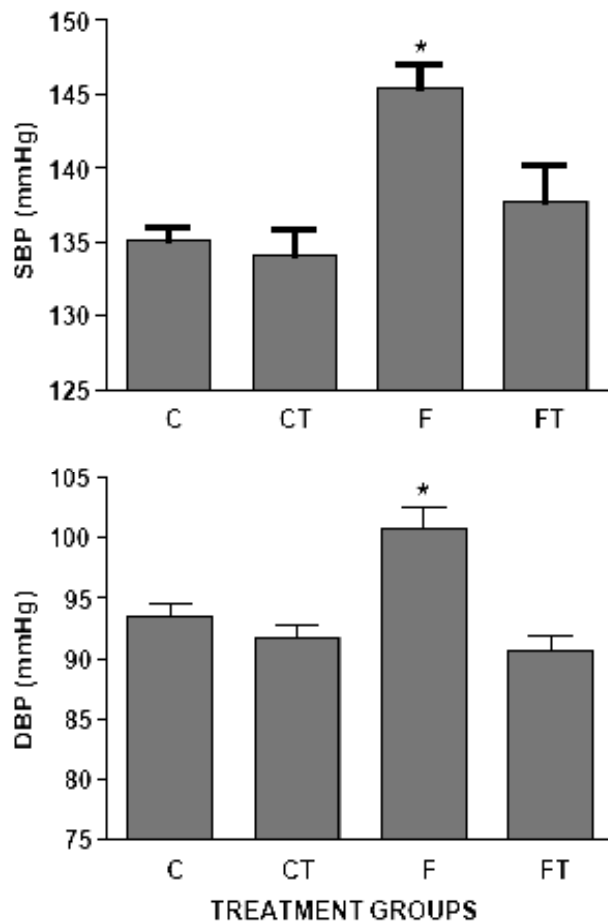


**Figure 3.4-1** Plasma glucose (mean  $\pm$  SEM) *versus* time (1A) and area under the curve (AUC, 1B) in groups of conscious, unrestrained rats after 10 weeks of treatment with normal chow (C), normal chow plus N-acetylcysteine (1.5 g/day per kg body weight; CT), fructose (60% of diet, F) and fructose plus N-acetylcysteine (FT). \* Significantly different ( $P < 0.05$ ) from all other groups.

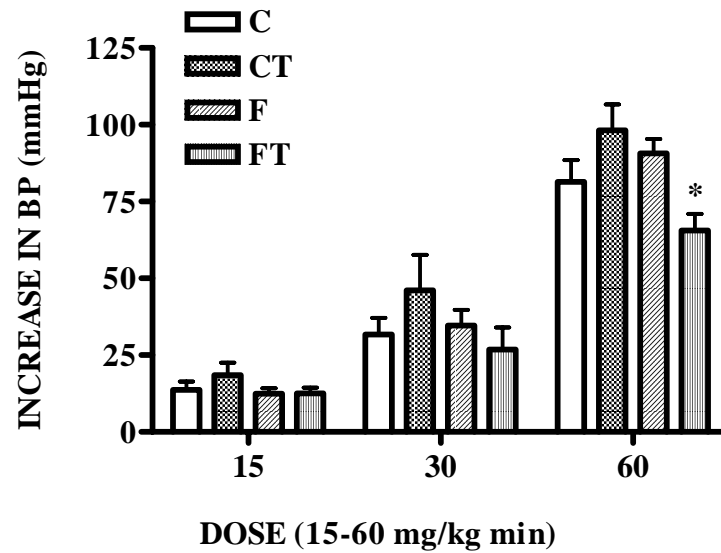




**Figure 3.4-2** Insulin sensitivity index (mean  $\pm$  SEM) in groups of conscious, unrestrained rats after 12 weeks of treatment with normal chow (C), normal chow plus N-acetylcysteine (1.5 g/day per kg body weight; CT), fructose (60% of diet, F) and fructose plus N-acetylcysteine (FT). <sup>a</sup>Significantly different ( $P < 0.05$ ) from F.



**Figure 3.4-3** Baseline intra-arterial systolic and diastolic blood pressure (SBP and DBP; mean  $\pm$  SEM) in groups of conscious, unrestrained rats after 12 weeks of treatment with normal chow (C), normal chow plus N-acetylcysteine (1.5 g/day per kg body weight; CT), fructose (60% of diet, F), and fructose plus N-acetylcysteine (FT). \*Significantly different from all other groups ( $P < 0.05$ ).



**Figure 3.4-4** Dose-response effects of methoxamine on systolic blood pressures (SBP mean  $\pm$  SEM) in groups of conscious, unrestrained rats after 12 weeks of treatment with normal chow (C), normal chow plus N-acetylcysteine (1.5 g/day per kg body weight; CT), fructose (60% of diet, F) and fructose plus N-acetylcysteine (FT). \*Significantly different from responses to the same dose of methoxamine in the other three groups ( $P < 0.05$ ).

## 4. DISCUSSION

### 4.1 Role of iNOS in cardiac contractile function in Zucker diabetic fatty rats.

At 12 weeks old, the body weight of the ZDF rats was greater than that of the control rats. At 20 weeks of age, the ZDF rats had hyperglycemia and were clearly in the chronic phase of diabetes. At this time, the ZDF rats had similar body weight to control rats despite their markedly greater consumption of food and fluid.

Baseline readings of MAP, LVDP, LVEDP and  $+dP/dt$  of the ZDF rats were similar to those of the control rats, but baseline HR (-12%) and rate pressure product (-12%) were lower in the ZDF rats relative to the controls. The results of echocardiographic evaluations elsewhere have shown that ZDF rats, at 19-20 weeks old, have reduced left ventricular fractional area shortening (Zhou et al., 2000), or increased fractional shortening and ejection fraction (Fredersdorf et al., 2004), which reflect reduced or increased baseline left ventricular contractile functions, respectively. Furthermore, *in vitro* investigation has shown that LVDP and RPP are similar between the Langendorff perfused hearts from 11-week-old ZDF rats and the corresponding control rats (Chatham and Seymour, 2002).

To further examine if cardiac contractile function was altered following challenge with an adrenoceptor agonist, the ZDF rats were tested with dobutamine (a selective  $\beta_1$ -adrenoceptor agonist). The results showed that the ZDF rats had reduced dose-dependent responses to dobutamine on LVDP,  $+dP/dt$ , HR and rate pressure product indicating compromised left ventricular contractile function when imposed with increased workload. This is the first report of reduced cardiac contraction to an adrenoceptor agonist in ZDF rats. We have previously shown that rats with STZ-induced type 1 diabetes for three weeks had reduced baseline LVDP,  $+dP/dt$  and HR readings as well as reduced LVDP,  $+dP/dt$  and HR responses to noradrenaline (Cheng et al., 2004).

The mechanisms responsible for depressed cardiac contractile function in type 1 or type 2 diabetes remain elusive. Reduced myocardial adrenoceptor density (Matsuda et al., 1999; Heyliger et al., 1982), alterations in contractile proteins (Golfman et al., 1999; Liu et al., 1997;

Liu et al., 1996; Malhotra et al., 1981) and impaired calcium cellular movements (Ganguly et al., 1983) have been postulated to be responsible for the pathogenesis of diabetes-induced heart dysfunction in rats with STZ- or alloxan-induced type 1 diabetes. Results from our laboratory and others have shown that overproduction of NO by iNOS may be involved in the development of cardiovascular abnormalities in rats with STZ-induced type 1 diabetes (Cheng and Pang, 2004; Nagareddy et al., 2005).

There is also evidence that iNOS is activated in type 2 diabetes. For example, it has been found that immunoreactivity for iNOS, iNOS expression and nitrotyrosine were higher in the retina of Goto-Kakizaki rats with type 2 diabetes (Carmo et al., 2000), and iNOS is activated in pancreatic  $\beta$ -cells in ZDF rats (Shimabukuro et al., 1997; Wang et al., 1998). Moreover, iNOS was identified in penile cavernosal smooth muscle and endothelium of diabetic patients (Seftel et al., 1997). In addition, nitrotyrosine and iNOS-dependent peroxynitrite were detected in the blood of humans (Ceriello et al., 2002b) and platelets (Tannous et al., 1999) of patients with type 2 diabetes. There is also evidence that increased iNOS gene expression is associated with increased incidence of nephropathy, neuropathy and retinopathy (Kumaramanickavel et al., 2002; Morris et al., 2002).

To find out if iNOS was activated and over-expressed in the ZDF rats as compared to the control rats, immunostaining and Western blot analysis of iNOS as well as measurement of iNOS activity were made in the hearts of the ZDF and control rats. Relative to the controls, the heart tissue of ZDF rats was found to have increased immunostaining intensity and increased iNOS protein expression as well as increased iNOS activity. Our results are in agreement with those of Zhou et al. which showed increased iNOS mRNA expression in the heart tissue of 20 week old ZDF rats (Zhou et al., 2000).

To further investigate if the induction of iNOS is associated with for the depressed cardiac function in ZDF rats, we examined the effect of 1400W (a selective iNOS inhibitor) on cardiac contractile function in the ZDF rats. Acute treatment with 1400W did not affect baseline readings of MAP, HR, LVDP, +dP/dt and LVEDP in either the ZDF or control rats.

The lack of changes under baseline readings of MAP, HR and left ventricular contractile function by 1400W suggests that the influence of iNOS was small in the baseline conditions of both the control and ZDF rats. Previous studies in this laboratory have shown that 1400W also did not alter baseline MAP or left ventricular function in control rats or rats with STZ-induced type 1 diabetes (Cheng et al. 2004). Inhibition of iNOS significantly increased responses of dobutamine on LVDP and RPP, but not  $+dP/dt$  or HR responses in the ZDF rats. In contrast to the ZDF rats, the responses of the control rats were not affected by 1400W. In a previous study, administration of 1400W also significantly increased left ventricular contractile function in rats with STZ-induced diabetes but did not affect left ventricular contractile function of control rats (Cheng et al., 2004). Furthermore, 1400W decreased the activity of iNOS in heart tissue from the ZDF but not control rats in the present study. These results confirm that iNOS was activated in the myocardium of ZDF rats and that it is associated with decreased cardiac contractile function to inotropic agents. Consistent with the results from previous studies (Cheng et al. 2003, 2004), 1400W did not alter MAP or left ventricular contractile function in the control rats, and this suggests that the functional role of iNOS is negligible in control rats.

There is substantial evidence that activation of iNOS depresses cardiovascular function. Activation of iNOS is well-known to cause the production of excessive amounts of NO, and subsequent formation of large quantities of cytotoxic products of RNS, namely, superoxide and peroxynitrite, which depress cardiac contractile function (Cheng et al. 2004; Ferdinandy and Schulz, 2003; Schulz et al., 1997). The results of the present results on immunostaining and Western blot analyses studies have shown that nitrotyrosine, an *in vivo* biomarker for oxidative damage induced by peroxynitrite and other RNS, was increased in the hearts of ZDF rats relative to the hearts of control rats. This indicates that the depressed cardiac contractile function in ZDF rats may be associated with the formation of peroxynitrite due to the excessive amounts of NO produced by iNOS. It has been shown that excessive production of NO can cause cellular injury by inhibiting DNA synthesis and suppressing mitochondrial respiration (Stuehr and Nathan, 1989). Furthermore, production of large amounts of NO by iNOS in

cultured cardiac myocytes inhibited  $\beta$ -adrenoceptor agonist-induced increases of intracellular cAMP and cGMP, thereby interfering with  $\text{Ca}^{2+}$ -mobilization and cardiac contractile function (Joe et al., 1998; Ungureanu-Longrois et al., 1995). Moreover, myocardial iNOS activation has been shown to be responsible for reduced response to  $\beta$ -adrenoceptor agonists in rats with volume-induced heart failure (Gealekman et al., 2002) and patients with left ventricular dysfunction (Hare et al., 1995). These results and ours are supportive of the premise that excessive production of NO through activation of iNOS in a variety of conditions can lead to reduced response to  $\beta$ -adrenoceptor agonists and decreased cardiac contractile function.

To summarize, the results of the present study show that 20-week old ZDF rats had depressed cardiac contractile response to dobutamine, and increased myocardial protein expression as well as activity of iNOS. The depressed cardiac contractile function in ZDF rats may involve the formation of peroxynitrite due to the excessive amounts of NO produced by iNOS. Administration of 1400W reduced the activity of iNOS and augmented cardiac contractile response to dobutamine. Our results are the first to demonstrate that iNOS activation is associated with functional impairment of response to  $\beta$ -adrenoceptor activation in ZDF rats with type 2 diabetes.

#### **4.2 Effect of selective *versus* non-selective suppression of NOS on regional haemodynamics in Zucker diabetic fatty rats.**

ZDF rats, at 18 weeks of age, have been shown to have unchanged SBP and decreased HR (Fredersdorf et al., 2004). In the present study, ZDF rats, at 20 weeks of age, had similar baseline MAP, CO and TPR, but reduced HR relative to the control rats. This is a first report on CO and TPR in the ZDF rats. Blood glucose in the ZDF rats was 4.4-fold that of the control rats. Although body weight of the ZDF rats was similar to that of the control rats, the weight of adipose tissue in the ZDF rats was 3.5-fold that of the control rats.

Our results showed that the ZDF rats had reduced BF to the intestine (67% of controls), kidneys (62%) and adipose tissue (42%), and similar BF to other organs and tissues (heart, liver,

stomach, caecum/colon, pancreas, skeletal muscle, skin, testes and brain). Since decreased blood flows to the intestine, kidneys and the adipose tissue were associated with increased organ/tissue weight (230, 170 and 350% of control rats, respectively), it is evident that reduced blood flow (per g tissue) was compensated for by cellular hypertrophy and/or hyperplasia to restore overall flow in these tissues. Indeed, total renal blood flow in the ZDF rats (18.7 ml/min per rat) was similar to that of the control rats (18.9 ml/min per rat). Similar mean arterial pressure, heart rate, cardiac output, total peripheral resistance and blood flow to splanchnic and skeletal muscle beds, but reduced renal blood flow and arterial conductance have been reported in the kidneys of sand rats at an early phase of type 2 diabetes (Hilzenrat et al., 1996). Renal and intestinal hyperplasia and hypertrophy have been reported in rats with type 2 (Fujita et al., 1998; Uriu et al., 1999) or type 1 (Gronbaek et al., 1995; Fedorak et al., 1990) diabetes. Increased mass of adipose tissue is related to genetic defect of the ZDF rats. Diabetes in the ZDF rats, similar to humans, is polygenetic: obesity is due to a mutation in the leptin receptor (*fa gene*), insulin resistance is due to *fa gene*'s faulty encoding for the insulin receptor, and diabetes is due to defective transcription of pancreatic  $\beta$ -cell gene to adequately increase insulin secretion to compensate for insulin resistance (Lee et al., 1994; Hirose et al., 1996; Griffen et al., 2001).

NO is likely a most important endogenous vasodilator. To further investigate the roles of iNOS and cNOS on BF regulation, the effects of 1400W (a selective iNOS inhibitor) and L-NAME (a non-selective NOS inhibitor) on haemodynamics were determined in the ZDF and control rats. Administration of 1400W did not significantly alter MAP, CO, HR, TPR or regional BF in either group of rats. These results show that iNOS does not participate in the regulation of BF in these rats. In sharp contrast to these results, 1400W in rats with STZ-induced type 1 diabetes significantly increased MAP and TPR, and generally decreased in BF to all organs and tissues, with significant reductions in the heart, stomach, intestine and caecum/colon (Cheng et al., 2004a). Similar to the present results, injection of 1400W did not alter BF in the control rats (Cheng et al., 2004a). There is thus a distinct difference in the



vascular role of iNOS in STZ-induced type 1 diabetes and ZDF rats with spontaneous type 2 diabetes.

L-NAME caused generalized systemic vasoconstriction in both groups of rats, exemplified by reductions in arterial conductance (BF normalized by MAP) in all organs and tissues, a large (2.5-fold) increase in TPR and a reduction in CO. All organs and tissues were affected although significant reduction in arterial conductance was not achieved in the liver of the ZDF rats, and the testes of both groups of rats. Lack of significant reduction in hepatic arterial conductance is likely related to marked vasoconstriction in intestinal organs. It has been shown by Lautt (1980) that hepatic arterial flow is regulated by the hepatic arterial buffer response (involving modulation of the release of the vasodilator adenosine) in an attempt to maintain constant total hepatic BF (hepatic arterial plus portal venous flow).

A comparison of constrictor responses in both groups show that L-NAME caused less vasoconstriction in the pancreas, but greater vasoconstriction in the heart and brain, thereby indicating reduced production of NO in the pancreas but greater NO availability in the heart and brain of the ZDF rats relative to the controls. Since selective inhibition of iNOS by 1400W did not affect BF in any organs or tissues, altered response to L-NAME reflects changes in the activities of eNOS and/or nNOS. It is well known that eNOS plays an important role in the regulation of peripheral circulation, however, a vasodilator role of nNOS has been established only in the central nervous system (Wang et al., 1995; Hudetz et al., 1998; Staunton et al., 2000; Kelly et al., 2000). Changes observed with L-NAME in the present study therefore likely reflect altered activity of eNOS. Our finding of greater constrictor response to L-NAME in the brain of the ZDF rats relative to the controls is in agreement of published results with increased eNOS expression in the cerebral arteries of 3-4 month old ZDF rats (Schwaninger et al., 2003a). To our knowledge, there is no published study that compares the expression or activity of iNOS or eNOS between pancreatic vascular tissues of control animals and animal models with type 2 diabetes.

To summarize, 20-week-old ZDF rats had hyperglycemia and similar MAP, CO and TPR,

but reduced HR relative to the control rats. The diabetic rats also had reduced BF to the intestine, kidneys and adipose tissue, and similar BF to other organs and tissues. Injection of 1400W did not alter MAP or haemodynamics in either the control or diabetic rat, and this shows a lack of involvement of iNOS on BF regulation. Administration of L-NAME caused similar increases in MAP and TPR, and similar reductions in CO and HR in the either the control diabetic rats, and this shows a lack of involvement of iNOS on BF regulation. Although L-NAME caused vasoconstriction of all beds in both groups of rats, its constrictor influence was greater in the heart and brain, but less in the pancreas, of the ZDF rats relative to the controls.

#### **4.3 Venous function and the role of NOS in conscious obese Zucker rats with metabolic syndrome.**

The present results show that the obese Zucker rats, by 14 weeks of age, had a metabolic syndrome-like condition characterized by obesity (1.5 times the body weight of the age-matched lean rats), increases in systolic BP, diastolic BP and MAP (+24, + 5 and +14 mmHg, respectively) relative to corresponding readings in the control lean rats. The obese rats also had markedly higher plasma concentrations of insulin (six-fold relative to controls) and triglycerides (five-fold that of control). Plasma glucose levels were also increased in the obese rats (+13%), although these remained within the normal range. These measurements are consistent with abnormalities of metabolic syndrome X, and are in accordance with those from other studies involving obese Zucker rats (Alonso-Galicia, Brands, Zappe, and Hall, 1996 Pamidimukkala and and Jandhyala 1996). It has been established that these abnormalities in the obese Zucker rats are the consequence of altered expression of the leptin receptor (*Ob-R*), which results in an approximately 10-fold reduction in the binding affinity of leptin for its receptor (Iida, Murakami et al., 1996; Rosenblum, Tota et al., 1996).

The obese rats, although moderately hypertensive, had a reduced pressor response to noradrenaline. The mechanism by which obesity causes hypertension is unclear, and the role of the sympathetic nervous system on vascular function in obesity has not yet been resolved.

Increased sympathetic nerve activity has been observed in obese patients (Grassi, Seravalle, Dell'Oro, Turri, Bolla, and Mancia, 2000) and obese Zucker rats (Pamidimukkala and Jandhyala, 1996; Morgan, Anderson, and Mark, 1995). However, interference with sympathetic nerve activity through concurrent administration of terazosin plus propranolol caused only a modest decrease in BP in conscious obese Zucker rats, thereby suggesting only modest involvement of the sympathetic nervous system (Alonso-Galicia, Brands, Zappe, and Hall, 1996). In contrast, ganglionic blockade by mecamylamine or hexamethonium caused a large decrease in MAP in obese Zucker rats that were conscious (Carlson, Shelton, White, and Wyss, 2000) or anaesthetized and artificially ventilated (Schreihöfer, Hair et al., 2005). There are also inconsistencies in the vascular response reported to  $\alpha$ -adrenoceptor agonists. As compared to lean rats, isolated aortic rings of obese Zucker rats had greater *in vitro* constriction to phenylephrine (Brooks-Asplund et al. 2002; Ouchi et al. 1996) and similar constriction to noradrenaline (Subramanian and MacLeod, 2003); however, constriction to noradrenaline was decreased in isolated mesenteric artery rings (Subramanian and MacLeod, 2003) as well as in the isolated perfused mesenteric artery (He and MacLeod, 2002). As well, constriction to noradrenaline was increased in the isolated gracilis artery and *in situ* cremaster arterioles of obese Zucker rats (Stepp and Frisbee, 2002). *In vivo* studies show that vasoconstriction to phenylephrine was greater in the hindlimb artery, unchanged in the renal artery, and reduced in the mesenteric bed of anaesthetized, artificially-ventilated and ganglia-blocked obese Zucker rats (Schreihöfer, Hair, and Stepp, 2005) compared to lean rats, and that the pressor response to noradrenaline was either greater (Zemel, Peuler et al., 1992) or unchanged (Schreihöfer, Hair, and Stepp, 2005). These differences in experimental findings among various studies, including our own, are likely due to varying experimental conditions, such as *in vitro* versus *in vivo* responses, use of different pharmacological agents, and conscious intact rats versus anaesthetized, ganglia-blocked rats.

The obese Zucker rats in this study also had lower MCFP responses to noradrenaline as compared to lean rats, indicating reduced venoconstriction. This is the first study to

demonstrate reduced *in vivo* constriction of capacitance vessels to noradrenaline in obese Zucker rats. MCFP is the driving force of venous return, and experimentally is the equilibrium pressure that exists in the circulation immediately after an abrupt cessation of BF (Guyton, 1963). Mathematically, at a constant blood volume, MCFP is inversely proportional to venous compliance (Grodins, 1959) and can be used as an index of body venous tone (Pang, 2000 and 2001). Our results show that baseline MCFP measurements were similar between the obese and lean rats, although the MCFP response to noradrenaline was less in the obese than in the lean rats. The mechanism responsible for the attenuated arterial and venous constrictions to exogenously administered noradrenaline is unclear, and may be due to down-regulation of vascular adrenoceptors secondary to increased sympathetic nerve activity and increased endogenous release of noradrenaline. It would be interesting to directly measure vascular sympathetic nerve activity or indirectly estimate sympathetic tone via measurement of plasma concentration of catecholamines. Alternatively, MCFP could be measured prior to and after ganglionic blockade to estimate the influence of the sympathetic nervous system, as ganglionic blocking agents have been shown to reduce MAP in obese Zucker rats (Carlson, Shelton, White, and Wyss, 2000; Schreihöfer, Hair, and Stepp, 2005; Zemmel et al. 1992).

Administration of noradrenaline caused similar bradycardia in the obese Zucker and lean rats. Although there is evidence that the obese Zucker rats have blunted baroreflex activity (Bunag and Barringer 1988; Barringer and Bunag 1989; Pamidimukkala and Jandhyala 1996), this was not detected in the present study due to concurrent direct activation of  $\beta_1$ -adrenoceptors by noradrenaline and reflex bradycardia secondary to noradrenaline-induced pressor response. The lower bradycardic effects of L-NAME in obese rats may have been the result of lower pressor effect of L-NAME in the obese rats, and does not necessarily reflect depression of the baroreflex system in the obese Zucker rats. L-NAME increased MAP in both the obese and lean Zucker rats; however, the pressor effect was markedly greater in the lean rats, indicating reduced baseline involvement of the NO/L-arginine pathway in the obese rats. Reduced arterial bioavailability of NO could be a cause of hypertension in the obese Zucker rats. These

results are in agreement with those of Bohlen and Nase (2002), which showed reduced *in situ* NO production in the intestinal arterioles of anaesthetized, mechanically ventilated obese Zucker rats relative to those of lean rats. In addition, rats with obesity due to chronic intake of a high fat diet were found to have reduced serum concentrations of nitrite/nitrate and precontracted aortic rings from these rats had reduced relaxation responses to acetylcholine, suggesting decreased arterial availability of NO (Shah and Singh, 2006). Our results also show that L-NAME increased MCFP in neither the obese nor lean rats, suggesting negligible influence of endogenous NO on baseline body venous tone. One possible mechanism for the difference in arterial and venous responses to L-NAME could be that NO production is much lower in the veins than in the arteries of obese Zucker rats. This is supported by studies done by Vallance which showed that inhibition of NOS did not change the diameter of the isolated jugular veins of rabbits (Vallance, Palmer et al., 1992) or the diameter of dorsal hand veins of human subjects (Calver, Harris et al., 1994), indicating negligible constitutive production of NO by capacitance vessels. However, increased MCFP response has also been found after NOS inhibition in both pigs (Magder and Kabsele, 1998) and Sprague-Dawley rats (Glick, Gehman et al., 1993). The inconsistency in results may be related to the type of venous vessels and research subjects (humans *versus* animals) and varying methodology (*in vivo versus. in vitro*) used for the evaluation of venous function.

It is important to point out that blood volume is a key factor to consider when interpreting the results of arterial/venous pressure and constrictor responses to noradrenaline and L-NAME. A recent study showed that at 15 weeks of age, obese Zucker rats, relative to lean rats, had comparable blood volumes, but lower volume-to-body weight ratio (Schreihöfer et al. 2005). Since the doses of noradrenaline and L-NAME administered were adjusted according to body weight, the obese rats were likely exposed to higher concentrations of drugs. As a consequence of this possibility, it is probable that the extent to which the impaired pressor and MCFP responses to noradrenaline, as well as the reduced pressor response to L-NAME, were actually underestimated in the obese rats.

In summary, the present study showed that the obese Zucker rats had impaired *in vivo* pressor and MCFP responses to noradrenaline, as well as reduced pressor response to L-NAME. The results indicate that increased MAP in the obese relative to the lean rats is not due to increased arterial and venous constrictor response to noradrenaline. Instead, the increased baseline arterial pressure of the obese rats may be related to a reduced influence of the NO/L-arginine system.

#### **4.4 Effects of chronic N-acetylcysteine treatment on fructose-induced insulin resistance and hypertension in rats**

The results of this study show that the feeding of a diet consisting of high fructose increases plasma insulin, triglycerides, 15-F2t-isoprostane and arterial pressure, and causes the development of insulin resistance. These changes induced by a high fructose diet are in accord with the findings of other studies (Damiano et al., 2002; Huang et al., 1997; Hwang et al., 1987; Bhanot et al., 1994; Ceriello et al., 2002), and are consistent with abnormalities of syndrome X. A possible initiating factor for the increase in arterial pressure in these rats is the generation of free radicals by a high fructose diet. A diet high in carbohydrates is associated with the generation of ROS (Fields, 1998). Exposure of human aortic endothelial cells to high glucose increases the generation of highly reactive oxidants such as peroxynitrite and  $O_2^-$  (Zou et al., 2002; Cosentino et al., 1997). Furthermore, elevated plasma levels of superoxide and hydrogen peroxide have been reported in patients with essential hypertension (Kumar and Das, 1993; Lacy et al., 1998) as well as the vasculature and plasma of hypertensive animals (Laursen et al., 1997; Swei et al., 1999). In the present study, chronic treatment with the antioxidant N-acetylcysteine prevented the increase in BP in the fructose-fed rats, but did not affect the BP of control rats given normal chow. N-acetylcysteine also reduced the pressor response to methoxamine in the fructose-treated rats but not normal chow-fed control rats, which indicates that the effects of N-acetylcysteine may involve the suppression of  $\alpha_1$  adrenoceptor-mediated vasoconstriction. These results are supportive of the role of free radicals in the pathogenesis of hypertension and increased vasoconstriction. There is evidence that the levels of free radical

scavengers such as vitamin E, glutathione and SOD are decreased in hypertensive patients (Kumar and Das, 1993; Sagar et al., 1992). Furthermore, a depletion of glutathione in rats via chronic administration of a glutathione synthase inhibitor has been shown to cause hypertension (Vaziri et al., 2000), and supplementation of vitamin E has been shown to decrease BP both in humans and experimental animals (Boshtam et al., 2002; Pezeshk and Derick Dalhouse, 2000; Vasdev et al., 2002). Fructose-fed rats, relative to the controls, had elevated plasma free 15-F2t-isoprostane as well as triglycerides. 15-F2t-isoprostane, a product of the non-enzymatic free-radical metabolism of arachidonic acid, is a reliable marker of lipid peroxidation and oxidative injury (Morrow and Roberts, 1997) as well as an efficacious vasoconstrictor (Mobert et al., 1997). Increased plasma concentrations of 15-F2t-isoprostane in this study therefore reflect an increase in oxidative stress, and both 15-F2t-isoprostane and oxidative stress may be contributing factors in increasing the BP in the fructose-fed rats. In addition to 15-F2t-isoprostane, dyslipidemia and increased plasma triglycerides are also risk factors of hypertension (Zicha et al., 1999) as well as insulin resistance (Agata et al., 1998).

In the present study, N-acetylcysteine, in addition to preventing the increase in BP in the fructose-fed rats, normalized the plasma levels of insulin and 15-F2t-isoprostane, and significantly reduced plasma triglycerides and increased insulin sensitivity. N-acetylcysteine is a thiol-containing nucleophile that plays an important role in the scavenging of reactive oxidative radicals such as peroxynitrite (De Vries and De Flora, 1993). N-acetylcysteine has been shown to increase the synthesis of reduced glutathione in cultured Chinese hamster ovary cells through the promotion of cysteine uptake (Issels et al., 1988), to increase intracellular concentration of glutathione in erythrocytes, liver cells and lung cells of rats (De Flora et al., 1985), as well as to replenish reduced hepatic glutathione stores following experimental depletion in mice (Nakata et al., 1996). Chronic administration of N-acetylcysteine was also shown to increase the content of reduced glutathione in the aorta of the spontaneous hypertensive rats, and reduced systolic but not diastolic BP (Cabassi et al., 2001). It is, however, not known if N-acetylcysteine prevents the development of hypertension in the

fructose-induced metabolic syndrome through the scavenging of reactive oxidative radicals and prevention of insulin resistance.

In recent years, the relationship between oxidative stress and insulin resistance has attracted much attention. There is evidence that free radicals induce insulin resistance (Paolisso et al., 1994; Paolisso and Giugliano, 1996). N-acetylcysteine is reported to exert a protective effect on pancreatic  $\beta$ -cells of diabetic db/db mice (Kaneto et al., 1999), Zucker diabetic fatty rats (Tanaka et al., 1999) and alloxan-induced diabetic weanling CD1 mice (Ho et al., 1999), and to reduce blood glucose and/or increase glucose-induced insulin secretion. In the present study, we have shown, for the first time that chronic treatment with N-acetylcysteine decreased plasma insulin and improved insulin sensitivity in fructose-induced metabolic syndrome. Interestingly, the administration of metformin has been shown to reduce plasma insulin and to prevent the increase in BP in rats given a high fructose diet, suggesting that hyperinsulinemia is also a risk factor of hypertension (Verma et al., 1994). Our results show that oxidative stress plays an essential role in the development of hyperinsulinemia and insulin resistance in the fructose-fed rats, and that the reduction in insulin secretion and insulin resistance may also be a contributing factor in the normalization of BP.

In summary, our results show that chronic treatment of rats with a diet high in fructose causes high BP, insulin resistance and oxidative stress as revealed by increased lipid peroxidation. Treatment of fructose-fed rats with N-acetylcysteine reduces oxidative stress and restores insulin sensitivity while inhibiting the increase in BP.



## 5. SUMMARY AND CONCLUSION

In summary, our results showed that:

- (1) In ZDF rats (an animal model of type 2 diabetes), baseline LVDP and  $+dP/dt$  were unchanged at 20 weeks of age, but HR and RPP were lower in the ZDF than control rats. The activity and protein expression of iNOS and nitrotyrosine were higher in the hearts of the diabetic than control rats. Dobutamine dose-dependently increased LVDP,  $+dP/dt$ , HR and RPP in both groups, but the responses were less in the diabetic than control rats. Selective inhibition of iNOS by 1400W did not alter responses to dobutamine in the control rats, but augmented the effects of dobutamine on LVDP and RPP, but not HR and  $dP/dt$ , in the diabetic rats.
- (2) ZDF rats showed decreased regional BF (normalized per g tissue) in the kidneys and intestines as compared to control rats. Treatment with 1400W did not significantly alter haemodynamic values in either group. Treatment with L-NAME significantly increased MAP and TPR, and decreased CO and HR, in both the ZDF and control rats. Regional BF and arterial conductance were decreased by L-NAME in all organs and tissues in both the ZDF and control rats; however, the reductions did not reach statistical significance in the testes and adipose tissue of the control rats, and in the testes and liver of the ZDF rats. Relative to the control rats, L-NAME-induced decreases in flow and conductance in the ZDF rats were markedly less in the pancreas, and greater in the hearts and brains.
- (3) The Zucker obese rats (an animal model of metabolic syndrome) had higher MAP, body weight, and plasma insulin and triglycerides, but reduced pressor and MCFP responses to noradrenaline ( $2.5\text{--}30 \times 10^{-9}$  mol/kg/min, i.v.) compared to the controls. The non-selective NOS inhibitor L-NAME (8 mg/kg, i.v.) did not alter MCFP in either group, but increased MAP of both groups, although the increase was markedly less in the obese than lean rats.
- (4) The fructose-fed rats had increased systolic BP, increased plasma insulin, triglyceride and 15-F2t-isoprostane, and decreased insulin sensitivity; these changes were prevented by

N-acetylcysteine. Maximal pressor response to methoxamine was attenuated in the fructose-fed rats given N-acetylcysteine relative to the other three groups.

We conclude that heart function is compromised and regional blood flow is altered in the ZDF rats in the chronic stage of type 2 diabetes. The depressed cardiac contractile function in ZDF rats is associated with the activation of iNOS and the formation of peroxynitrite due to the excessive amounts of NO produced by iNOS. Regional BF and conductance are altered in the ZDF rats with type 2 diabetes. However, iNOS plays a negligible role in regulating regional BF in the ZDF rats. There appears to be greater eNOS- derived NO-mediated vasodilation in the heart and brain, but less in the pancreas of rats with type 2 diabetic relative to controls. In the Zucker obese rats with genetic metabolic syndrome, the venous response to noradrenaline is altered but NOS may be not involved in basal venous tone regulation. The increased baseline arterial pressure of the Zucker obese rats may be related to reduced influence of the NO/L-arginine system. Furthermore, in the fructose-fed rats, abnormalities of diet-induced metabolic syndrome such as hyperinsulinemia, insulin resistance and BP elevation are associated with oxidative stress and increased lipid peroxidation. Our results suggest that activation of iNOS plays an important role in suppressing heart function in type 2 diabetes. Oxidative stress is associated with heart dysfunction in type 2 diabetes as well as the development of insulin resistance and BP elevation in metabolic syndrome as revealed by formation of peroxynitrite due to activation of iNOS in ZDF rats and increased lipid peroxidation in animal models of metabolic syndrome. Treatment with the selective iNOS inhibitor (1400W) improves heart contractile function in type 2 diabetes and pretreatment with the antioxidant (N-acetylcysteine) protects against the development of metabolic abnormalities in metabolic syndrome.

## **6. LIMITATION OF PRESENT STUDY AND FUTURE STUDY DIRECTIONS**

In the present studies, we were only able to show the acute effect of iNOS inhibition on cardiac contractile function in one genetic type of type 2 diabetes. Not enough work has been done to address the further mechanisms involved in the progression of functional changes of type 2 diabetes (for example, how the induction of iNOS in type 2 diabetes is related to the changes of carbohydrate and lipid metabolism as well as insulin sensitivity). In future studies, it will be of great interest and value to investigate how chronic treatment with selective iNOS inhibitors and antioxidants such as NAC (started from an age before hyperglycemia develops) affect cardiac function and other abnormalities such as insulin resistance in animal models of type 2 diabetes and how these treatments influence carbohydrate and lipid metabolism.

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## **APPENDIX: LIST OF PUBLICATIONS AND MANUSCRIPTS THAT CONTRIBUTE TO THIS THESIS**

1. Song, D., Hutchings, S., Pang, C.C.Y. Chronic N-acetylcysteine prevents fructose-induced insulin resistance and hypertension in rats. *Eur J Pharmacol* 2005;508:205-210.
2. Song, D., Hutchings, S.R., Pang, C.C.Y. Impaired arterial and venous constriction in rats with metabolic syndrome. *Naunyn-Schmiedeberg's Arch Pharmacol*, 2006; 373(6):451-456.
3. Song, D., Kuo, K.H., Yao, R., Hutchings, S.R., Pang, C.C.Y. Inducible nitric oxide synthase depresses cardiac contractile function in Zucker diabetic fatty rats. *Eur J Pharmacol* 2008; 579:253-259.
4. Song, D., Yao, R., Pang, C.C.Y. Altered vasodilator role of nitric oxide synthase in the pancreas, heart and brain of rats with spontaneous type 2 diabetes. Submitted to *Eur J Pharmacol*.