

**THE ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) ON
CARDIOVASCULAR FUNCTION IN RATS WITH STREPTOZOTOCIN-INDUCED
DIABETES**

by

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**A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

in

**THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF PHARMACOLOGY & THERAPEUTICS
FACULTY OF MEDICINE**

We accept this thesis as conforming
to the ~~required~~ standard

THE UNIVERSITY OF BRITISH COLUMBIA

March 2004

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ABSTRACT

Cardiovascular complications are leading causes of death in Type 1 and Type 2 diabetes mellitus. Experimental studies and clinical trials have firmly established a link between hyperglycemia and cardiovascular complications. The precise mechanism involved, however, remains elusive. Recently, inducible nitric oxide synthase (iNOS) and oxidative stress have been implicated as factors that initiate cardiovascular complications in diabetes. However, there is a lack of direct evidence that link these biological changes with abnormalities in cardiovascular function.

The three primary goals of the present investigation were: **1)** to investigate if rats with streptozotocin (STZ, 60 mg/kg i.v.)-induced Type 1 diabetes had functional abnormalities of the cardiovascular system, and if these changes were associated with the activation of iNOS, formation of peroxynitrite, and reduced myocardium antioxidant capacity; **2)** to examine if treatment with 1400W (inhibitor of iNOS) improved cardiovascular response to noradrenaline (NA) concurrently with inhibition of the activation of iNOS and formation of peroxynitrite in diabetes; **3)** to examine if the chronic treatment of the antioxidant N-acetylcysteine (NAC, antioxidant and inhibitor of iNOS) reduced activation of iNOS, decreased formation of peroxynitrite, increased antioxidative capacity, and improved cardiac contractile function in diabetes.

The results show that diabetic and control rats had similar mean arterial pressure (MAP) and total peripheral resistance (TPR) at three weeks after injection of STZ. NA increased *in vivo* MAP and TPR in both groups; however, the responses were markedly less in the diabetic than control rats. Rats with diabetes, relative to the controls, had reduced potency (increased ED₅₀) for the pressor (2.5-fold of control) and mean circulatory filling pressure (MCFP, an index of venous tone, 4.3-fold of control) response to NA, as well as reduced maximum pressor response (efficacy) to NA. Diabetic rats also had reduced potency (ED₅₀, 5-fold of control) of the pressor response to angiotensin II. Therefore, arterial and venous constrictions are impaired at an early phase of STZ-induced Type I diabetes.

The diabetic rats in the present study also had reduced heart rate (HR) and maximal rate of increase as well as decrease of left ventricular pressure (\pm dP/dt of LV) relative to the controls at three weeks after injection of STZ. In addition, the diabetic rats had reduced inotropic and chronotropic responses to NA and dobutamine (β_1 -adrenoceptor agonist), exemplified by the

decreases in the efficacy (reduced E_{\max}) and potency (increased EC_{50}) of HR, and left ventricular developed tension as well as contractility ($\pm dP/dt$ of LV) responses to dobutamine.

The activation of iNOS and oxidative stress were confirmed in myocardium at three weeks after injection of STZ, evidenced by **1)** detection of RT-PCR products (RNA) of iNOS in the hearts of the diabetic but not control rats; **2)** the three-fold increase of activity of iNOS in the hearts of diabetic rats relative to the controls, and inhibition of this increase by 1400W; **3)** clear identification of immunostaining (proteins) of iNOS and NT (*in vivo* marker of peroxynitrite, an oxidant and cytotoxic mediator) in the hearts of the diabetic rats, but not the controls; **4)** similar baseline lipid peroxidation (formation of 15-F_{2t}-isoprostane), but decreased tissue antioxidation capacity (increased thiobarbituric acid reactive substances, thiobarbituric acid reactive substance) upon peroxidation challenge.

Acute treatment with 1400W (3 mg/kg followed by 3 mg/kg/h, i.v) did not alter baseline hemodynamic variables or responses to NA in the control rats but completely restored the efficacy (E_{\max}) and insignificantly increased the potency (reduced ED_{50}) of MAP response to NA. This drug also completely restored the TPR response to NA, and increased MCFP response to a high dose of NA. Moreover, treatment with 1400W enhanced the influence of NA on cardiac contractility (LVP, LV $\pm dP/dt$) in the diabetic rats and concurrently reduced the activity of iNOS in the diabetic myocardium. Therefore, an activation of iNOS contributes to depressed cardiovascular contractile response to NA at the acute phase of STZ-induced diabetes. Selective inhibition of iNOS partially restores cardiovascular responses to NA.

Chronic treatment with the antioxidant NAC (1.2 g/day/kg) did not affect any measured variables in the control rats but improved cardiac contraction to dobutamine (1-57 $\mu g/min/kg$), exemplified by increased efficacy (E_{\max}) and potency (EC_{50}) of HR and left ventricular developed tension (LVP) as well as contractility ($\pm dP/dt$ of LV) responses to dobutamine. NAC also concurrently reduced immunostaining of iNOS and NT, as well as decreased myocardial level of 15-F_{2t}-isoprostane. Therefore, antioxidant supplementation is beneficial in the management of cardiac contractile dysfunction in diabetes mellitus.

To our best knowledge, this is the first study that links cellular activation of iNOS in diabetes to abnormal *in vivo* adrenoceptor-mediated responses in the heart and blood vessels, and demonstrates the restoration of cardiovascular function after selective inhibition of the activity of iNOS. These results provide direct evidence that link depressed cardiac contractility with the activation of iNOS as well as the production of peroxynitrite and its associated nitration

of tyrosine and reduction of antioxidative capacity. These results also provide the first evidence that NAC, an antioxidant/iNOS inhibitor, improved cardiac function concurrently with the reduction of myocardial level of 15-F_{2t}-isoprostane at three week after the induction of diabetes. Our results suggest that antioxidant therapy or inhibition the activity of iNOS is beneficial in the management of cardiac contractile dysfunction at an early phase of diabetes, even when baseline levels of lipid peroxidation products are not yet elevated.

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LIST OF ABBREVIATION

AGEs	Advanced glycosylation end products
cAMP	Adenosine 3',5'-cyclic monophosphate
<i>t</i> -BHP	<i>t</i> -butylhydroperoxide
CI	Cardiac index
CO	Cardiac output
+dP/dt	Maximal rate of increase of left ventricular pressure
-dP/dt	Maximal rate of decrease of left ventricular pressure
ET	Endothelin
EDRF	Endothelial-derived relaxing factor
GC	Guanylate cyclase
GSH	Reduced glutathione
GSH-Px	GSH peroxidase
GSSG	glutathione disulfide
GST	glutathione S-transferase
HR	Heart rate
IDDM	Insulin-dependent diabetes mellitus, type 1 diabetes
LPS	Lipopolysaccharide
LV	Left ventricular
LVEDP	LV end diastolic pressure
LVP	LV systolic pressure
MAP	Mean arterial pressure
MCFP	Mean circulatory filling pressure
NA	Noradrenaline
NAC	N-acetylcysteine
NT	Nitrotyrosine
L-NAME	N ^G -monomethyl-L-arginine
NIDDM	Non-insulin-dependent diabetes mellitus, type 2 diabetes
NF κ B	Nuclear transcription factor κ B

NOS	NO synthase
eNOS	Endothelial NOS
iNOS	Inducible NOS
nNOS	Neuronal NOS
cNOS	Constitutive NOS, including eNOS and nNOS
ONOO ⁻	Peroxynitrite
PKC	Protein kinase C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOS	Superoxide dismutase
O ₂ ⁻	Superoxide anion
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substances,
TPR	Total peripheral resistance
WHO	World Health Organization
1400W	N-3-aminomethyl-benzyl-acetamidine

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor and my mentor, Dr. Catherine CY Pang, for giving me the opportunity to fulfill my dream—earning a Ph.D. degree—under her professional guidance, as well as her full support at all times. It is my pleasure to have met her in my life.

I am very grateful to my supervisory committee member Dr. Michael Walker for his sense of humor, support, and professional guidance. I am thankful to another member of my supervisory committee, Dr. Brian Rodrigues, for his professional guidance and valuable contributions to my thesis. Many thanks also to Dr. Morley Sutter for his kindness and support.

I appreciate very much all the assistance I received from Ms. Su Lin Lim. It has been a pleasure working with her.

I also appreciate the friendship and emotional support from Dr. Lewei Zhang. She has always been there whenever I need her.

Finally, I would like to express my special thanks to my family, my husband Tom Karoutsos and our daughter Xingxing, for their special support, unbelievable patience, and understanding.

DEDICATION

To

My lovely daughter Xing Xing

My caring husband Tom

My loving parents

1. INTRODUCTION

1.1 Diabetes mellitus

Diabetes mellitus is a common disease characterized by elevated levels of blood glucose. The name of diabetes mellitus comes from the Greek words “diabetes” for a siphon, meaning “to pass through” and “mellitus” meaning “honey” (Pickup and Williams, 2003). Diabetes was first described in the Egyptian papyrus *Ebers* in 1500BC. It currently affects approximately 150 million people worldwide (Zajaz et al., 2002).

Diabetes mellitus is a growing public health problem throughout the world, especially in North America, and has become a heavy global public health burden (King et al., 1998; Vinicor, 1994; Vinicor, 1998). Diabetes mellitus is reported to be among the five leading causes of death in most countries. The disease and its associated morbidity and mortalities contribute to enormous personal, public and economic costs (Venkat Narayan et al., 2000). According to a survey in Ottawa in 1986, the average annual averaged routine care costs were \$962 per person annually (McKendry, 1989). In 1998, the total economic burden (in US\$) of diabetes and its chronic complications (principally cardiovascular diseases) in Canada was estimated to be between 4.76 and 5.23 billion dollars (Dawson et al., 2002). The prevalence of diagnosed diabetes worldwide has increased from 30 million in 1985 to 135 million people in 1995. In 2000, the number increased again to 150 million, and the projected number of cases of diabetes in the world in 2025 is 300 million, meaning that the prevalence of diabetes will be 5.4% of the

population (Amos et al., 1997; King et al., 1998). Approximately 3.5%, or 0.9 million, of Canadians aged 12 or older were diagnosed with diabetes in 1998/1999 (Statistics Canada, 1998-1999). Considering the fact that there are many undiagnosed cases of diabetes (Leiter et al., 2001), as many as 7.0 % of Canadian adults may presently have diabetes mellitus. This number is believed to escalate to 3 million by 2010 (Meltzer et al., 1998).

Therefore, diabetes mellitus is a public-health disorder with three essential characteristics that define a public-health disorder: disease burden, a rapid change in disease incidence (suggesting preventability), and public concern about risk (Vinicor, 1994; Vinicor, 1998). More efforts must be made to prevent the development of the disease and its associated morbidities and mortalities.

1.1.1 Definition, classification, pathogenesis and diagnostic criteria

Definition: Diabetes mellitus is a heterogeneous group of metabolic disorders with the phenotype of hyperglycemia and disturbances of carbohydrate, fat, and protein metabolism resulting from absolute or relative impairment of the secretion or action of insulin. The effects of diabetes mellitus include long-term damage, dysfunction and failure of many organs.

Classification and pathogenesis: The first widely accepted classification of diabetes mellitus was published in 1980 and modified in 1985 by the World Health Organization (WHO) (World Health Organization, 1985). This classification, based on clinical descriptive criteria and treatment, included two major types of diabetes: insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM).

The American Diabetes Association published its final recommendation in 1997 (American Diabetes Association, 1997); the revised scheme was adopted by WHO as well as the Canadian Diabetes Association in 1998 (Alberti and Zimmet, 1998; Meltzer et al., 1998). The new classification encompassed both clinical stages and etiological types of hyperglycemia and was supported by numerous epidemiological studies. The terms IDDM and NIDDM were eliminated and replaced by the terms Type 1 and Type 2, respectively.

The current classification was further modified by the International Expert Committee in 2001, under the sponsorship of the American Diabetes Association, and it contains four major categories: 1) Type 1 diabetes is due to autoimmune destruction of pancreatic β -cells, and is characterized by an absolute deficiency in insulin (5-10% of all diabetes); 2) Type 2 diabetes ranges from predominant insulin resistance with relative insulin deficiency to a predominant secretory defect with or without insulin resistance (nearly 90% of all individuals with diabetes); 3) other specific types of diabetes, mainly associated with specifically genetic defects of the secretion or action of insulin, or is related to other diseases or drug use; 4) gestational diabetes is related to glucose intolerance with the onset during pregnancy (Alberti and Zimmet, 1998; American Diabetes Association, 2001; Meltzer et al., 1998).

Diagnostic criteria: Diagnosis of diabetes is established by the documentation of abnormal glycemic values and can be made by three methods: 1) random plasma glucose concentration ≥ 200 mg/dl (11.1 mmol/L) in the presence of diabetic symptoms; 2) fasting plasma glucose concentration ≥ 126 mg/dl (7.0 mmol/L); 3) two hour plasma glucose concentration ≥ 200 mg/dl (11.1 mmol/L) during the Oral Glucose Tolerance Test (75 g). To establish the

diagnosis, each abnormal glucose value must be confirmed a subsequent day using another one of the above three criteria (American Diabetes Association, 2001).

1.1.2 Diabetes and its complications

Since the advent of insulin treatment in 1940s, the clinical impact of the complications of diabetes has been changed from complications arising from acute diabetes to complications from chronic diabetes (Tooke, 1999). Regardless of the etiology, long-term metabolic abnormalities initiated by long-term hyperglycemia in chronic diabetes mellitus affect various organs, thus leading to various late complications. Although tight metabolic control has reduced the incidence of complications within the past three decades, the incidence of complications is still very high (35-70%) in the USA (The Diabetes Control and Complications Trial Research Group, 1993). Clinically, these late complications may be classified as follows:

- Cardiovascular complications, which will be discussed later.
- Diabetic retinopathy and cataracts, which threaten vision, and are the leading cause of blindness in Americans of working age (24-74 years old) (Patz and Smith, 1991). Proliferate retinopathy is the most common cause of blindness in Type 1 diabetes. Maculopathy is a very common form of retinopathy in Type 2 diabetes.
- Increased incidence of diabetic nephropathy, which occurs in 30-35% of patients with Type 1 and Type 2 diabetes. Diabetic nephropathy is closely associated with cardiovascular diseases and hypertension (Gnudi and Gruden, 2003), and can lead to end-stage renal failure, one of the most frequent causes of death in Type 1 diabetic patients under 30 years of age (Rosenstock and Appel, 2002).
- Diabetic neuropathy, a nonspecific term that implies an abnormality of peripheral nerves, is a common chronic complication of diabetes. It is often synonymously and imprecisely termed polyneuropathy, which is the most common form of diabetic neuropathy (Chin and Rubin, 2002). Diabetic neuropathy involves nerve damage in almost all body organs and systems, and may affect neuropathy of autonomic nerves that innervate the cardiovascular and gastrointestinal systems. Autonomic neuropathy is particularly stressful in the form of impotence and disabling in the form of postural hypotension.

1.2 Cardiovascular complications in diabetes

Cardiovascular complications are leading causes of death in Type 1 and Type 2 diabetes mellitus. A survey of the WHO multinational study in diabetes in ten countries around the world has demonstrated that cardiovascular diseases account for 44 and 52%, respectively, of deaths in Type 1 and Type 2 diabetes mellitus (Morrish et al., 2001). In another study conducted in the United Kingdom, diabetes has almost double the risk of cardiovascular diseases. Cardiovascular disorders cause approximately 35% of deaths in non-diabetics, compared to 60% in Type 2 diabetics, and 67% in Type 1 diabetics over 40 years old (Watkins, 2003). In 1998, diabetes-related cardiovascular complications cost roughly 5 billion (US\$) in Canada (Dawson et al., 2002). Diabetic patients exhibit multiple cardiac and vascular abnormalities; the latter impacts on the function of almost all organs and systems (Shaw, 1996). Therefore, from the point of view of cardiovascular medicine it may be appropriate to say that diabetes is a cardiovascular disease (The Diabetes Control and Complications Trial Research Group, 1993). A better understanding of the mechanisms by which the cardiac and vascular pathologies develop in diabetes will aid the development of future treatment strategies (Guest and Sperling, 2000).

Cardiovascular complications in diabetes can be divided into three categories on the basis of pathological criteria: macrovascular pathology, microvascular pathology and cardiomyopathy.

1.2.1. Macrovascular pathology

Macrovascular pathological changes may lead to the development of **atherosclerosis**, **coronary heart disease**, and **cerebrovascular disease**, which can culminate in hypertension, stroke and myocardial infarction, or the development of **peripheral vascular disease** that increases the susceptibility of infection, ulceration, poor healing, gangrene and amputation of the periphery (Williams and Airey, 1999).

1.2.2 Microvascular complications

Microvascular pathological changes may contribute to the development of diabetic **retinopathy**, the leading cause of blindness in working age Americans (Patz and Smith, 1991); **nephropathy**, the most important cause of end-stage renal disease in the US (Rosenstock and Appel, 2002); **cardiomyopathy** and **neuropathy**. In addition to peripheral somatic nerves, autonomic nerves can also be affected. Cardiovascular autonomic neuropathy is characterized

by resting tachycardia, impaired exercise-induced cardiovascular responses, cardiac denervation, orthostatic hypotension, and impaired reflex arterial and venous constrictions (Vinik et al., 2000 and 2003).

1.2.3 Diabetic cardiomyopathy

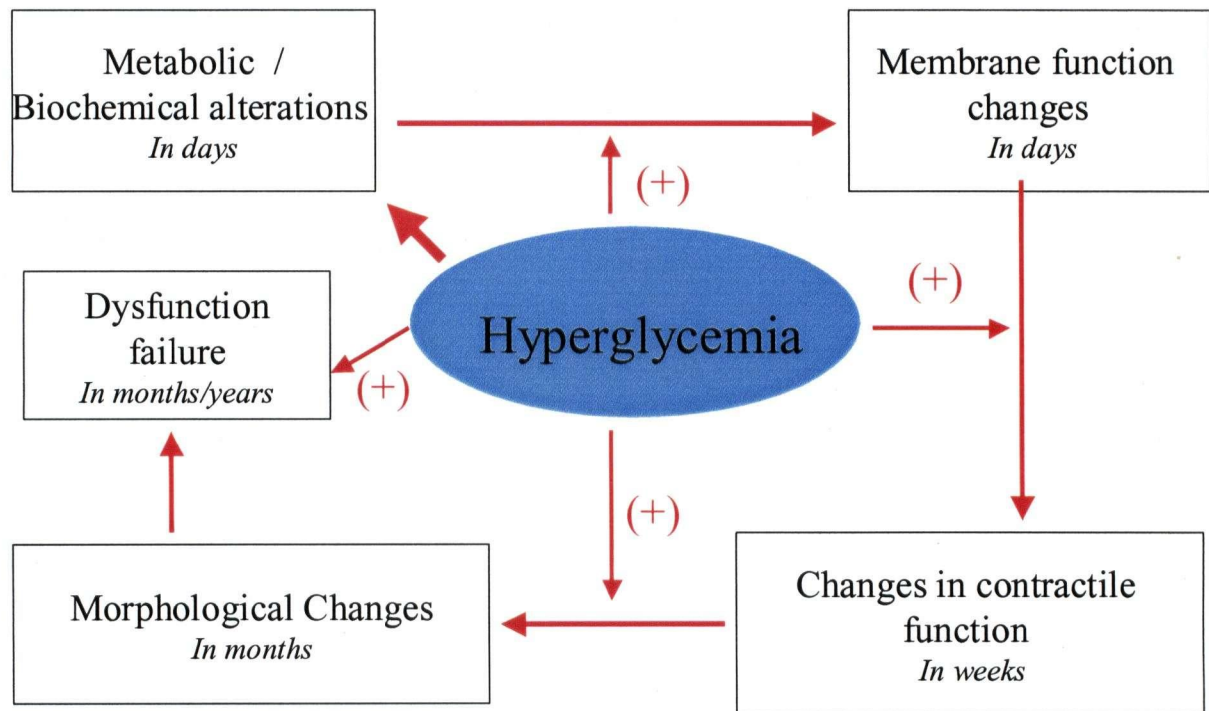
Cardiomyopathy is defined as cardiac dysfunction in the absence of dominant pathophysiology (Davies, 2000; Richardson et al., 1996). Cardiomyopathy independent of coronary atherosclerosis, macroangiopathy and autonomic neuropathy has been recognized in diabetes since 1972, when Rubler et al. (1972) reported the existence of diabetic patients with congestive heart failure but no evidence of valvular, congenital, hypertensive disease or coronary atherosclerosis (Rubler et al., 1972). Diabetic patients have an increased risk of congestive heart failure (Kannel et al., 1974; Kannel and McGee, 1979). Hyperglycemia, as an independent risk factor, can directly cause cardiac damage which culminates as diabetic cardiomyopathy (Cai and Kang, 2001). There were, however, histological changes in the heart, for example, the accumulation of peroxidase acid Schiff (PAS)-positive material (Regan et al., 1977) as well as lipid accumulation within the cardiomyocytes (Yokoyama et al., 2003). Furthermore, diabetic cardiomyopathy is associated with defects in myocardial cellular organelles such as myofibrils, mitochondria, sarcoplasmic reticulum and sarcolemma. In addition, calcium handling and adrenergic function of the heart are impaired. The above derangements adversely affect cardiac contraction and relaxation, and can ultimately lead to cardiac dysfunction and failure (Lagadic-Gossman et al., 1996; Malhotra et al., 1981; Matsuda et al., 1999; McNeill, 1996).

1.2.4 Potential underlying mechanisms: an overview

Multicenter, randomized, prospective, and controlled-clinical studies (the Diabetes Control and Complications Trial, DCCT) have established a cause and effect relationship between chronic hyperglycemia and diabetic complications (DCCT Research Group, 1987; DCCT Research Group, 1993; DCCT Research Group, 1995; Tamborlane and Ahern, 1997). Hyperglycemia-induced cardiac complications may range from metabolic or biochemical alterations to functional changes in the cell membrane, contractile defect, morphological changes, and finally cardiac dysfunction (Fig. 1-1). Although chronic hyperglycemia may be the most important cause of cardiac complications in diabetes, the mechanism by which it

causes functional and morphologic changes is not clear. Four major theories have been postulated to explain the etiology and pathogenesis of diabetic complications (Caprio et al., 1997; Nishikawa et al., 2000a; Sima and Sugimoto, 1999).

Figure 1-1 Stages of diabetic cardiac effects



Adapted from *Cardiovascular Toxicology*, 1:183 (Cai and Kang, 2001).

1.2.4.1 Advanced glycosylation end products (AGEs)

Hyperglycemia and ensuing increased intracellular glucose may lead to increased nonenzymatic glycosylation of amino groups on proteins and the formation of AGEs. AGEs have been shown to cross-link proteins such as collagen and extracellular matrix proteins, thereby causing morphological and functional cardiovascular abnormalities, such as the thickness of the basement membrane, increased production of reactive oxygen species (ROS), and the development of atherosclerosis (Chappey et al., 1997; Lapolla et al., 2001).

1.2.4.2 Polyol pathway

Hyperglycemia increases the conversion of glucose to sorbitol via aldose reductase. Increased cellular level of sorbitol may decrease myoinositol formation, decrease the activity of

cytosolic Na^+/K^+ ATPase, and alter redox potential, the latter is through a decrease in the concentration of NADPH which leads to GSH depletion and increased oxidative stress. All these changes ultimately lead to cellular dysfunction (Pickup and Williams, 2003).

1.2.4.3 Increases in diacylglycerol (DAG) level and activated protein kinase C (PKC)

It has been proposed that hyperglycemia increases the formation of DAG which causes the activation of the β isoform of PKC (Meier and King, 2000). PKC_β may in turn modulate the activities of a variety of enzymes that include Na^+/K^+ -ATPase and increase the expression of proteins such as extracellular matrix proteins and contractile proteins. Several growth factors and other pathways may also be involved in this pathogenesis. The transform of growth factor β (TGF_β) appeared to play an important role in the activation of the PKC_β isoform (Caprio et al., 1997). As well, abnormal amount of basement membrane matrix can lead to vascular dysfunction and atherosclerosis (Ishii et al., 1998). It is of interest that transgenic mice with an over-expression of PKC_β isoform in the heart also developed cardiac failure (Wakasaki et al., 1997). These findings show a link between PKC_β activation and cardiovascular abnormalities in diabetes (Inoguchi et al., 1992) (King et al., 1996; Koya and King, 1998).

1.2.4.4 Oxidative stress

The autooxidation of glucose in diabetes has been shown to indirectly cause oxidative stress through activation of three seemingly independent biochemical pathways (see above) and/or through attenuation of the scavenging capacity for reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Cai and Kang, 2001; van Dam, 2002). Furthermore, hyperglycemia-induced overproduction of superoxide from the mitochondria has been shown to enhance each of the three pathways described above (Du et al., 2000; Koya and King, 1998). Oxidative stress may be a single unifying mechanism that links hyperglycemia to functional and morphological changes that define diabetic complications (Nishikawa et al., 2000a).

1.3 Diminished cardiac contractility and changes of hemodynamics in diabetes

1.3.1 Diminished cardiac contractility

The contraction and relaxation phase of ventricular myocytes (shortening and relengthening) were impaired at four to six days (short term) and five to eight weeks (long term) following the injection of streptozotocin (STZ) in rats (Okayama et al., 1994; Ren and Davidoff, 1997a; Yu et al., 1994b). These abnormalities may be due to an intrinsic depression

of excitation-contraction coupling, such as defective Ca^{2+} storage or release in the sarcoplasmic reticulum and/or contractile dysfunction of the myocytes. Isolated diabetic rat hearts had significantly lower left ventricular (LV) systolic pressure (LVP), slower maximal rate of increase of left ventricular pressure (LV+dP/dt), lower heart rate (HR), and slower isovolumic relaxation at four to eight weeks after injection of STZ (El-Omar et al., 2003; Rodrigues et al., 1986; Smith et al., 1997; Verma and McNeill, 1994). There are also reports of decreases in *in vivo* resting HR, LVP, and LV \pm dP/dt (maximal rate of increase and decrease of left ventricular pressure), and increases in LVEDP (LV end-diastolic pressure) as well as the time constant of LV relaxation, at 26 days or four to eight weeks after the induction of diabetes by STZ (Dowell et al., 1986; Litwin et al., 1990; Okayama et al., 1994).

In addition, adrenoceptor-mediated inotropic responses are also depressed in the hearts of diabetic rats. The sensitivity of response to β -adrenoceptor agonists such as isoproterenol and noradrenaline (NA) was decreased in LV trabecular muscles at four weeks after injection of STZ (Banyasz et al., 1996). As well HR, LVP and LV \pm dP/dt responses to NA and dobutamine were impaired (Paulson et al., 1986) *in vivo* and *ex vivo* (Smith et al., 1997) at eight week after injection of STZ. It has been reported that the number but not the affinity of β -adrenoceptors was reduced in the cardiac membranes of diabetic rats at two to ten weeks after injection of STZ (Atkins et al., 1985; Beenen et al., 1997; Nishio et al., 1988; Ramanadham and Tenner, 1987).

In summary, there seems to be a strong link (Kashiwagi et al., 1989) between hyperglycemia and cardiac abnormalities. There were contractile defects identified in the hearts of animals with a deficiency of insulin, and these range from slower shortening and relaxation kinetics, elevated LVEDP, to reduced cardiac contractility, cardiac output and resting HR. In addition, there was a reduction in the number or density of β -adrenoceptors in cardiac membranes. All these abnormalities were reversed following treatment with insulin (Litwin et al., 1990).

1.3.2 Changes of hemodynamics in diabetes

1.3.2.1 Changes in vascular contractile function

In vitro experiments. Several studies have shown that vascular preparations from diabetic animals have abnormal relaxation or contraction response to vasoactive agents as follows. The superior mesenteric artery from rats with STZ-induced diabetes for 12-14 week had reduced

efficacy (maximum response) but similar potency (ED_{50}) of response to NA (Bardell and MacLeod, 2001). As well, the efficacy of contraction to ET-1 and dilatation to IRL-1620 (ET_B receptor agonist) were reduced in mesenteric artery preparations from rats injected with STZ for 10 week (Makino and Kamata, 1998a). The efficacy but not potency of contraction to ET-1 was attenuated in aorta from rats treated with STZ for two weeks (Utkan et al., 1998). Aortic preparations from rats with alloxan-induced diabetes for four weeks had decreased potency of response to a variety of constrictor agents, namely, KCl, angiotensin II, and $CaCl_2$; both potency and efficacy of responses to these agents were, however, reduced at eight weeks after the onset of diabetes (Turlapaty et al., 1980). The importance of disease progression was highlighted in a recent study which showed that endothelium-dependent relaxation of aortic rings was increased at 24 h, normal after one and two weeks, but impaired at 8 weeks after injection of STZ (Pieper, 1999). These results show that vascular relaxation and contraction are impaired at the acute phase of diabetes.

In vivo studies. Reduced pressor responses to NA were reported in pithed rats at two weeks (Foy and Lucas, 1976), (Lucas, 1985) and in conscious rats at four to six weeks after injection of STZ (Jackson and Carrier, 1983; Yu and McNeill, 1992). Reduced pressure responses to angiotensin II was also been reported in conscious rats that were intact (Jackson and Carrier, 1983) or ganglionic-blocked (Hebden et al., 1987) at three to five weeks after injection of STZ. The impairment of responsiveness is time-dependent and dependent on the severity of the disease. Hypotensive responses to ACh or bradykinin as well as the pressor responses to NA were unaffected at two weeks, but reduced at five weeks after injection of STZ, whereas pressor responses to ET-1 was decreased at both times (Guillon et al., 1998). In addition, depressor responses to ACh and sodium nitroprusside were decreased at six week after injection of STZ (Yu and McNeill, 1992).

Possible mechanism. Various hypotheses have been proposed to account for the abnormalities of vascular relaxation or contraction to various vasoactive agents. Attenuated contraction to ET-1 and vasodilatation to ET_B -receptor agonist in the mesenteric arterial bed was attributed to desensitization of ET receptors (Makino and Kamata, 1998a). Reduced efficacy to NA in the mesenteric artery was attributed to activation of iNOS (Bardell and MacLeod, 2001), or via NO-independent mechanisms (Tatchum-Talom et al., 2000).

Capacitance vessels. A serious consequences of autonomic neuropathy in diabetics is orthostatic hypotension (Stansberry et al., 1997; Vinik et al., 2000; Watkins and Thomas, 1998)

which is due to diminished constriction of capacitance vessels in the splanchnic and leg regions during a change from the supine to upright posture (Pang, 2001). Despite importance of the venous vasculature in the regulation of cardiac output and blood pressure, there is limited research on venous response in diabetes. Turlapaty et al. (1980) reported that the potencies of responses to angiotensin II and CaCl_2 in isolated portal vein were decreased at four and eight weeks, respectively, after injection of STZ; the magnitudes of responses to NA, angiotensin II, KCl, CaCl_2 were, however, unaffected at 1-8 weeks, whereas spontaneous phasic contractions of the portal vein increased progressively at this time (Turlapaty et al., 1980). Relative to healthy controls, forearm venous compliance decreased in patients with Type 2 diabetes with or without (incipient) nephropathy (Schaper et al., 1994). More studies are needed to elucidate responses and the cause of abnormal responses in venous vasculatures and the venous system in diabetes.

1.3.2.2 Changes in cardiac output and regional blood flow

Diabetes mellitus is associated with a reduction in cardiac output (CO). There is, however, a paucity of information on how diabetes affect the regional distribution of blood flow to various organs and tissues. CO and hindquarter arterial flow were decreased at an early stage (six days) of STZ-induced diabetes, (Brands and Fitzgerald, 1998; Brands et al., 2000). At eight weeks after the onset of diabetes, CO as well as flows to the skeletal muscle and skin beds were reduced, but flows to the kidneys and intestine were increased (Hill and Larkins, 1989). In contrast, Litwin reported the maintenance of CO despite impaired cardiac systolic function at four week after injection of STZ (Litwin et al., 1991). In pithed rats, TPR as well as constrictor responses to NA in the intestine and kidneys were attenuated in rats with STZ-induced diabetes for two weeks (Lucas, 1985). Diabetes is expected to differentially affect blood flow to different beds according to the extents of autonomic sympathetic neuropathy and/or local release of vasodilator or constrictor mediators at the particular sites. More studies are needed to elucidate how the progression of diabetes affects the distribution of blood flow, as well as how neurotransmitters, vasoconstrictors, vasodilators and drugs (pharmacological agents) affect the distribution of flow to different organs and tissues.

1.3.3 Potential mechanisms of cardiac abnormalities

The cause of diabetic cardiomyopathy is unclear, and may involve mechanical defects in the myocardium, a reduction in the affinity or number of β -adrenoceptors, and/or defective

β -adrenoceptor-mediated signaling (Beenen et al., 1997; Cai and Kang, 2001; Malhotra and Sanghi, 1997a; Ramanadham and Tenner, 1987; Wold et al., 2001; Yu et al., 1994a).

Abnormalities in the structure and function of contractile proteins and regulatory proteins (such as myosin ATPase and myosin ATPase) could be the cause of mechanical defects in the diabetic heart. Calcium-stimulated myosin ATPase activity was decreased in the heart of rats at one and two week after injection of STZ (Malhotra et al., 1981). In the spontaneously (Type 1) diabetic Bio-Breeding Worcester (BB/W) rat, calcium-stimulated myosin ATPase activity was depressed at age of four and seven months, and actin-activated myosin ATPase was depressed at one, four and seven months (Malhotra et al., 1985). Cardiac myosin isoenzyme pattern shifted from the normally predominant V1 to V3 in the hearts of rats with STZ-induced diabetes for five to six weeks (Takeda et al., 1988) and those of BB/W rats (Malhotra et al., 1985). This shift in cardiac myosin heavy chain could contribute to the impairment of cardiac function. Decreased cardiac myofibrillar ATPase activity was also reported in rats after induction of diabetes with STZ for four weeks (Malhotra et al., 1995). There was a report that the phosphorylation of TnI, a protein that regulates muscle contraction and calcium sensitivity (discussed later in this Chapter), was higher in the hearts of rats treated with STZ for eight weeks, and that this change contributed to the depression of cardiac myofibrillar ATPase activity in diabetics (Liu et al., 1996).

Reduction in contractile response to β -adrenoceptor agonists could be due to a change in the number or affinity of β_1 -adrenoceptors or an alteration in signal transduction. A reduction of the number of β -adrenoceptors, particularly high affinity receptors, has been observed in the cardiac membrane of rats with STZ-induced diabetes for two to ten weeks (Atkins et al., 1985; Beenen et al., 1997; Nishio et al., 1988; Ramanadham and Tenner, 1987). Rats with STZ-induced diabetes for four to six week had decreased density of β -adrenoceptor mRNA in the ventricular myocardium (Matsuda et al., 1999). Radioligand binding analysis revealed a specific decrease in the population of the high affinity state of β -adrenoceptors, a necessary intermediate for adenylate cyclase activation, and this occurred simultaneously with reduced response to isoproterenol and increased circulating level of adrenaline (Ramanadham and Tenner, 1987). All these abnormalities occurred concurrently with depressed cardiac contractile and adenylate cyclase responses to isoproterenol and dobutamine (Atkins et al.,

1985; Beenen et al., 1997). Recently, a change in the expression of β -adrenoceptor subtype was reported in the hearts of rats with STZ-induced diabetes (Dincer et al., 2001).

Impaired ventricular contraction in diabetes could be the result of defective post-receptor regulation of intracellular Ca^{2+} ; it is however, unclear if a Ca^{2+} -deficient or overload state exists in the resting diabetic hearts (Pierce and Russell, 1997b). Isolated cardiomyocytes from diabetic rats had decreased maximum increase in intracellular Ca^{2+} (but no change in sensitivity) in response to isoproterenol as well as 8-bromo-cAMP -- these results suggested the presence of post receptor defects in the myocardium (Yu et al., 1994a). There is evidence that myocardial sarcoplasmic reticulum intracellular Ca^{2+} content, uptake and release are defective in diabetics. Indeed, caffeine-induced Ca^{2+} release (Yu et al., 1994b) (Lagadic-Gossman et al., 1996) (Tamada et al., 1998) and rapid-cooling contracture (Yu et al., 1994b), indices of sarcoplasmic reticulum Ca^{2+} content, were reduced in myocytes from rats with STZ-induced diabetes for three to six weeks. Yu et al. (1994) also reported that Ca^{2+} uptake and release in the sarcoplasmic reticulum were reduced in the diabetic myocardium (Yu et al., 1994b). There was also the report of decreased diastolic and peak systolic intracellular Ca^{2+} as well as the decay phase of the systolic intracellular Ca^{2+} transients, which resulted in the prolongation of the duration of intracellular Ca^{2+} transients in diabetic myocytes from rats with STZ-induced diabetes for three to four week (Lagadic-Gossman et al., 1996). The underlying mechanism may involve the following:

- 1) A reduction in the number of high-affinity ryanodine binding sites on sarcoplasmic reticulum Ca^{2+} -release channels in the myocardium of rats with STZ-induced diabetes six week (Yu et al., 1994b), and this could reduce the availability of intracellular Ca^{2+} for contraction.
- 2) Decreased activities in sarcoplasmic reticulum Mg^{2+} -ATPase and Ca^{2+} - Mg^{2+} -ATPase in the myocardium of diabetic rats at four to five and nine weeks after injection of STZ (Penpargkul et al., 1981). Myocardial Ca^{2+} -ATPase activity and affinity for Ca^{2+} were decreased at 12 week after injection of STZ (Kim et al., 2001), and this would decrease the rate of sarcoplasmic reticulum Ca^{2+} uptake. Furthermore, STZ-treated diabetic transgenic mice with over-expression of sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) had greater *in vitro* myocardial contractility (LVP and $\pm\text{dP/dt}$) as well as

active force and the maximum speed of relaxation of the papillary muscles than the corresponding responses in the wild-type mice (Trost et al., 2002).

- 3) Decrease in the activities of the sarcolemmal Ca^{2+} pump (Hattori et al., 2000b) and the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (Makino et al., 1987) during STZ-induced diabetes, and this could reduce intracellular Ca^{2+} and cardiac performance.
- 4) Other factors that affect intracellular Ca^{2+} homeostasis include increased expression of phospholamban in the hearts of rats at 12 weeks after injection of STZ, which could enhance the inhibition of sarcoplasmic reticulum Ca^{2+} uptake via sarcoplasmic reticulum Ca^{2+} -ATPase (Kim et al., 2001), and a shift of contractile regulatory proteins from TnT1 to TnT3 in cardiac trabeculae of diabetic rats at three to six month after injection of STZ and this could change length-dependence of Ca^{2+} sensitivity (Akella et al., 1995).

It is worthy to note that most of these abnormalities could be corrected following treatment with insulin, and this implies a link between hyperglycemia and cardiovascular complications.

1.4 Nitric oxide, nitric oxide synthase and diabetes

1.4.1 Overview

Nitric oxide (NO) was first described as an endothelial-derived relaxing factor (EDRF) by Furchgott and Zawadzki in 1980 (Furchgott and Zawadzki, 1980). NO is synthesized by NO synthase (NOS) that catalyzes the oxidation of L-arginine to L-citrulline in the presence of oxygen, NADPH, and cofactors FAD, FMN, haem, thiol and tetrahydrobiopterin. There are three major isoforms of NO synthase (NOS), namely, endothelial NOS (eNOS, type III) in platelets and endothelial cells, neuronal NOS (nNOS, type I) in neurons, and iNOS (Type II) in vascular endothelial cells, smooth muscle cells, cardiomyocytes, macrophages and mast cells (Colasanti and Suzuki, 2000; Mayer and Andrew, 1998). Both eNOS and nNOS are constitutive (and known as cNOS), and they require Ca^{2+} for activation. The activity of iNOS, however, is independent of Ca^{2+} , but requires an inducer, e.g. invading microorganism, endotoxin or cytokines which may be activated by inflammation, diabetes, or other disorders. After induction, iNOS produces vast quantities of NO, which is substantially larger than those produced by eNOS and nNOS. The activity of iNOS continues until the substrate is exhausted.

NO, at 100-500 nM, is an endogenous activator of soluble guanylate cyclase (GC) which converts guanosine triphosphate (GTP) to cyclic 3',5'-guanosine monophosphate (cGMP), a second messenger in many cells (Beckman and Koppenol, 1996b). At the nanomolar level, NO is carried within the circulation through interaction with hemoglobin. At a higher steady state concentration (1-3 μM), NO effectively competes with superoxide dismutase (SOD) for superoxide ($\text{O}_2^{\cdot -}$) to form of ONOO^- because of its rapid rate constant (approximately three times faster than SOD for scavenging $\text{O}_2^{\cdot -}$) (Beckman and Koppenol, 1996b) (Fig. 1-2)(Beckman, 1996a). NO can also be metabolised to produce a diverse mixture of toxic nitrogen intermediates, named reactive nitrogen species (RNS), which include nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3). RNS oxidizes and nitrates virtually all classes of biomolecules that include lipids, DNA, thiols, amino acids and metals, thereby causing oxidative stress, and ultimately cytotoxicity.

Figure 1-2 Cellular mechanisms of NO, superoxide and peroxynitrite actions

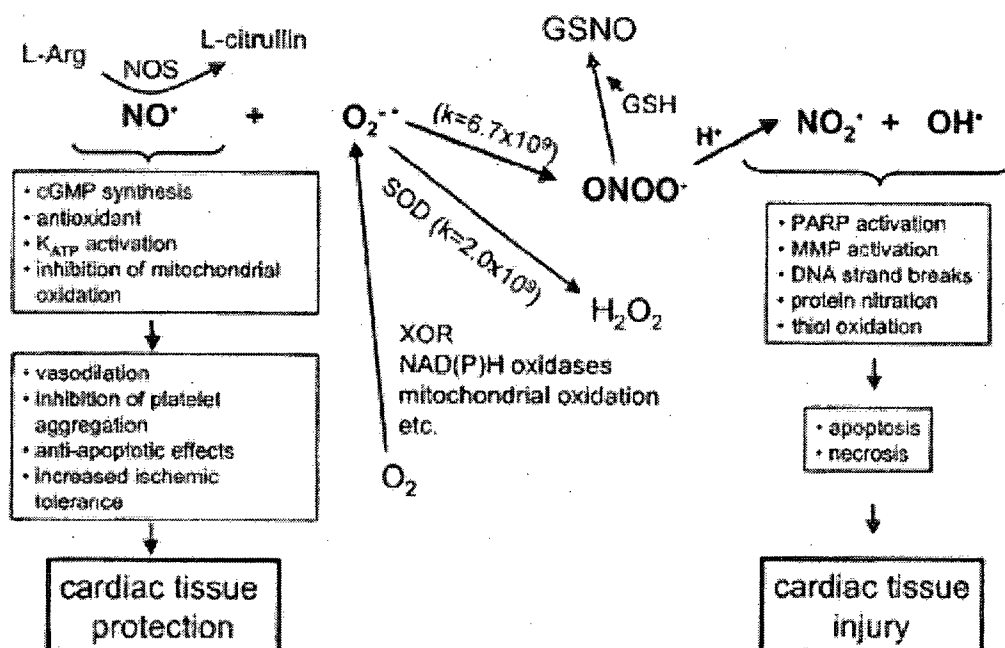


Fig. 1-2 Nitric oxide (NO) is essential for normal cardiovascular function via its vasodilator, antioxidant, anti-platelet, and anti-neutrophil adhesion actions. NO is, however, detrimental when combines with $\text{O}_2^{\cdot -}$ to form ONOO^- , which rapidly decomposes to highly reactive oxidant species that

can cause tissue injury. In the presence of superoxide dismutase (SOD), O_2^- is converted to H_2O_2 , which has less oxidant potential than O_2^- and $ONOO^-$. There is thus a critical balance between cellular concentrations of NO, O_2^- , and SOD. $ONOO^-$ is detoxified when combines with reduced glutathione (GSH) or other thiols to form S-nitrosoglutathione or nitrosothiols that can serve as donors of NO. MMP (matrix metalloproteinase); PARP (poly-ADP ribose synthase); XOR (xanthine oxidoreductase). Adapted from *British Journal of Pharmacology*, 138: 532-543, 2003 (Ferdinandy and Schulz, 2003).

1.4.2 NO and cardiovascular function

Under physiological conditions, eNOS is the major isoform of NOS within the cardiovascular system, and exists primarily in endothelial cells, but is also present in cardiac myocytes and platelets. NO released from the endothelium plays an important role in the regulation of vascular tone, inhibition of platelet and leukocyte adhesion and aggregation, and inhibition of cell proliferation (Garg and Hassid, 1989; Moncada et al., 1991a). NO synthesized by constitutively expressed NOS participates in the regulation of spontaneous beating rate, contractile responsiveness of rat ventricular myocytes to muscarinic (carbachol) and β -adrenoceptor (isoproterenol) agonists (Balligand et al., 1993a), as well as control of *in vivo* myocardial contractility in response to β -adrenoceptor agonist (e.g. dobutamine) in ganglionic-blocked dogs (Keaney et al., 1996).

Within the vasculature, eNOS is activated physiologically by physical (e.g. shear stress) and agonist-dependent (e.g. ACh) stimuli that increase the intracellular concentration of calcium. Upon activation, eNOS stimulates the production of NO, which diffuses from the endothelium to the underlying smooth muscle cells to activate soluble GC and the formation of cGMP. The latter activates protein kinase G which in turn decreases the cytosolic concentration of calcium thereby causing the vascular relaxation. NO-dependent regulations of other processes occur through similar mechanisms. For instance, NO production through eNOS activation is linked to other signal-transduction cascades such as the negative inotropic effect of acetylcholine (muscarinic agonists) in the heart (George et al., 1970) and the reduction of Ca^{2+} influx and the amplitude of muscle shortening by carbamylcholine (muscarinic and nicotinic agonist) in rat ventricular myocytes (Balligand et al., 1995).

In pathological conditions, iNOS is activated in a variety of cells, such as vascular endothelial cells, smooth muscle cells, cardiomyocytes, macrophages and mast cells (Colasanti

and Suzuki, 2000; Mayer and Andrew, 1998). The large amounts of NO produced by iNOS have beneficial effects such as antibacterial and anti-inflammatory actions; however, the large amount of NO produced by iNOS also impairs contractile function of myocytes (Balligand et al., 1993b; Brady et al., 1992; Roberts et al., 1992a; Roberts et al., 1992b; Schulz et al., 1992a) and vascular smooth muscle cells (Fleming et al., 1991b; Knowles et al., 1990; Rees et al., 1990).

There is evidence that iNOS is induced in hearts from animals with heart failure, such as rats with experimental autoimmune myocarditis, rats with volume-overload heart failure and mice with myocardial infarction (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). The selective blockade of iNOS with 1400W improved contractile function in the papillary preparations. Depressed *in vivo* myocardial contractility was also observed in rats with experimental autoimmune myocarditis; contractility was restored after the administration of aminoguanidine, a selective inhibitor of iNOS (Ishiyama et al., 1997). *In vitro* left ventricular contractility at baseline condition as well as in response to dobutamine were higher following myocardial infarction in mice with iNOS(-/-) mutant relative to those of the wild-type mice (Feng et al., 2001). Hare et al. (1995) also reported that in humans with LV dysfunction, NO produced in the heart caused the attenuation of positive inotropic response to dobutamine (β -adrenoceptor agonist) (Hare et al., 1995c). The possible mechanism by which iNOS depresses cardiovascular function may involve the following: 1) NO produced by iNOS attenuates the increase of intracellular cAMP and increases intracellular cGMP in response to β -adrenoceptor stimulation thereby interferes with Ca^{2+} -mobilization (Gealekman et al., 2002; Joe et al., 1998; Ungureanu-Longrois et al., 1995a); 2) NO increases the production of RNS which causes oxidative stress (Ishiyama, Hiroe et al., 1997); and 3) NO directly and indirectly (via the formation of RNS) depresses vascular reactivity (Fleming et al., 1991a; Szabo et al., 1995).

In summary, NO is important for normal physiological function of the cardiovascular system, but may also contribute to cardiovascular functional abnormalities in pathophysiological conditions such as heart failure and myocarditis.

1.4.3 Nitric oxide and nitric oxide synthase in diabetes

The vascular endothelium was damaged in both clinical and experimental diabetes, and this altered the bioavailability of endothelium-derived mediators and impairs endothelium-dependent relaxation of vascular smooth muscles (Farkas et al., 2000). Vasodilator mediators that may be affected by diabetes include NO, the most important mediator of endothelium-dependent vasodilatation, prostacyclin, and endothelium-derived hyperpolarizing factor. In clinical as well as experimental Type 1 and 2 diabetes, endothelium-derived NO formation or action has been shown to be increased, unchanged or decreased (Chan et al., 2000; Farkas et al., 2000). Conflicting data among studies may be due to the variations in the model, the duration and severity of diabetes, the methods of measuring the concentration or effect of NO, and the amount of inactivators of NO, such as superoxide that destroys NO or glycation end-products that quench NO (Chan et al., 2000; Farkas et al., 2000). Furthermore, the particular vascular bed or vessel (conduit or resistance artery) being examined also affects the results. As an example, acetylcholine-induced relaxation was attenuated in the perfused mesenteric bed but unchanged in the aortic rings of the same rat with STZ-induced diabetes, relative to the responses of control rats (Taylor et al., 1994). Furthermore, the potency of response to NA was reduced in the aorta but normal in the mesenteric microvessels of rats with alloxan-induced diabetes (Fortes et al., 1983). To better understand the role of NO in diabetes, it is important to examine the functional role of NO in intact organisms.

There is evidence that iNOS is activated in clinical and experimental diabetes:

- 1) *In hearts*: Isolated rat hearts perfused with high glucose (33.3 mmol/l) for two hours had increased iNOS expression but unchanged eNOS expression (Ceriello et al., 2002a). The total activity of NOS and mRNA encoded eNOS were increased in the hearts of rats with STZ-induced diabetic rats for four to six weeks (Stockklauser-Farber et al., 2000). iNOS was detected in the cardiac myocytes of diabetic rats (at four to six week or eight-week after STZ injection) but not control rats (El-Omar et al., 2003; Smith et al., 1997). The content of eNOS in the cardiac myocytes is unchanged (El-Omar et al., 2003) or increased (Smith et al., 1997) in diabetic rats relative to the controls. Furthermore, contractility was decreased in isolated perfused hearts from diabetic rats compared to controls, which was improved by L-NAME and L-NIL (El-Omar et al., 2003; Smith et al., 1997). These results suggest that NO derived from NOS is related to the depressed myocardial performance in STZ-induced diabetes.

2) *In vessels*: Inducible NOS was detected in the mesenteric artery of rats with STZ-induced diabetes for 12-14 weeks, but not in control rats (Bardell and MacLeod, 2001). Furthermore, the selective inhibition of iNOS by S-ethylisothiourea reduced EC_{50} of the concentration-contractile response to NA in endothelium-denuded mesenteric arteries of diabetic but not control rats. Therefore, it appears that iNOS is activated in blood vessels from diabetic rats and it reduces the potency of response to NA.

3) *In cells*: High glucose level (25 mmol/l) has been shown to enhance stimulated iNOS promoter activity and iNOS mRNA expression upon challenge with lipopolysaccharide (LPS) and interferon- γ in a murine macrophage cell line (RAW 264.7) and murine glomerular mesangial cells (Sharma et al., 1995). High glucose level, in the presence of interleukin-1 β , greatly increased the level of iNOS in these cells (Xu et al., 1999). Glycated human serum albumin has also been shown to activate nuclear factor- $\kappa\beta$ and the iNOS promoter in vascular smooth muscle cells of rats (Hattori et al., 1999). These results show that hyperglycemia, in the presence of cytokines, stimulates the cellular production of iNOS.

4) *In kidneys*: The iNOS level was increased significantly in the kidneys of STZ-induced diabetic rats from the 15th to 20th day while renal eNOS level remained unchanged (Cosenzi et al., 2002a). iNOS may be responsible for the increased urinary excretion of NO metabolites, which may be related to vasodilatation of the afferent arteriole and glomerular hyperfiltration observed in kidneys from rats with STZ-induced diabetes (Cosenzi et al., 2002b; Cosenzi et al., 2002a; Du et al., 2002)

5) *In patients*: iNOS was detected in the platelets of patients with Type 1 and 2 diabetes, but not in those of control subjects (Tannous et al., 1999). Furthermore, *in vitro* L-arginine/NO-dependent peroxynitrite production in the platelets was higher in these diabetic patients than in controls. It was suggested that iNOS-dependent peroxynitrite production is the cause of platelet damage observed in diabetes.

6) *Hyperglycemia-iNOS-eNOS-nNOS*:

The production of NO and the expression of iNOS mRNA in macrophage were increased in rats or mice with autoimmune (Type 1) as well as STZ-induced diabetes. These increases were abolished by treatment with insulin (Stevens et al., 1997).

Hyperglycemia increases the production of NO, expression of iNOS and formation of NT in the retina, which have been detected *in vivo* in retinal homogenates of rats with STZ-induced diabetes for eight to ten weeks, and *in vitro* in bovine retinal endothelial cells and a transformed

retinal glial cell line incubated in 25 mmol/ml glucose (Du et al., 2002). The expression of eNOS by cultured retinal microvascular endothelial cells was reduced after five days of exposure to high glucose and glycated albumin (Chakravarthy et al., 1998). In contrast, the other constitutive isoform of NOS, nNOS, is decreased in the retina after one week, and continued to decrease after three to 32 weeks in rats with STZ-induced diabetes (Roufail et al., 1998). Since the induction of iNOS was associated with reduced expression of the eNOS gene (Lamas et al., 1992), it has been suggested that decreased expression of eNOS is a consequence of the up-regulation of iNOS (Chakravarthy et al., 1998). Furthermore, an overproduction of NO by iNOS may inhibit the synthesis of NO by eNOS or/and nNOS through negative feedback inhibition of synthesis or the exhaustion of substrates.

The above studies show that iNOS is activated in various cells in Type 1 and Type 2 diabetes, and that this may be a consequence of hyperglycemia.

1.4.4 Inhibition of NOS in diabetes

Most of the NOS inhibitors used in studies in diabetic complications, such as L-NAME (Cosenzi et al., 2002b; El-Omar et al., 2003; Smith et al., 1997) and N(5)-(1-iminoethyl)L-ornithine (Bardell and MacLeod, 2001), lacked selectivity. Relative selective inhibitors, such as aminoguanidine (Corbett and McDaniel, 1996; Du et al., 2002), S-methyl-isothiurea (Bardell and MacLeod, 2001), and L-N⁶-(1-iminoethyl)lysine (El-Omar et al., 2003), have also been used for the elucidation of the pathophysiological role of iNOS in diabetes. Non-selective inhibition of the activities of both inducible and constitutive NOS is undesirable because the small amount of NO synthesized by eNOS and nNOS is important for normal physiological function (Fig. 1-2). The first available selective inhibitor of iNOS was aminoguanidine, which was 50-fold more effective in inhibiting iNOS than eNOS and nNOS (Corbett and McDaniel, 1996). Unfortunately, it had low *in vivo* potency and other actions, such as the inhibition of redox-active enzymes such as diamine oxidase, catalase and horseradish peroxidase as well as the formation of advanced glycosylation end-products (Mayer and Andrew, 1998; Monnier, 1996; Rahbar et al., 1999; Southan and Szabo, 1996a). Its low potency (up to 400 mg/kg *in vivo*) and non-selectivity limited its suitability for the evaluation of the *in vivo* role of iNOS (Cameron and Cotter, 1996; Kihara et al., 1991). Two other compounds, S-methyl-isothiurea and L-N⁶-(1-iminoethyl)lysine, have relatively low selectivity (20 and 30 fold, respectively) for iNOS over eNOS (Mayer and Andrew, 1998). The best available drug is 1400W (N-3-

aminomethyl-benzyl-acetamidine, Calbiochem, San Diego). The selectivity of 1400W in inhibiting iNOS over eNOS has been demonstrated *in vitro* (Garvey et al., 1997; Young et al., 2000) as well as *in vivo* (Garvey et al., 1997; Laszlo and Whittle, 1997).

In rats, 1400W is >1000-fold (*in vitro*) and >50-fold (*in vivo*) more selective in inhibiting iNOS than eNOS, and is also very potent -- a single injection (0.3 mg/kg s.c., ED₅₀ dose) of 1400W reversed delayed LPS (iNOS)-induced vascular injury in rats (Garvey et al., 1997). In a rat model of LPS-induced endotoxic shock, 1400W restored blood pressure (Wray et al., 1998; Hamilton & Warner, 1998). We have found that 1400W (3 mg/kg iv, followed by 3 mg/kg.h) is efficacious and selective for iNOS. When given to thiobutabarbital-anesthetised rats at 2.5 h after i.v. injection of LPS (10 mg/kg), 1400W prevented the decrease in mean arterial pressure (MAP) but not CO, and had no effect on control rats. In contrast, L-NNA (non-selective inhibitor of NOS) markedly decreased MAP and CO in LPS-treated rats and increased MAP and decreased CO in controls (Cheng et al., 2003a).

1.5 Oxidative stress in diabetes

1.5.1 Overview: oxidative stress

Oxidative stress refers to a serious imbalance between the production of free radicals [such as superoxide anion (O_2^-) & hydroxyl radicals (OH^\cdot), peroxy radical ROO^\cdot in the ROS group, NO^\cdot , NO_2 , peroxynitrite ($ONOO^-$) in RNS group] and antioxidant capacity. Thus oxidative stress can be a result of either an increased formation of ROS/RNS and/or a diminished ability to inactivate ROS/RNS, thereby leading to tissue and organ damage and malfunction (Halliwell, 1995). Packer (1996) defined antioxidant as a metabolic intermediate, a substrate that protects biological tissues from radicals induced damage, and that undergoes recycling or regenerating via interaction with biological reductants, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSSGRed), catalase, glutathione (GSH), vitamin C or vitamin E (Packer and Tritschler, 1996).

ROS/RNS, in addition to their direct cytotoxic effects, have recently gained attention as physiological and pathological important cellular messengers (Sen and Packer, 1996). There is evidence that ROS/RNS may act as signalling molecules that regulate gene expression, cell proliferation, and cell death (Palmer and Paulson, 1997). ROS/RNS appear to be a key factor that initiate the activation of nuclear transcription factor κB (NF κB) (Muller et al., 1997). Once activated, NF κB binds to nuclear DNA and modulates the expression of several genes

encoding for adhesion molecules such as selectins, intercellular adhesion molecule 1 and vascular adhesion molecule 1, and up-regulates the production of various proinflammatory cytokines such as IL-2, IL-6, tumor necrosis factor, and iNOS (Ho et al., 1999; Xie et al., 1994).

1.5.2 Biomarkers of oxidative damage *in vivo*

It is well known that in the conditions of oxidative stress, ROS/RNA and their derivatives are able to damage DNA, proteins, and cause lipid peroxidation. Currently, the most important available *in vivo* biological markers of oxidative stress are **3-nitrotyrosine (NT)** for protein damages, **F₂-isoprostanes**, **malondialdehyde (MAD)** and/or **thiobarbituric acid reactive substances (TBARS)** for lipid peroxidation damages, and **8-hydroxydeoxyguanosine** for DNA damages (Rosen et al., 2001).

The presence of NT indicates the nitration of proteins, which can be receptors, regulatory proteins, transport proteins, and enzymes, including enzymes for antioxidant defence or repair. NT is generated after the attack of tyrosine residues in proteins by various RNS (such as ONOO⁻, NO[•]) and is therefore a biomarker for RNS-induced oxidative damages under *in vivo* conditions rather than an indication of the presence of ONOO⁻ (Frustaci et al., 2000; Halliwell, 1997; Kajstura et al., 2001). NT can be detected by immunological techniques (i.e. immunohistochemistry, western blot or ELISA), HPLC or gas chromatography-mass spectrometry. Immunohistochemistry is, however, the most popular method for the detection of NT in human and animal tissues (Beckman et al., 1994; Halliwell, 1997; Ischiropoulos, 1998).

F₂-isoprostanes, a group of biologically active compounds, are generated principally by non-enzymatic free radical oxidation of arachidonic acid in the membrane phospholipids and lipoproteins under the conditions of increased oxidative stress in animals and human (Barden et al., 1996; Davi et al., 1999; Morrow et al., 1999). It appears to be the most reliable marker of lipid peroxidation *in vivo* for the quantification of oxidative stress (Mezzetti et al., 2000; Morrow et al., 1999; Morrow and Roberts, 1997).

One of the most abundant isomers of F₂-isoprostanes is 15-F_{2t}-isoprostane, previous name: 8-epi prostaglandin F_{2α} or 8-epi-PGF_{2α}, which not only is a reliable marker of lipid oxidation, but also has biological activities such as coronary vasoconstriction and myocardial depression (Kromer and Tippins, 1999; Mobert et al., 1997). The quantification of 15-F_{2t}-Isoprostane is also a useful way of evaluating the effects of antioxidant interventions. Several

methods exist to quantify F_2 -isoprostanes and one of which is gas chromatographic/negative ion chemical ionization mass spectrometry. Mass spectrometry is a highly sensitive method of measuring isoprostanes and yields quantitative results in the low picogram range. However, high cost and technical difficulties limit its use. A recently developed alternative method to quantify isoprostanes is via an immunological approach (Morrow et al., 1999). Antibodies have been generated against 8-iso-PGF 2α and immunoassay kits are commercially available, which make this technique affordable and relatively easy to perform. Moreover, purification of plasma and tissue homogenate performed by solid phase extraction prior to direct ELISA has yielded reliable and acceptable results (Xia et al., 2003).

1.5.3 Potential sources of oxidative stress in diabetes

Nishikawa et al. (2000a) proposed that hyperglycemia is associated with increased production of ROS/RNS as well as the activation of each of the three major pathways (AGE pathway, polyol pathway and DAG-PKC pathway) that cause complications in diabetes (Nishikawa et al., 2000a). There is convincing experimental and clinical evidence that ROS/RNS is increased in both Type 1 and 2 diabetes, and this may be via the autooxidation of glucose (Wolff and Dean, 1987), the formation of AGEs (Bunn, 1981) and their subsequent interaction with their receptors or binding proteins (Yan et al., 1994), the activation of NADPH-oxidase (Rosen et al., 1998), the depletion of NADPH and GSH caused by the polyol pathway, protein glycation (Giugliano et al., 1996), and the activation of iNOS. At same time, there are disturbances in the antioxidant defence systems in diabetes, namely, reduction of SOD, GSHpx and GSH in tissues including the hearts of rats injected with STZ (Matkovics et al., 1997-1998), and decrease of antioxidative capacity of plasma or erythrocyte in Type 1 and Type 2 diabetes (Rocic et al., 1997; Varvarovska et al., 2003; Zaltzberg et al., 1999). More interesting is the similar levels of 15- F_{2t} -Isoprostane in the kidneys of control and diabetic rats at three weeks after the induction of diabetes, but a reduction in the level of 15- F_{2t} -Isoprostane in the kidneys of diabetic rats after treatment with the antioxidant DL- α -lipoic acid (Obrosova et al., 2003). Also, although the baseline plasma levels of 15- F_{2t} -Isoprostane were similar in control and diabetic subjects, plasma 15- F_{2t} -isoprostane increased during myocardial ischemia following heart surgery in high-risk patients with Type 1 and 2 diabetes relative to the corresponding levels in low-risk patients. Increased plasma level of 15- F_{2t} -Isoprostane was

correlated with worse post-operative cardiac function (Ansley et al., 2003). All the above observations are supportive of the existence of oxidative stress in diabetes

1.5.4 Oxidative stress in clinical and experimental diabetes

1.5.4.1 *In vitro studies*

The exposure of human serum albumin with glucose (25 and 50 mM) caused oxidative reactions in a concentration-dependent manner, as revealed by the electron paramagnetic resonance spectra. This was further confirmed by the measurement of the carbonyl content of human serum albumin, an indirect marker of oxidative damage (Finotti et al., 2001). Inoguchi reported that both high glucose level (400 mg/dl, for 72 h) and free fatty acids (FFA, 200 micromol/l of palmitate) stimulated ROS production, as detected by electron spin resonance spectroscopy, through PKC-dependent activation of NAD(P)H oxidase in both cultured aortic smooth muscle cells and endothelial cells (Inoguchi et al., 2000). Recently Zou also reported that exposure of human aortic endothelial cells to high glucose (30 or 44 mmol/l) for seven to ten days increased the release of $O_2^{\cdot-}$ in response to the calcium ionophore A23187 and nitrate, a breakdown product of peroxynitrite, in parallel with a decline in cGMP, which may be related to the loss of the modulatory role of the endothelium that is important in proper function of the vasculature (Zou et al., 2002a). AGE albumin or AGEs immuno-isolated from diabetic plasma caused oxidative stress in endothelial cells, as reflected by the generation of TBARS and activation of NF κ B, each of which was blocked by antibodies to AGE receptor polypeptides as well as by antioxidants (Yan et al., 1994).

1.5.4.2 *In vivo studies*

Levels of iNOS and NT immunoreactivity were higher in blood vessels and the retina of rats with new onset (two to six days after the onset of hyperglycemia) diabetes, and the levels were higher in rats at the chronic phase (4-18 months after the onset of hyperglycemia), which suggested that iNOS and peroxynitrite may contribute to retinal damage (Ellis et al., 2002). Sano et al. (1998) evaluated oxidative stress in diabetes through the measurement of electron paramagnetic resonance of ROS and free radicals *in vivo*. They found that a diabetic state enhanced the generation of free radicals *in vivo* at two, four and eight weeks as well as increased the urinary excretion of malondialdehyde after injection of STZ. Glycemic control through insulin treatment and antioxidant treatment with vitamin E could reduce oxidative stress (Sano et al., 1998). One of the consequences of oxidative stress is lipid peroxidation,

which was indicated by an increase in the concentration of 4-hydroxyalkenal in the retina of rats with STZ-induced diabetes for six weeks (Obrosova et al., 2000) and an increase in TBARS in the liver of rats with STZ-induced diabetes for four week (Feillet-Coudray et al., 1999a). Antioxidative defence activities, including SOD, GSHPx, GSSGRed and glutathione transferase, also decreased in the retina of rats with STZ-induced diabetes relative to that of the controls (Obrosova et al., 2000).

1.5.4.3 In human studies

NT (0.251 ± 0.141 mM) was detected by ELISA in the plasma of patients with Type 2 diabetes, but not in the plasma of healthy control subjects, with the plasma values of NT correlated with that of glucose. Moreover, total antioxidant parameter values were reduced in diabetic patients relative to controls (Ceriello et al., 2001). Recently, there is much attention on postprandial hyperglycemia as a cardiovascular risk factor in diabetes. Ceriello's study showed a direct correlation between postprandial hyperglycemia and plasma NT in patients with Type 2 diabetes. They found that fasting NT was higher in diabetic patients and was further increased during meal tests in diabetic subjects but not normal subjects (Ceriello et al., 2002b). More intense NT immunostaining was apparent in the vascular endothelium and villous stroma (both $P < 0.02$) in placentas from patients with Type 1 diabetes (Lyall et al., 1998). Platelets from patients with Type 1 or Type 2 diabetes, monitored with dichlorofluorescein acetate with a combination of confocal microscopy and steady-state fluorescence, exhibited a higher production of L-arginine/NO-dependent peroxynitrite than that of the controls (4.07 ± 2.88 in Type 1; 1.82 ± 2.12 in Type 2 vs 0.58 ± 0.36 nmol/mg protein in controls). Consistent with this finding, iNOS was immunologically detected in the platelets in both types of diabetic patients. It was concluded that **iNOS-derived peroxynitrite is a cause of platelet damage in diabetes** (Tannous et al., 1999). Plasma NT and other RNS were elevated in patients with early Type 1 diabetes, and have been suggested to be the cause of peripheral nerve dysfunctions (Hoeldtke et al., 2002).

The level of iso-PGF_{2α} excretion was higher in patients with Type 2 and Type 1 diabetes than in age-matched control subjects, and appeared to have a linear relationship with the levels of blood glucose (Davi et al., 1999). During an oral 75 g glucose tolerance test, plasma isoprostane concentrations rose from 0.24 ± 0.10 to 0.33 ± 0.17 ng/l after 90 min, thereby suggesting that free radical-mediated oxidative damage is associated with acute hyperglycemia (Sampson et al., 2002). Also, Type 2 diabetes is associated with increased

oxidative stress as reflected by the high level of plasma hydroperoxides (ROOHs) relative to that of the control subjects (Nourooz-Zadeh et al., 1997).

1.5.5 Reduced glutathione (GSH) and N-acetylcysteine (NAC) in diabetes

1.5.5.1 GSH and NAC

The detrimental effects of peroxynitrite on the myocardium was inversely related to antioxidative capacity (Vinten-Johansen, 2000). For example, ONOO^- could be detoxified following interaction with GSH or other thiols to form S-nitrosoglutathione or other nitrosothiols, which are NO donors (Ferdinandy and Schulz, 2003; Wu et al., 1994). Nakamura et al. (2000) reported that in cardiopulmonary bypass in anesthetized dogs, the addition of GSH (500 μM) in crystalloid cardioplegia detoxified ONOO^- and converted it to cardioprotective nitrosoglutathione. This resulted in the attenuated neutrophil adherence and neutrophil-mediated damage, as well as selective endothelial protection (Nakamura et al., 2000). Hence, it is logical to expect that the presence of glutathione (GSH) may convert ONOO^- to a product that is non-toxic or one with cardioprotective properties (Fig. 1-2).

GSH is a cysteine-containing tripeptide and is the most abundant nonprotein thiol in mammals. It is a substrate of the ROS defense enzyme GSH-Px and the GST family of detoxification enzymes that maintain the cellular redox balance. GSH also protects sulfhydryl groups from oxidation. In addition, GSH modulates cellular metabolism and gene expression by affecting the cellular thiol redox status. GSH (0.3 mM) has been reported to prevent the activation of iNOS, generation of superoxide, formation of NT, and cardiac cell apoptosis, following high glucose (33.3 mM) perfusion of isolated rat hearts for two hours (Ceriello et al., 2002a). Also, GSH completely prevented the effects of hyperglycemia on increments of systolic and diastolic blood pressure, HR and plasma catecholamine in healthy subjects (Marfella et al., 1995) as well as in patients with Type 2 diabetes (Marfella et al., 2000).

NAC is a precursor of GSH. It has been shown that NAC treatment of HIV infected individuals for eight weeks replenishes the level of GSH in blood and T cells, which are depleted in individuals infected with HIV (De Rosa et al., 2000). Furthermore, NAC is the antidote of choice in the management of acetaminophen overdose when hepatic intracellular GSH store is depleted (Kelly, 1998). NAC has also been reported to inhibit *in vivo* induction of iNOS in rats treated by LPS, but had no effect when iNOS was already induced. NAC may inhibit iNOS induction by inhibiting the expression of iNOS protein (Bergamini et al., 2001).

or/and inhibiting the DNA binding activity of NF κ B in the peripheral blood cells (Rota et al., 2002) or LPS- treated mouse macrophages (Xie et al., 1994).

1.5.5.2 GSH changes in the cardiovascular system of diabetics

GSH is important for regulating the redox state, and a decline of GSH in tissue is regarded as increased oxidative stress. Incubation of the rat aorta with high glucose for 48h has been shown to reduce the intracellular level of GSH (Hattori et al., 2000a). GSH level decreased in the hearts of rats at eight to twelve weeks after injection of STZ (Doi et al., 2001). In addition, the level of GSH in the myocardium of rats was decreased at 48h after the injection of STZ even when the plasma GSH level was still normal (Matkovics et al., 1997-1998). Moreover, there is evidence that GSH in the myocardium seems to be depleted before other myocardial antioxidative components are changed. Volkovova reported that the level of GSH and the activity of glutathione S-transferase (GST) decreased significantly whereas the activities of SOD, CAT and GSH-Px were unchanged or increased in the myocardium of rats at eight days after the injection of STZ (Volkovova et al., 1993). Clinically, the levels of GSH and NADPH as well as NADPH/NADP⁺ and GSH/glutathione disulfide (GSSG) ratios were lower in the erythrocytes of patients with Type 2 diabetes than in the erythrocytes of the control subjects. This change is believed to be related to the activation of the polyol pathway (Bravi et al., 1997). Furthermore, the level of plasma thiol and red cell GSH were significantly lower in patients with Type 1 diabetes (16 \pm 4 years old) with no clinical diabetic angiopathy (Elhadd et al., 1999).

1.5.5.3 NAC supplement in diabetes

NAC attenuated glomerular damage in rats with STZ-induced diabetes (Odetti et al., 2003). Supplementation with NAC (500 mg/kg) reduced alloxan-induced hyperglycemia as well as NF κ B and iNOS activation by ROS in pancreatic beta cells of Weanling CD1 mice (Ho et al., 1999). NAC also retained glucose-stimulated insulin secretion and moderately decreased blood glucose in diabetic C57BL/KsJ-db/db mice (Kaneto et al., 1999). Oral administration of NAC (1g/kg/day) corrected the decreased GSH levels in erythrocytes, the increased lipid peroxide levels in plasma, and the increased lipopolysaccharide-induced TNF activity in sera, thereby inhibiting the development of functional and structural abnormalities of peripheral nerves in rats with STZ-induced diabetes (Sagara et al., 1996). Also, the addition of NAC to the culture medium normalized the morphology and 8-epi-PGF2a concentration of rat embryos exposed to high glucose (Wentzel et al., 1999).

In human studies, the incubation of the ketone bodies acetoacetate, which was elevated in patients with Type 1 diabetes, with normal erythrocytes for 24 h caused marked GSH depletion, oxidized glutathione accumulation, hydroxyl radical generation, and membrane lipid peroxidation which was blocked by vitamin E and NAC (Jain and McVie, 1999). NAC was also capable of counteracting alterations of erythrocyte ultrastructure, spectrin cytoskeleton, and insulin receptor distribution in patients with Type 2 diabetes (Straface et al., 2002).

It has also been reported that NAC, along with a selective PKC inhibitor GF109203X, suppressed TNF α -induced NF κ B activation caused by high glucose (27.5 mmol/l) in smooth muscle cells of the rat aorta (Hattori et al., 2000a). NAC was shown to be more potent than SOD in normalizing the electrically stimulated responses and improving substance P responses by modulating peripheral endothelial and smooth muscle-dependent vascular responses in rats at four week after the injection of STZ (Bassirat and Khalil, 2000). Treatment with NAC (1.2 g/day for one month) decreased the expression of plasma vascular cell adhesion molecule-1 and the level of intraerythrocytic GSSG but increased the concentration of GSH and GSH:GSSG ratio in patients with Type 2 diabetes without complications. These results suggest that NAC may slow down the progression of vascular damage by the inhibition of the upregulation of endothelial adhesion molecules and improvement of redox status (De Mattia et al., 1998).

In summary, NAC was experimentally and clinically shown to have beneficial effects on diabetics. To the best of our knowledge, there is **no study that addresses the effects of NAC on cardiac functions in early diabetes.**

1.6 NO and peroxynitrite: contributing factors of cardiovascular abnormalities in diabetes?

1.6.1 The link between ONOO⁻ or/and NO and cardiac abnormalities

NO is essential for normal physiological function via its vasodilator, antioxidant, antiplatelet, and anti-neutrophil adhesion actions. It becomes detrimental if released in large quantities, especially when combined with O⁻₂ to form ONOO⁻ and other toxic products. There is a critical balance between cellular concentration of NO and O⁻₂, which physiologically favours O⁻₂ to be converted to H₂O₂ by SOD but pathologically results in ONOO⁻ formation (Fig. 2-1) (see Chapter 1.4.1).

Peroxynitrite has been reported to have a direct toxic effect to cardiac cells at a relatively high concentration (100 μ M), but an indirect protective effect at a much lower

concentration (1 and 3 μM) (Ma et al., 2000). In contrast, Ferdinandy and Schulz (2003) reported that there was no literature showing any tissue protective action of endogenously formed ONOO^- (Ferdinandy and Schulz, 2003). There is much evidence that endogenously form of ONOO^- in the myocardium contributes to cardiac pathologies:

1) The activation of iNOS was associated with the depression of cardiac contractility and contractile responses to β -adrenergic agonists during myocardial infarction in mice (Feng et al., 2001), autoimmune myocarditis in rats (Ishiyama et al., 1997), and systemic inflammatory response syndrome in rats (Ungureanu-Longrois et al., 1995a).

2) Increased iNOS activity and peroxynitrite generation/enhanced NT formation were immunohistochemically detected in the rat myocardium with experimental autoimmune myocarditis (Ishiyama et al., 1997) and in the dog myocardium after the injection of $\text{IL-1}\beta$ into the left main coronary artery (Oyama et al., 1998), as well as chemically detected in the perfusate of cytokine-treated isolated hearts (Ferdinandy et al., 2000) and normal isolated rat hearts during 60-120 min of perfusion when cardiac work spontaneously declined (Ferdinandy et al., 1999).

3) Correlation analyses revealed a highly significant inverse relationship between cardiac NT level and *in vivo* left ventricular fractional shortening in the mouse model of doxorubicin-induced cardiotoxicity (Weinstein et al., 2000) and *in vivo* left ventricular ejection fraction in dogs after the injection of $\text{IL-1}\beta$ (Oyama et al., 1998).

4) In humans, peroxynitrite has been reported to play a role in inflammation-associated myocardial dysfunction (Kooy et al., 1997).

5) The infusion of L-NAME (an inhibitor of NOS) augmented contractility to dobutamine in patients with heart failure (Hare et al., 1998). Overexpression of metallothionein (an antioxidant protein) in hearts inhibited diabetes-induced reduction of contractility in isolated ischemic mouse hearts (Liang et al., 2002), prevented reduced cardiac contractility, and slowed intracellular Ca^{2+} decay in mouse myocytes (Ye et al., 2003). An inhibitor of ONOO^- mediated tyrosine nitration, 3-mercapto-2-methylpentan-1, inhibited ONOO^- mediated cellular toxicity in human hepatoma cell line (Rose et al., 2003).

In summary, there is a link between ONOO^- or/and NO and cardiac abnormalities.

1.6.2 Potential mechanisms of peroxynitrite-induced cardiovascular abnormalities

The biological actions of ONOO^- has received considerable attention since ONOO^- , at physiological pH, was reported to protonate peroxynitrous acid (HOONO), an unstable compound that transfers an oxygen atom or hydroxyl group to biological targets such as lipids, carbohydrates, nucleic acids, and iron sculpture centers. Nitration reactions, such as the nitration of tyrosine in proteins, could also occur (Gordge, 1998). The ability to hydroxylate and nitrate can induce cell injury via several pathways:

- 1) Initiating lipid peroxidation (Radi et al., 1991) to produce 4-hydroxynonenal, malondialdehyde, and other metabolites. Once formed, these peroxidation metabolites can alter metabolic processes. A variety of lipid peroxidation products have relatively long half-lives within the cells, which allow for multiple interactions with cellular components, thereby indirectly or directly causing cytotoxic alterations (Keller and Mattson, 1998).

- 2) Causing DNA breakage and apoptosis (Salgo et al., 1995a and b). HOONO has been shown to irreversibly inhibit mitochondrial respiratory chain (Brown, 2001; Xie and Wolin, 1996) and to trigger the apoptosis of cardiac myocytes (Beckman, 1999).

- 3) Inducing protein modifications including **protein oxidation** (of methionine, cysteine, tryptophan or tyrosine residues) and **nitration** (of tyrosine or tryptophan residues) (Beckman and Koppenol, 1996b), which alter the activity of key proteins and enzymes, thus leading to cellular and organ dysfunction (Lopez et al., 1997; Ma et al., 1997). Moreover, enzymes containing a redox active transition metal center (iron/sulfur centers), zinc fingers, and protein thiols, such as SOD, GSH-Px, and ATPase, are prime targets of oxidation (Beckman and Koppenol, 1996b). Many enzymes and proteins that affect cardiovascular signal transduction pathways as well as the function of cardiovascular contractile proteins are targets of nitration, since the largest targets of nitration are tyrosine residues which are present in approximately 4 mol% of mammalian proteins. Nitration alters the structure and function of proteins and accelerate its rate of proteolytic degradation (Ischiropoulos, 1998). It has been reported that succinyl-CoA:3-oxoacid CoA-transferase (SCOT), which enables the heart to use ketone bodies as an alternative source of energy, was susceptible to tyrosine nitration in the hearts of rats treated with STZ. After four and eight weeks of diabetes progression, the decrease in SCOT catalytic activity was accompanied by an accumulation of NT in SCOT protein (Turko et al., 2001). More recently, proteins with nitrated tyrosine residues were detected in the mitochondria of the hearts of mice four weeks after the onset of alloxan-induced diabetes under

in vitro and vivo conditions of oxidative stress. Functional studies showed that protein nitration adversely affect functions, and that protection against nitration protects the functional properties of the proteins. Since proteins involved in major mitochondrial functions, such as energy production, antioxidant defense and apoptosis, were nitrated, it was concluded that the nitration of mitochondrial proteins may lead to dysfunctional mitochondria in diabetes (Turko, 2003), thus contributing to myocardial contractile depression. In addition, an increase in the nitration of prostacyclin synthase in cultured human aortic endothelial cells exposed to high glucose (30 or 44 mmol/l) for seven to ten days was associated with a decrease in its activity (Zou et al., 2002a).

4) Depletion of important antioxidants such as GSH and cysteine (Ferdinandy and Schulz, 2003) which, with the inhibition of antioxidative enzymes discussed above, augments oxidative stress.

1.6.3 Peroxynitrite in diabetic cardiovascular system

There is evidence that peroxynitrite is formed in the diabetic state (see Chapter 1.5.4). NT has been detected in the plasma, cardiac cells, and other tissues of diabetic patients, concurrently with increased apoptosis of myocytes, endothelial cells and fibroblasts in the heart biopsies (Frustaci et al., 2000). Furthermore, the formation of NT was related to the activation of iNOS, generation of superoxide, and detection of cardiac cell apoptosis in isolated rat hearts perfused for two hours with high glucose (33.3 mM) (Ceriello et al., 2002a). Mice with STZ-induced diabetes had progressively depressed ventricular contractility starting from the 7th day to the 30th day after the injection of STZ, a trend characterized by increased presence of NT and apoptosis (Kajstura et al., 2001). The marked depression of *in vivo* LV function in mice with STZ-induced diabetes for nine weeks was reversed by pharmacologic neutralization of peroxynitrite toxicity with FP15, a novel potent peroxynitrite decomposition catalyst (Szabo et al., 2002).

1.6.4 NO and peroxynitrite—a link between hyperglycemia and abnormal cardiovascular function?

A number of studies suggest that iNOS is activated (see Chapter 1.4.4) and that peroxynitrite is formed in diabetic states (see Chapter 1.5.4 and 1.6.3). The toxic action of NO is postulated to be mediated via its metabolic byproduct ONOO⁻, which rapidly decomposes

into highly reactive oxidant species (Fig. 1-2, Beckman and Koppenol, 1996b; Ferdinandy and Schulz, 2003). In addition, ONOO^- and O_2^- could be directly generated by iNOS in mouse macrophages (Xia and Zweier, 1997) and purified mouse iNOS (Xia et al., 1998), even when L-arginine is depleted. As well, ONOO^- may increase the level of iNOS via the activation of NF_κB (Cooke and Davidge, 2002). In summary, ONOO^- may be generated directly or indirectly from NO or other RNS. Collectively, derivatives of NO and ONOO^- play an important pathophysiological role in causing cardiovascular injury in diabetes.

The contractile function of the heart is dependent on its high metabolic demand, i.e. the mitochondrial system is highly active. A series of oxidation-reduction reactions continually takes place in cardiomyocytes and this results in much formation of ROS/RNS (Ferdinandy et al., 1999). Therefore, a decreased capacity of the antioxidant defence system (such as SOD, catalase, glutathione peroxidase, GSH and vitamin E), triggered by hyperglycemia, can lead to cellular oxidative injury. Indeed, hyperglycemia may decrease antioxidative capacity in diabetic hearts. Decreased level of GSH was reported in the heart of rats 48 hours or eight and twelve weeks after the injection of STZ (Doi et al., 2001; Matkovics et al., 1997-1998). Reduced activity of SOD and increased production of TBARS in the myocardium of rats were reported at 48 hours after the injection of STZ (Matkovics et al., 1997-1998). The decreased antioxidative capacity in the diabetic state would be expected to aggravate peroxynitrite toxicity.

There is evidence that the endogenous formation of ONOO^- in the myocardium contributes to cardiac pathologies (see Chapter 1.6.1). NT was detected in the myocytes of diabetic patient (Frustaci et al., 2000), in isolated hearts perfused with high glucose for two hours (Ceriello et al., 2002a), and in the hearts of mice with STZ-induced diabetes (Kajstura et al., 2001). Furthermore, hyperglycemia may increase the production of ROS/RNS, which are transferred to **peroxynitrite and its derivatives**, and **decrease the oxidative capacity** in the diabetic myocardium and vasculature. The increased production of ROS/RNS and decreased oxidative capacity may contribute to **myocardial and vascular damages** at the molecular and cellular levels (discussed in Chapter 1.6.2), which, in turn, **cause morphological and functional abnormalities (Fig. 1-1)**. If such a hypothesis was true, then pharmacological targeting of ONOO^- formation and decomposition, such as the inhibition of the endogenous formation of ROS/RNS (including NO) or the enhancement of cardiac antioxidant status, could

prevent cardiovascular abnormalities and improve functions (Ferdinandy and Schulz, 2001a; Rosen et al., 2001). Indeed, several studies have demonstrated the beneficial effects of the inhibition of NO formation and/or peroxynitrite decomposition in the cardiovascular system in experimental diabetes (Bardell and MacLeod, 2001; El-Omar et al., 2003; Smith et al., 1997; Szabo et al., 2002). However, there is a lack of direct evidence that link iNOS activation and peroxynitrite formation to depressed cardiovascular contractility at the early phase of diabetes mellitus.

1.7 Selective inhibition of iNOS in septic shock

1.7.1 Sepsis and sepsis shock

Sepsis is defined as a systemic response to infection, the most common cause being the contamination of the blood with bacteria (Bone, 1994). Septic shock (sepsis with hypotension) develops in almost half of the patients with a mortality rate of 40-60%; and this is despite aggressive treatments (Thiemermann, 1997). The development of shock results in impaired tissue perfusion and oxygen extraction of the vital organs of the body (Thiemermann, 1997). Key symptoms and signs of sepsis include a severe fall in blood pressure (hypotension) with hyporeactivity to vasoconstrictor agents (vasoplegia), which may lead to reduced perfusion of important organs, namely the lungs, liver, kidneys and brain (multiple organ dysfunction), and ultimately death (Hewett and Roth, 1993; Thiemermann, 1997). Cardiovascular abnormality in severe sepsis may include decreased cardiac contractility and output, reduced vascular tone, and inadequate tissue perfusion. These changes are secondary to the release of various inflammatory mediators that include NO.

It is established that bacterial endotoxin, a lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria, is the major mediator of the high morbidity and mortality rates in septic shock. Administration of endotoxin experimentally to animals mimics the symptoms of a septic shock.

1.7.2 NO produced by iNOS and septic shock

It is evident that nitrate production is increased in endotoxemia. Plasma and urinary nitrite and nitrate concentrations were increased in patients with sepsis, and the levels were higher in the presence of multiple organ dysfunction (Gomez-Jimenez et al., 1995). Nitrite and nitrate production in mammalian tissues was via NO synthase-catalyzed conversion of L-

arginine to NO, and the subsequent degradation of NO into nitrite and nitrate, the stable end-products of NO metabolism (Moncada et al., 1991b). There is a quantitative and qualitative relationship between NO production, endotoxemia, hemodynamic dysfunction and multiple organ dysfunction in human septic shock (Gomez-Jimenez et al., 1995). During endotoxemia, iNOS expression has been observed in a variety of cell types, which includes endothelial cells, macrophages, Kupffer cells, hepatocytes, vascular smooth muscle cells, kidney cells, chondrocytes, cardiac myocytes, pancreatic islets and fibroblasts (Moncada et al., 1991b; Morris and Billiar, 1994; Salter et al., 1991).

In recent years there have been suggestions that the overproduction of NO is responsible for the pathophysiology of septic shock and multiple organ dysfunction. The induction of NO synthase, with the subsequent excessive formation of NO, has been proposed to be a major cause of pathologic vasodilatation and tissue damage in septic shock (Ceppi et al., 1997; Thiernemann, 1997). There is evidence that NO induced by iNOS may be the most important hypotensive mediator released in sepsis. Indeed, mice with iNOS gene-knockout experienced minor hypotension when challenged with endotoxin (MacMicking et al., 1995; Wei et al., 1995).

1.7.3 NO and the cardiovascular system in septic shock

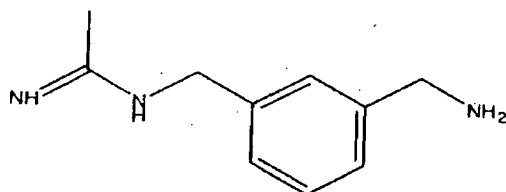
The induction of iNOS and excessive NO synthesis have been postulated to be the cause of cardiovascular dysfunction in septic shock (Thiernemann, 1997; Titheradge, 1999). NO caused excessive vasodilatation via both cGMP-dependent and cGMP-independent mechanisms (Kilbourn et al., 1997). NO produced in the vascular smooth muscle and endothelial cells activated soluble guanylate cyclase by binding to the heme moiety, thereby causing an increase in cGMP formation, cGMP-dependent reduction of intracellular Ca^{2+} , and vascular relaxation (Kilbourn et al., 1997; Knowles and Moncada, 1992). cGMP-independent mechanisms, on the other hand, operate through a direct effect of NO or OONO^- on the vascular smooth muscle, leading to reduced contractility by energy depletion and the activations of Ca^{2+} -dependent K^+ channels and ATP-sensitive K^+ channels (Kilbourn et al., 1997). An induction of NOS in the myocardium has also been shown to be responsible for cardiac dysfunction in endotoxemia. Reduced myocardial performance contributed to hypotension in sepsis (Schulz et al., 1992b).

1.7.4 Inhibition of NOS in cardiovascular system in sepsis

Several studies have focused on the potential benefits of non-selective inhibitors of NOS to restore blood pressure and vascular responsiveness during sepsis (Cai et al., 1996; Szabo et al., 1993; Thiernemann et al., 1995). However, non-selective inhibitors of NOS may increase systemic vascular resistance at the cost of compromising cardiac output (CO) and organ blood flow (Cheng and Pang, 1998; Klemm and Moody, 1998; Millar and Thiernemann, 1997; Mulder et al., 1994). The results from these studies suggest that the very large amounts of NO produced by iNOS during septic shock may be detrimental, and that selective inhibition of iNOS, but not eNOS may be beneficial, because the small amount of NO synthesized by eNOS and nNOS are important for normal physiological function.

It should be mentioned that there are several selective inhibitors of iNOS, e.g., L-canavanine, L-N-iminoethyl-lysine, S-methyl-isothiurea and aminoguanidine. All these inhibitors prevent hypotension, either maintain CO or attenuate the decrease of CO, and restore vasodilatation to acetylcholine or attenuate vasoconstriction caused by acetylcholine or bradykinin in LPS-treated animals (Fischer et al., 1999; Rosselet et al., 1998; Strunk et al., 2001; Wolfard et al., 1999). However, they have low *in vivo* potency and less than 30-fold selectivity for iNOS over eNOS (Southan and Szabo, 1996a). Aminoguanidine, in particular, has other actions that include the inhibition of redox-active enzymes and the formation of advanced glycosylation end-products (Mayer and Andrew, 1998; Monnier, 1996; Rahbar et al., 1999; Southan and Szabo, 1996b). A new highly selective inhibitor of iNOS, 1400W, is a non-amino acid analog of L-arginine (Fig. 1-1), and it has been shown to be >1000- (*in vitro*) and >50-fold (*in vivo*) more selective in inhibiting iNOS rather than eNOS in rats (Garvey et al., 1997). On the basis of its greater selectivity, 1400W was chosen over the other inhibitors of iNOS for this study.

Figure 1-3 The structure of 1400W



N-(3-(aminomethyl)benzyl)acetamidine

1.8 Animal model of diabetes

Diabetic patients are particularly prone to cardiovascular disorders. Mortality from cardiovascular disease is almost three times higher in patients with diabetes mellitus than the general population (Jarrett, 1989). Consequently, there has been much interest in the development of animal models for the study of cardiovascular complications in diabetes. Animal models of Type 1 diabetes include the spontaneously diabetic BioBreeding (BB) Wistar rat and NOD mouse. Popular Type 2 models include the *fa/fa* Zucker diabetic fatty (ZDF) rat, db/db mouse, and Chinese hamster (McNeill, 1999). Beside these genetic diabetic models, chemicals, such as STZ and alloxan, have also been used to induce diabetes. These chemicals specifically cause β -cell necrosis, thereby decreasing the production of insulin, as in Type 1 diabetes. As a tool for studying the etiology of cardiovascular disorders in diabetes, the chemical-induced models are devoid of some of the problems associated with the genetic models, such as the presence of more than one inborn defects in the ZDF model, e.g. obesity caused by a mutation in the leptin receptors, peripheral insulin resistance caused by the *fa* gene's faulty encoding for insulin receptors, and a defective transcription of the β -cell gene resulting in an inability to adequately compensate for insulin resistance brought about by the *fa* gene (Griffen et al., 2001). The STZ-induced model of diabetes is at present the most popular method for inducing diabetes. Relative to alloxan, STZ has a greater selectivity of β -cell and lower mortality rate (Hoftiezer and Carpenter, 1973; Junod et al., 1969).

Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosourea) 1-D-glucopyranose) is an antibiotic extracted from *Streptomyces achromogenes*, which only destroy β -cells in some species, but not in humans. It contains a highly reactive methyl nitrosourea side chain bound to the C2 position of 2-deoxy-D-glucopyranose. The nitrosourea moiety is responsible for the cytotoxic

action of STZ (Schnedl et al., 1994). Three mechanisms have been proposed to cause the β -cell cytotoxic effects of STZ: the methylation of DNA, free radical production, and NO generation (Robbins et al., 1980; Tanaka et al., 1995; Uchigata et al., 1982).

STZ (i.v.) was first reported by Rakieta in 1963 to cause diabetes mellitus in rats and dogs (Rakieta et al., 1963). Junod's group (1967) further showed that STZ caused massive β -cell degranulation and necrosis and an increase in the serum levels of insulin and hypoglycemia. This was followed by prolonged hyperglycemia (1-28 days) that coincided with a reduction in pancreatic insulin level to less than 5% of the normal value. There was no histological evidence of the toxic effect of STZ in the liver and kidneys. Moreover, rats treated with a dose of STZ that destroys all the β -cells (100 mg/kg) survived as long as they were given insulin (Junod et al., 1967). Therefore, it was concluded that STZ was selectively toxic to the pancreatic β -cells.

The diabetogenic effects of STZ were found to be dose-dependent (Junod et al., 1969), which ranged from a mild state of diabetes following a dose of 35 mg/kg, to a severe ketotic state, which led to death within two to three days after a dose of 100 mg/kg. An intermediate dose of STZ (55-65 mg/kg, used in most cardiovascular studies) caused the rats to increase blood glucose levels to three to four times that of normal rats. The rats would not gain weight, but would still be able to survive without insulin supplement and would not develop ketonuria, since the remaining pancreatic β -cells could release enough insulin for survival. The diabetogenic actions of STZ in rats also appeared to be influenced by the route of administration as well as the strain, age and gender of the rats (Rodrigues et al., 1999). A single intravenous injection of STZ (50-75 mg/kg) induced stable diabetes in male Wistar rats without requiring insulin supplement for survival (Rodrigues et al., 1999).

The STZ-induced diabetic rats display many of the characteristic symptoms and signs seen in human subjects with uncontrolled diabetes mellitus, including hyperglycemia, hypoinsulinemia, elevated plasma lipid levels, polydipsia, polyuria and weight loss (Hebden et al., 1986; Tomlinson et al., 1989). They also exhibit cardiovascular abnormalities similar to those in human (see Chapters 1.2 and 1.3). Although many diabetic patients suffer from chronic insulin deficiency, investigations of short-term STZ-induced diabetes provide valuable information regarding the pathophysiological changes that lead to chronic diabetic complications. The STZ-induced diabetic rat model has also been used extensively to evaluate

the efficacy of new chemicals to ameliorate cardiovascular dysfunctions in diabetes (Tomlinson et al., 1992).

1.9 Limitation of present study

It is well known that diabetes mellitus is a metabolic disease that involves abnormalities in carbohydrate, protein and fat metabolism secondary to insufficiency in insulin secretion and/or action. This study focused on the role of iNOS and antioxidants on cardiovascular function in diabetes. There was no attempt to elucidate the effects of the iNOS inhibitor 1400W and antioxidant NAC on derangements in protein and fat metabolism in diabetes mellitus.

2. RESEARCH OUTLINE

2.1 1400W-LPS

The effects of highly selective inhibitor of inducible nitric oxide synthase (iNOS) on the cardiovascular system of rats with LPS-induced endotoxemia

2.1.1 Research direction, objectives and rationales:

The late phase of severe septic shock is associated with reduced cardiac contractility and output, reduced vascular tone, and inadequate tissue perfusion, which are secondary to the release of various inflammatory mediators that include nitric oxide. Excessive NO synthesis and induction of iNOS have been implicated in the cardiovascular dysfunction observed in both patients with septic shock and animal models of endotoxemia (Thiemermann, 1997; Titheradge, 1999). A number of clinical and experimental studies used nonselective or poorly-selective inhibitors of NOS to block the action of iNOS. These inhibitors had variable effects on septic shock since they inhibited the actions of iNOS as well as cNOS, the basal release of NO synthesized by cNOS is, however, important for normal physiological function. The aim of this study was to examine if a highly selective inhibitor of iNOS, 1400W, improve hemodynamics better than the nonselective NOS inhibitor, L-NNA, in LPS-induced sepsis.

Compound 1400W has been shown to be >1000- (*in vitro*) and >50-fold (*in vivo*) more selective in inhibiting iNOS than eNOS in rats (Garvey et al., 1997). Indeed, a single injection of 1400W (≈ 0.3 mg/kg, s.c., ED₅₀ dose) reversed delayed LPS-induced vascular injury in rats (Garvey et al., 1997). Furthermore, 1400W restored the blood pressure of endotoxemic rats when given either before (Hamilton and Warner, 1998) or at 2 h after (Wray et al., 1998) LPS challenge. To the best of our knowledge, there are no published reports on the effects of 1400W on CO and the regional distribution of blood flow in endotoxemia. The hemodynamic effects of 1400W were compared with those of L-NNA in anesthetized rats with *Escherichia coli* LPS-induced endotoxemia.

2.1.2 Hypothesis:

A highly selective iNOS inhibitor, 1400W (N-3-aminomethyl-benzyl-acetamidine), but not a nonselective NOS inhibitor improve cardiovascular function and regional hemodynamics in LPS-induced endotoxemic rats.

2.2 1400W-NAC-Diabetes

The effects of a selective inhibitor of iNOS (1400W) and an antioxidant (N-acetylcysteine) on cardiovascular contractility and hemodynamics at an early stage of STZ-induced diabetes in rats

2.2.1 Direction of research and rationales

Diabetes is associated with functional abnormalities in the cardiovascular system. These abnormalities may start from metabolic and/or biochemical alterations in cells and develop into functional changes in membrane, contractility changes in the heart and blood vessels, morphological changes, and finally dysfunctions in the cardiovascular system. Cardiac dysfunction caused by cardiomyopathy independent of vascular diseases has been recognized for many years, though the pathogenesis of diabetic cardiomyopathy remains unclear. It is uncertain what the primary cause of depressed cardiac function and reduced vasoconstriction at the acute phase of diabetes is, as this condition is associated with multiple endocrinal and metabolic changes. Several studies have shown that iNOS is activated in various cells in Type 1 and Type 2 diabetes, and this may be a consequence of hyperglycemia (see Chapter 1.4.3). Recently, more attention has been paid to the fact that the combination of the overproduction of NO by iNOS and oxidative stress that is well known to exist in diabetes may favor the formation of peroxynitrite, thereby initiating cardiovascular complications in diabetes by causing the oxidation and nitration of amino acid residues of proteins and guanine of DNA, lipid peroxidation, DNA cleavage, and the depletion of antioxidants. Pharmacological targeting of ONOO⁻ formation and decomposition, such as the inhibition of the endogenous formation of ROS/RNS (including NO) or the enhancement of cardiac antioxidant reserve via supplementing with exogenous antioxidants, would be strategies in preventing and/or improving cardiovascular abnormalities (Ferdinandy and Schulz, 2001a; Rosen et al., 2001). However, there is a lack of direct evidence that link the activation of iNOS, the formation of peroxynitrite, and depressed antioxidative capacity to depressed cardiovascular contractility in early diabetes mellitus in intact animals.

The three primary goals of this thesis were 1) to investigate if rats with STZ-induced diabetes for three weeks developed abnormalities in the cardiovascular system and if these abnormalities were associated with activation of iNOS, formation of peroxynitrite, and reduced myocardium antioxidant capacity; 2) to examine if 1400W improved cardiovascular responses

to NA while inhibiting the activation of iNOS and formation of peroxynitrite; and 3) to examine if antioxidant NAC reduced the activation of iNOS, decreased the formation of peroxynitrite, increased antioxidative capacity, and improved cardiac contractile function. These studies were conducted in the STZ-induced Type 1 diabetes rats, which is widely employed in diabetic research.

2.2.2 Hypothesis

iNOS is activated in early diabetics. The increased production of NO accompanied by the formation of peroxynitrite and decreased antioxidative capacity may be principally responsible for diminished cardiac contractility and depressed cardiovascular responses to vasoconstrictors. The administration of a selective inhibitor of iNOS or an antioxidant/iNOS inhibitor may therefore improve cardiovascular functions in diabetes by blocking the formation of NO and peroxynitrite as well as restoring the antioxidative capacity.

2.2.3 Research objectives

2.2.3.1 Objective 1:

To determine the effects of diabetes (versus control) on cardiac function (LVSP, \pm dp/dt, HR), cardiac index (CI), MAP, TPR (total peripheral resistance), regional blood flow, and body venous tone before and during the infusion of vasoactive substances. These studies were conducted in anaesthetized rats at two or three weeks after the injection of STZ.

2.2.3.2 Objective 2:

To find out if iNOS is activated and if peroxynitrite is formed (through the detection of NT, a product of protein nitration, and 15-F2t-isoprostane, a marker of lipid oxidation, in the myocardium of rats at three weeks after the injection of STZ. As well, to find out if there are changes in the total antioxidant capacity in the myocardium of rats with STZ-induced diabetes.

2.2.3.3 Objective 3:

To determine if acute administration of 1400W ameliorates cardiovascular abnormalities at three weeks after the injection of STZ.

2.2.3.4 Objective 4:

To determine if chronic administration of NAC at the time of injection of STZ ameliorates cardiac abnormalities and increases cardiac antioxidative capacity in rats at three weeks after the injection of STZ.

2.3 Research design

Study 1: Selective versus non-selective suppression of nitric oxide synthase on regional hemodynamics in rats with or without LPS-induced endotoxemia.

The overall aim was to determine the effects of a selective inhibitor, 1400W. To ensure that 1400W is selective and efficacious in inhibiting the activation of iNOS, a first study was carried out to find out if 1400W alters hemodynamics of anaesthetized rats with LPS-induced septicemia.

Study 2: The effects of 1400W on arterial and venous constrictions to NA and angiotensin II in conscious rats at three weeks after injection of STZ or vehicle.

Study 3: The effects of 1400W on hemodynamic responses to NA in anaesthetized rats at three weeks after injection of STZ or vehicle.

Study 4: The effects of 1400W on cardiac contractility, cardiac contractile responses to NA, and content of iNOS in anaesthetized rats at three weeks after injection of STZ or vehicle.

Study 5: The effects of antioxidant NAC on cardiac contractility and contractile responses to dobutamine as well as on activation of iNOS, formation of peroxynitrite, and myocardium antioxidative capacity in anaesthetized rats at three weeks after injection of STZ or vehicle.

3. MATERIALS AND METHODS

All the experiment has been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institute of Health.

3.1 Induction of diabetes and treatment of animals

3.1.1 Induction of diabetic rats

Male Wistar rats (300-350g) were injected with streptozotocin (STZ, Sigma, 60 mg/kg, i.v.) or the vehicle (0.9% NaCl) via the tail vein under light halothane anesthesia. The rats were considered to be diabetic and used for the study if they had hyperglycemia (>15 mmol/l) at 48 h after injection of STZ, as detected by AccuSoft test strips (Hoffmann-La Roche Ltd) (Sambandam et al., 2000; McNeill, 1999; Rodrigues et al., 1999). The rats were studied at two or three weeks after injection of STZ. Plasma glucose level was measured by the glucose oxidase method (Sigma, Trinder 100 kit) via the use of a Spectrainbow (ART F039039, Austria).

3.1.2 Acute administration of 1400W

The rats were injected with 1400W (i.v. 3 mg/kg over 5 min), followed by infusion of 1400W (i.v. 3 mg/kg/h) after the rats recovered from the cardiovascular responses to NA. At 60 min from the start of administration of 1400W, the cardiovascular responses to NA were measured again.

3.1.3 Chronic administration of NAC

At 48 h after the injection of STZ, half of the control and diabetic rats were given NAC (Sigma, 1-2 g/L) which was added to the drinking water, and the remaining rats were given tap water. The concentration of the drug in the water was adjusted daily according to the amount of water consumed the previous day. The amount of NAC consumed by the rats during the study (19-21 days) was 1.2 ± 0.1 g/day per kg body weight.

3.2 Methodology

3.2.1 Surgical Procedures

3.2.1.1 Anesthetized rats

The rats were anesthetized with sodium pentobarbital (60 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). Polyethylene cannulae (PE50), filled with heparinized saline (0.9% NaCl, 25 I.U./ml), were inserted into the right iliac vein for the administration of drugs, right iliac artery for the withdrawal of a reference blood sample by a withdrawal/infusion pump to measure cardiac output via the microspheres technique (Pang, 1983). The left iliac artery was cannulated for the measurement of MAP by a P23DB pressure transducer (Gould Statham, CA, USA) and withdrawal of a blood sample to measure the blood level of glucose. Heart rate (HR) was derived from the upstroke of the arterial pulse pressure by a tachograph (Grass, Model 7P4G). A PE50 cannula was inserted into the left ventricle (LV) through the right carotid artery for the injection of microspheres and recording of left ventricular peak systolic pressure (LVP) by a pressure transducer. All pressure recordings were displayed on a polygraph (Grass 7D, Grass Instruments, Quincy, MA, USA). A differentiator (Model 7P20C, Grass instrument, Quincy, Mass, USA) was used to derive maximal rate of increase (+dP/dt) and decrease (-dP/dt) of left ventricular pressure during contraction and relaxation, respectively.

3.2.1.2 Conscious rats

The rats were surgically prepared under halothane anesthesia. A saline-filled, balloon-tipped catheter was inserted into the right atrium through the right external jugular vein to transiently stop circulation for the measurement of body venous tone (Pang, 2000). Cannulae were also inserted into an iliac artery for the measurement of mean arterial pressure, the right iliac vein for the withdrawal of blood (0.1 ml) for the measurement of plasma glucose and infusion of drugs, and into the inferior vena cava via the left iliac vein for the measurement of central venous pressure. All cannulae were tunnelled subcutaneously to the back of the neck and exteriorised. The rats were recovered from surgery and anesthesia for at least 6 h prior to the study.

3.2.2 Microspheres technique

The following three sets of radioactively-labelled microspheres were sequentially injected into each rat in a random order: ^{57}Co , ^{113}Sn and ^{103}Ru (15 μm diameter, Perkin-Elmer Canada Inc., Ontario). Each determination involves injection into the left ventricle over 10 s of a well-stirred suspension (250 μl) containing 20,000-25,000 microspheres. A reference blood sample was withdrawn (Harvard pump) at 0.35 ml/min for 45 s from the iliac artery into a syringe containing heparinized saline, starting 10s before the injection of each set of microspheres. The blood was returned to the rats after the counting of radioactivity by a gamma counter with a three inch NaI crystal (1185 series, Nuclear-Chicago, IL, USA). At the end of the experiments, both kidneys were removed, weighed and counted for radioactivity. Any experiments in which the counts of any set of microspheres between the two kidneys differed by more than 15% were rejected, as it was assumed that the microspheres were inadequately mixed within the circulation. After termination of the experiments, organs and tissues were removed for the counting of radioactivity. Muscle samples were taken from the fore and hind limb and the abdomen. Skin samples were taken from between the fore and hind limb, were pooled and weighed. The radioactivity of tissues and reference samples was measured in a γ -counter (1185 series, Nuclear-Chicago, IL, U.S.A) with a three inch NaI crystal for two mins with discriminator windows set to cover the regions of interest (containing the γ -peaks of isotopes). The activity per isotope was calculated after background subtraction and spill-over correction, and was used for the calculation of CO (ml/min), total peripheral resistance (TPR, mmHg min/ml), tissue blood flow (BF, ml/min) and conductance (ml /min mmHg) as follows.

$$\text{CO} = \frac{\text{rate of withdrawal of blood} \times \text{total injected c.p.m.}}{\text{c.p.m. in withdrawn blood}}$$

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

$$\text{TPR} = \frac{\text{MAP}}{\text{CO}}$$

$$\text{Conductance} = \frac{\text{BF}}{\text{MAP}}$$

The radioactive microsphere method is the only method that permitting simultaneous measurements of flow in many organs. The methods is accurate if the following conditions are satisfied: sufficient numbers of microspheres per organ without causing hemodynamic changes, adequate mixing of the microspheres with blood, and steady state at the time of withdrawal of reference blood and injection of microspheres (Stanek et al., 1985; Flaim et al., 1978; Ishise et al., 1980; Tsuchiya et al., 1977). In our study, each rat received less than 85,000 microspheres in total to prevent hemodynamic changes (Tsuchiya et al., 1977), but all the samples contained more than 200 spheres for each isotope to ensure adequate sampling and accurate counting of radioactivity. Adequacy of mixing was checked by comparing left and right renal perfusion, and accepting the results from rats with right and left renal flow differencing by less than 15%. The different isotopes were injected to rats only when blood pressure and heart rate were stable, which indicate the presence of a steady state condition.

3.2.3 Mean circulatory filling pressure measurement (MCFP)

The MCFP is an index of body venous tone. The MCFP technique involves recording steady-state readings of MAP and central venous pressure at 4-5 s following inflation of the atrial balloon (Pang, 2000). Central venous pressure was measured after transient stopping of the circulation through injection of a small volume of fluid into the right atrial balloon. Within 5 sec following inflation of the balloon, MAP decreased to a plateau value (referred to as final arterial pressure, FAP), while central venous pressure increased to a plateau value (referred to as venous plateau pressure, VPP). To eliminate the need to rapidly equilibrate arterial and venous pressures during circulatory arrest, the arterial pressure contributed by the small amount of trapped arterial blood is corrected by the following equation: $\text{MCFP} = \text{VPP} + 1/60 (\text{FAP} - \text{VPP})$, using 1/60 as the ratio of arterial to venous compliance (Rothe, 1993; Yamamoto et al., 1980).

3.2.4 Assay of NOS activity

The hearts were excised, cleaned in cold saline and flash frozen in liquid nitrogen. Myocardial tissues were stored at -70° C. Frozen tissues were homogenized with a Polytron homogenizer in 5 volumes (W/V) of cold (4°C) homogenization buffer that contains 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 centrifuged at 10,000g at 4° C for 20 min. The supernatant was kept over ice for the assays of NOS and protein. NOS activity of the supernatant was quantified by measuring the formation of radiolabeled [3 H]-L-citrulline from [3 H]-L-arginine as described (Wu et al., 2001) but with slight modifications. The supernatant (50 μ l) was incubated in a reaction mixture that contains Tris-HCl (25 mM, pH 7.4), flavin adenine dinucleotide (10 μ M), tetrahydrobiopterin (10 μ M), L-arginine (0.2 μ Ci), L-[3 H]-arginine (Amersham), NADPH (1 mM), CaCl₂ (2 mM) and calmodulin (10 μ g/ml, Roche Diagnostics) in a total volume of 100 μ l for 1 h at 37° C. The reaction was terminated by the addition of cold, stop buffer (100 μ l, 50/6 mM of HEPES-EDTA, pH < 5.5). Dowex 50W (200-400, 1 ml 8% cross-linked, Na⁺ form; 1:1 w/v in water) was added to the solution to remove excess L-[3 H]arginine. To determine the amount of L-citrulline formed, the samples were centrifuged at 5,000 g for 10 min. An aliquot (500 μ l) 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. Ca²⁺-independent activity (iNOS) was measured by the addition of 1 mM of EDTA and EGTA (instead of Ca²⁺ and calmodulin), whereas cNOS activity was derived from the difference between total NOS activity and Ca²⁺-independent activity. 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. All reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

3.2.5 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction and RT-PCR were performed according to the method of Kawai (Kawai et al., 1999) with minor modifications. The frozen ventricular tissue was pulverised using mortar and pestle under liquid nitrogen. Samples of the powder (50-100 mg) were placed in individual tubes containing Trizol reagent (1 ml). After vortex mixing, the samples were left at room temperature to completely dissociate nucleoprotein complexes. Afterwards, chloroform (200 μ l) was added to each tube. The mixture was vortexed vigorously for 3 min,

followed by incubation at room temperature for 3 min, and centrifugation at 12,000g for 15 min at 4°C. The aqueous phase was transferred to a different tube, and RNA was precipitated with ice-cold isopropanol (0.5 ml at 4°C, 10,000g for 10 min), and then washed with 75% ethanol (1 ml at 4°C, 6000 g for 5 min). The resultant RNA was air-dried in the fume hood and dissolved in Tris buffer (50 µl of 10 mM). The samples were kept in the fridge overnight to completely dissolve the RNA pellet. Tissue RNA was quantified by a spectrophotometer (optical density when $\lambda=260$, OD₂₆₀). The purification, quality and integrity of the extracted RNA were monitored by observing the ratio of OD₂₆₀/OD₂₈₀ (> 1.9), the presence of two distinct bands (28S and 18S ribosomal RNA) from formaldehyde/agarose gel electrophoresis, and the sharpness of the bands.

RNA was reversely transcribed to cDNA as follows. RNA (2 µl of 1 µg/µl), oligo(dT)₁₂₋₁₈ primer (1 µl) and diethylpyrocarbonate (DEPC)-treated water (6 µl of 0.1%) were mixed, incubated at 65°C for 10 min, and rapidly chilled on ice. Upon addition of the reaction mixture (10 µl), the final reaction mixture contained the following: 1X PCR buffer, dNTP mix (0.5 mM), MgCl₂ (5 mM), dithiothreitol (5 mM), oligo(dT) primer (0.5 µg) and deoxyribonuclease I (1 U). The reaction mixture was incubated at 37°C for 30 min, then at 65°C for 5 min (to denature the deoxyribonuclease I), and followed by cooling on ice. After adding SuperScript II reverse transcriptase (100 U/µl) or DEPC-water (1 µl, negative control) and mixing, the reaction mixture was incubated at 42°C for 20 min and kept on the ice for the PCR assay.

The PCR reactions were performed in a total volume of 25 µl, and the solution contained the following: 1X PCR buffer, dNTPs (0.2 mM), MgCl₂ (2 mM), Taq DNA polymerase (2U) and the specific primer pairs (25 pmol) for eNOS and β -actin, or iNOS and β -actin. β -Actin was used as a positive control in each PCR reaction. The reactions for iNOS were thermocycled 35 times among temperature of 95°C (denaturation) for one min, 54°C (annealing) for one min, and then 72°C (extension) for two min. The reactions were extended for an additional seven min at 72°C after the last cycle was completed. The reactions for eNOS were thermocycled 28 times among temperature of 95°C (denaturation) for 30 s, 60°C (annealing) for 45 s, and 72°C (extension) for one min. The reactions were extended for an additional seven min at 72°C after the last cycle was completed. Negative control PCR reactions with a substitution of dissection solution for the RNA sample were performed in

parallel. The primer pairs were chosen from the published cDNA sequences of rat iNOS (576bp) and eNOS (189bp) and rat β -actin. (314 bp). The primer sequence for iNOS is GTG TTC CAC CAG GAG ATG TTG (sense) and CTC CTG CCC CCT GAG TTC GTC (antisense), for eNOS is TGC ACC CTT CCG GGG ATT CTG GCA (sense) and GGA TCC CTG GAA AAG GCG GTG AGG (antisense), and for β -actin is CGTAAAGACCTCTATGCCAA (sense) and AGCCATGCCAAATGTGTCAT (antisense). The PCR products were separated on agarose gel (1.5%) with ethidium bromide (16 μ g/100 ml), (1 \times TBE electrophoresis buffer, at 75 V for 2 h), visualized by ultraviolet-induced fluorescence and photographed. The photographs were screened, and the ratio was calculated by quantitation analysis computer software (Quantity One, USA). All the reagents were from Invitrogen (life technologies, USA). Primers were custom made by Oligonucleotide Synthesis Laboratory (Applied Biosystems, Canada).

3.2.6 Immunostaining of NT, iNOS and eNOS

The tissues were rinsed in phosphate-buffered saline (PBS, 0.1 M) and cut into small blocks (5 x 5 x 5 mm). The tissue blocks were embedded in Tissue-Tek OCT, frozen in liquid nitrogen and stored at -70° C. Sections (8 μ m thick) of the tissues were cut at -20° C, collected on slides, and fixed with liquid nitrogen-cooled acetone. Immunohistochemical staining for iNOS involved incubation of the sections for 3 min in hydrogen peroxidase (0.88 mol/L) to quench endogenous peroxidase activity; treatment with a blocking solution (Vectastain universal quick kit, Vector Laboratory) for 30 min; incubation with polyclonal anti-iNOS antibody [rabbit anti-iNOS, 1:500, pH 7.2, containing 0.1% bovine serum albumin (BioMol)] for 60 min; incubated with biotinylated panspecific universal secondary antibody for 10 min; and incubated with streptavidin-peroxidase complex (Vectastain universal quick kit, Vector Laboratory) for 20 min. A five min wash with PBS (0.1 M, pH 7.2) was performed between each step and a solution of 3, 3'-diaminobenzidine (0.5 mg/ml in 0.1 M PBS, pH 7.2; Sigma Chemical) was used as the chromogen. Some sections were treated with mouse nonspecific immunoglobulin G (1:200, Vector Laboratory) instead of the primary antibody and they serve as negative controls. The immunohistochemical staining protocols for NT and eNOS were similar to that for iNOS except that polyclonal anti-NT antibody (rabbit anti-nitrotyrosin, 1:250, pH 7.2, containing 0.1% bovine serum albumin; Upstate Biotechnology) and the

polyclonal anti-eNOS antibody (rabbit anti-eNOS, 1:500, pH 7.2, containing 0.1% bovine serum albumin; BioMol), respectively, served as the primary antibodies.

3.2.7 15-F_{2t}-isoprostane assay procedure

Extraction: The extraction and enzyme immunoassay procedures used for the measurement of tissue 15-F_{2t}-isoprostane were as described by the manufacturer [8-Isoprostane EIA (enzyme immunoassay) Kit, Cayman Chemical, Ann Arbor, MI], but with slight modifications as follows. The ventricular tissues (0.5 g) were homogenized in phosphate-buffered saline (PBS, 1.0 ml). 15-F_{2t}-isoprostane in the homogenate was extracted with ethanol (2.0 ml) containing butylated hydroxytoluene (BHT, 0.005%) to prevent oxidation, and centrifuged (2,000g) for 10 min. The supernatant was decanted into a test tube to which potassium hydroxide (15%) was added for the determination of total 15-F_{2t}-isoprostane by base hydrolysis. The samples were incubated at 40°C for 1 h, and diluted 10-fold with ultra pure water. The pH was adjusted to 7.0–7.5 with HCl for purification.

Purification: Each sample was passed through a 4 ml 15-F_{2t}-isoprostane (8-isoprostane) affinity column (Cayman Chemical). The affinity column was first washed with column buffer (2 ml), UltraPure water (2 ml), then elution solution (2 ml) to elute 15-F_{2t}-isoprostane from the columns. The elution solution was collected in test tubes and evaporated to dryness under a stream of dry nitrogen. Afterwards, enzyme immunoassay buffer (0.5 ml) was added immediately to dissolve 15-F_{2t}-isoprostane its quantification by enzyme immunoassay as described (Xia et al., 2003). In brief, standards (50 µl) and samples were added in duplicates to a 96-well plate, and this was followed by the addition of 15-F_{2t}-isoprostane acetylcholinesterase tracer and antibody. The prepared plates were incubated overnight at room temperature. The next day, the plates were washed 5 times with the wash buffer, followed by the addition of Ellman's reagent. After optimal development for 80–100 min, the plates containing 15-F_{2t}-isoprostane were read at 405 nm with the operator blinded.

The average sample recovery rate was 90 ± 5.1 % (mean \pm SEM, $n = 8$) as estimated using the 15-F_{2t}-isoprostane standards. All measured values were corrected by this recovery rate. Tissue 15-F_{2t}-isoprostane content was expressed as pg/g tissue.

3.2.8 Myocardium antioxidant capacity determination

Myocardial tissue antioxidant capacity was determined by exposure of the tissue homogenates to *t*-butylhydroperoxide (*t*-BHP, peroxidizing agent), and this results in the formation of lipid by-products, which form chromogens when incubated with thiobarbituric acid and are collectively termed TBARS. A lower tissue antioxidant capacity results in the formation of a greater amount of TBARS in the presence of *t*-BHP. The level of TBARS in the sample was estimated from the absorbance at 532 nm. Ventricular myocardial samples (300 mg) were thawed and homogenized on ice in Tris-EDTA buffer (0.05-0.1M) using a Polytron homogenizer (PT-10, Brinkman Instruments, Canada) for 30 s at 25% power. The homogenates were used for *in vitro* forced peroxidation using *t*-BHP and subsequent determination of TBARS, as described by Runzer (2002). In brief, tissue homogenate (400 µl) was combined with *t*-BHP (400 µl in 0.9% saline with 2 mM sodium azide to produce final concentrations of *t*-BHP, 0.5 to 5 mM). The suspensions were incubated for 30 min at 37°C, after which 400 µl of cold trichloroacetate (28% w/v)-sodium arsenite (0.1M) was added. The mixture was centrifuged at 12,000g for 5 min at 4°C. Supernatant (800 µl) was removed and added to thiobarbituric acid (400 µl of 0.5% in 25 mM NaOH). The samples were boiled for 15 min, and the absorbance was measured spectrophotometrically at 532 nm. All reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

3.3 Experimental protocols

3.3.1 Study 1: 1400W-LPS-induced endotoxemic rats

Selective versus non-selective suppression of nitric oxide synthase on regional hemodynamics in rats with or without LPS-induced endotoxemia

Protocol: The rats were divided into six groups ($n = 6$ each). Baseline hemodynamic measurements were taken at 30 min after the completion of surgery. Three groups were pretreated with normal saline (0.9% NaCl, i.v.), and were designated as normal rats. The other three groups were pretreated with LPS (10 mg/kg; 0.3-0.4 ml i.v. injected over two min), and were designated LPS-treated rats. Hemodynamic measurements were taken again at 2.5 h after pre-treatment with saline or LPS. Afterwards, one group each of normal and LPS-treated rats were given 1400W (3 mg/kg, i.v. injected over five min, followed by 3 mg/kg h). This dose of 1400W (3 mg/kg) was shown to reverse delayed LPS-induced vascular injury in rats (Garvey *et al.*, 1997), and to restore blood pressure in LPS-induced endotoxic shock (Wray *et al.*, 1998).

As well, 1400W, s.c. injected at either 1 or 5 mg/kg, abolished vascular leakage in the ileum and colon at 4-5 h after LPS challenge (Garvey et al., 1997; Laszlo and Whittle, 1997). Another group each of normal and LPS-treated rats were given L-NNA (8 mg/kg, i.v. bolus, followed by 3 mg/kg h; ED₈₀ dose on MAP (Wang et al., 1991). The remaining groups (of normal or LPS-treated rats) were given normal saline. Hemodynamics were measured a third time (4 h after injection of LPS or saline). Afterwards, the anesthetized rats were exsanguinated by removing the heart.

3.3.2 Study 2: 1400W-vascular constriction to NA and angiotensin II STZ-induced diabetes in conscious rats

Arterial and venous constrictions to NA and angiotensin II as well as effects of 1400W on arterial and venous constrictions to NA at an early stage of conscious rats with STZ-induced diabetes

Protocol I: After recovery from surgery and anesthesia for at least 6 h, each rat was placed in a small cage and allowed to wander freely during the study. The arterial and venous catheters were connected to pressure transducers (P23DB, Gould Statham, Oxnard, CA). The rats were equilibrated for 1 h prior to the study. Two groups of rats (diabetics and controls, n = 6 each) were first pretreated with propranolol (8×10^{-7} mol/kg i.v. bolus, followed by 3.4×10^{-7} mol/kg/min continuous infusion) to prevent the stimulation of β -adrenoceptors by NA. At 10 min later, dose-response curves of NA (2.2 - 300×10^{-9} mol/kg/min) were constructed in both groups. Another two groups of diabetic and control rats (n = 7 each) were infused with angiotensin II (1.3 - 550×10^{-11} mol/kg/min). MAP and central venous pressure were measured at the baseline condition, at 10 min after the start of infusion of propranolol (if applicable), and at the plateau phase of response (two to ten min after the start of infusion) to various doses of NA and angiotensin II. Each dose of drug was followed by a recovery period of 10-15 min.

Protocol II: Another two groups of diabetic and control rats (n = 6 or 7 each) were placed in a small cage and allowed to wander freely during the study. After equilibration for one hour, the rats were first pretreated with propranolol (8×10^{-7} mol/kg i.v. bolus followed by continuous infusion at 3.4×10^{-7} mol/kg/min) to block β -adrenoceptors. At 15 min after the start of administration of propranolol, dose-response curves of NA (6, 16, 45 and 122×10^{-9} mol/kg/min) were constructed in the diabetic and control rats. MAP and HR measurements were taken prior to and at 10 min after the start of infusion of propranolol, and at the plateau

phase of response to NA (three to ten min after the start of infusion) whereas MCFP readings were taken at the baseline and the plateau phase of response to the 2nd and 3rd doses of NA (16 and 45×10^{-9} mol/kg/min). Each dose of NA was followed by a recovery period of 10-15 min. After recovery of the response to the last dose of NA, the rats were given 1400W (3 mg/kg followed by 3 mg/kg/h, i.v.). At 1 hour later, a second dose-response curve of NA was constructed in both groups of rats. Hemodynamic measurements were again taken at the baseline condition (five min prior to the infusion of NA) and the plateau phase of response to NA.

3.3.3 Study 3: 1400W-hemodynamic response to NA in STZ-induced diabetic rats

The effect of 1400W on hemodynamic responses to NA at an early stage of STZ-induced diabetes in anesthetized rats

Protocol: At one hour after the completion of surgery, the pentobarbitone-anesthetized rats (diabetic and control, $n = 6-7$ each group) were pretreated with propranolol (i.v. 8×10^{-7} mol/kg followed by 3.4×10^{-7} mol/kg/min) to block β -adrenoceptors. At 10 min later, a baseline reading of CO and MAP was taken. NA (16.5×10^{-9} mol/kg/min) was infused for six min, and CO was measured at five min after the start of infusion. At 10 min after the termination of NA infusion, 1400W (i.v. 3 mg/kg over five min, followed by 3 mg/kg/h) was given. At 60 min from the start of administration of 1400W, NA was again infused for six min. CO was measured at five min after the beginning of NA infusion. Afterwards, organs and tissues were removed for the counting of radioactivity.

3.3.4 Study 4: 1400W-cardiac contractility in STZ-induced diabetic rats

The effect of inhibition of iNOS by 1400W on cardiac contractility and contractile responses to NA as well as activation of iNOS at an early stage of STZ-induced diabetes in anesthetized rats

Protocol I: The the pentobarbitone-anesthetized control and diabetic rats ($n = 6-7$) were given one hour equilibration following surgery. After baseline recordings were taken, the rats were infused with NA (16.5×10^{-9} mol/kg/min) for 6 min. MAP, HR, LVP, LV +dP/dt and LV -dP/dt were taken at 5 min after NA infusion. At 10 min after the termination of NA infusion, 1400W was i.v. injected (3 mg/kg over 5 min) and followed by continuous infusion (3

mg/kg/h) till the end of experiment. At 60 min from the start of administration of 1400W, the rats were infused with NA again for 6 min and the third measurement of cardiac contractility was taken. Afterwards, the hearts from the 1400W-treated control and diabetic rats were excised, cleaned in cold saline, weighed and flash frozen in liquid nitrogen for the measurement of the activity of NOS. The hearts were stored at -70°C until assayed within one month.

Protocol II: Two the pentobarbitone-anesthetized groups of control and diabetic rats (time-controls) were prepared as in protocol I except that the rats were given normal saline instead of 1400W. The hearts were excised and cleaned in cold saline and divided into two-halves. One-half was subdivided again so that the tissue was either flash frozen in liquid nitrogen for the extraction of RNA for RT-PCR of iNOS and eNOS ($n = 3$, each group), or embedded in optimal cutting temperature compound (OCT media, Tissue-Tek 4583) and quick frozen in liquid nitrogen for immunostaining of NT, iNOS and eNOS ($n = 3$, each group). The other halves of the hearts ($n = 6-7$, each group) were weighed and flash frozen in liquid nitrogen for the assay of NOS activity. All tissues were stored at -70°C until assayed within one month.

3.3.5 Study 5: NAC-cardiac contractility in STZ-induced diabetic rats

The effect of antioxidant NAC on cardiac contractility and contractile responses to dobutamine as well as on activation of iNOS, formation of peroxynitrite, and myocardium antioxidative capacity at an early stage of STZ-induced diabetes in anesthetized rats

Protocol I: The the pentobarbitone-anesthetized rats were divided into four groups ($n = 6$ each) as follows: control rats, control rats given NAC, diabetic rats, and diabetic rats given NAC. Baseline readings of MAP, HR, LVP and $\text{LV } \pm \text{dP/dt}$ were taken at 1 h after the completion of surgery. Dose-response curves of dobutamine ($1-57 \mu\text{g/min/kg}$, 1-2 min duration) were constructed in each group with a recovery period of 10-15 min between doses. Plateau responses were used for statistical analysis.

Protocol II: Another four groups rats (as in protocol I) were anesthetized with sodium pentobarbital (60 mg/kg , i.p.). The hearts were excised and cleaned in cold saline. Myocardial samples were weighed and flash frozen in liquid nitrogen for 15-F2t-isoprostane assay, determination of myocardium antioxidant capacity, and immunostaining of NT, iNOS and eNOS using the Vectastain universal quick kit. All samples were stored at -70°C until assayed.

3.4 Statistical analyses

All the results are presented as mean \pm S.E.M except for data with $n = 3$ in the RT-PCR results of study 3 where the results are presented as mean \pm range.

ED₅₀ and E_{max} values were obtained by analysis of dose response curves using the GraphPad Prism program. ED_{2.5 mmHg} values (doses that increased MCFP by 2.5 mmHg) were obtained in instances where maximum responses could not be attained. All data of ED₅₀ were log transformed prior to statistical analysis. ED₅₀, ED_{2.5 mmHg} and E_{max} values before and after drug treatment were analyzed by one -way analysis of variance (ANOVA) with $P < 0.05$ selected as the criterion for statistical significance.

One analysis of variance (ANOVA) and T-test (paired and no paired) were used for statistical analyses of data obtained within the same rat, and between groups of rats, respectively; Tukey's test was used for multiple comparison of group means (SigmaStat, Jandel Scientific software, USA). Dose-response relationships were analyzed by repeated measures analysis of variance followed by the Tukey test. In all cases, a probability of error ($P < 0.05$) was selected as the criterion for statistical significance.

3.5 Drugs and chemicals

N-3-aminomethyl-benzyl-acetamidine (1400W) was obtained from Calbiochem (San Diego, CA, USA). ⁵⁷CO, ¹¹³Sn and ¹⁰³Ru (15 μ m diameter) microspheres were from Perkin-Elmer Canada Inc., Ontario. Noradrenaline, propranolol, *Escherichia coli* lipopolysaccharide (LPS, #0127-B8), N^G-nitro-L-arginine (L-NNA), streptozotocin [STZ, 2-deoxy-2-(3-methyl-3-nitrosourea) 1-D-glucopyranose] and N-acetylcysteine (NAC) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All drugs were dissolved in normal saline (0.9% NaCl).

All the other reagents for RT-PCR, assay of NOS, immunostaining, assays of 15-F2t-isoprostane and thiobarbituric acid reactive substances (TBARS) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated (see Chapter 4.2).

4. RESULTS I (1400W-LPS)

4.1 Study 1: 1400W-LPS-induced endotoxemic rats

The effects of highly selective inhibitor of iNOS on the cardiovascular system of rats with LPS-induced endotoxemia

Baseline and time-control readings There are no significant differences in baseline values of MAP, HR, CO, TPR, as well as hemodynamics among the six groups of rats at 0 h (Tables 4-1). In the three groups of normal rats pretreated with saline, the second set of baseline readings at 2.5 h are similar to those of the first set taken at 0 h (Table 4-1 and 4-2).

Effects of 1400W and L-NNA on MAP, HR, CO, TPR and hemodynamics in normal rats

Neither the vehicle nor 1400W, given at 2.5 h into two groups of normal rats (immediately after obtaining the second set of baseline values), altered MAP, HR, CO, TPR (Table 4-1) or regional BF at 4 h (Table 4-2). Due to the lack of changes on MAP and regional BF, arterial conductance (ratio of BF to MAP) of the various organs and tissues at 2.5 and 4 h are also similar to the respective baseline readings at 0 h in the groups given the vehicle or 1400W (results not shown). In contrast, L-NNA, given at 2.5 h after obtaining the second set of baseline readings, increased MAP (Fig. 4-1A) and TPR, decreased CO, but did not alter HR at 4 h (Table 4-1). L-NNA also decreased BF and conductance of all tissues at 4 h (Table 4-2).

Effects of LPS Rats injected with LPS at 0 h and saline at 2.5 h had a biphasic decrease of MAP, which fell from 95 ± 2 to 70 ± 2 mmHg at 1 h, slowly recovered to 83 ± 2 mmHg at 2.5 h, and gradually declined to 59 ± 3 mmHg at 4 h (Fig. 4-1B, Table 4-3). Along with the hypotension are increases in TPR and progressive reductions in CO at 2.5 as well as 4 h (Table 4-3).

The three groups of rats injected with LPS at 0 h had identical treatments prior to the injection of saline, 1400W or L-NNA (Table 4-3, 4 and 5). Thus, MAP and CO were similarly reduced, and TPR was similarly increased at 2.5 h in all three groups. Furthermore, arterial flow and conductance of the stomach, skeletal muscle and skin were decreased (Table 4-4 and 5), whereas flow and conductance of other beds were not significantly altered at this time. There was a tendency of hepatic arterial conductance to be increased (20-35%, $P > 0.05$), and flow to the kidneys to be decreased (-14 to -21%, $P > 0.05$) at 2.5 after injection of LPS (Table 4-5).

At 4 h after injection of LPS, BF to all organs and tissues was decreased, but significant reductions were only obtained in the heart, kidneys, stomach, skeletal muscle and skin, relative

to the baseline readings at 0 h (Fig. 4-2A, significant differences not shown). When BF was normalized by MAP to reveal intrinsic vascular tone independent of perfusion pressure, LPS was found to increase arterial conductance of the heart, brain and liver, and decrease conductance of stomach, skeletal muscle and skin (Fig. 4-2B).

Effects of 1400W and L-NNA on MAP, HR, CO, TPR and hemodynamics in LPS-treated rats

In rats given LPS at 0 h, treatment with 1400W at 2.5 h (after obtaining the second set of baseline) prevented the second phase decrease in MAP (Fig. 4-1B); however, MAP at 4 h was still lower than baseline MAP at 0 h (Table 4-3). In the 1400W-treated rats, CO further decreased, and TPR further increased at 4 h; the reading is similar to that at 4 h in the LPS-treated rats given saline (Table 5-3). Administration of L-NNA, however, did not prevent the second phase decline in MAP in the LPS-treated rats (Fig. 4-1B, Table 4-3). Furthermore, CO drastically decreased, and TPR markedly increased at 4 h after injection of L-NNA; these changes are markedly greater than those in the endotoxemic rats given saline (Table 4-3).

At 4 h after injection of LPS, all values of flow and conductance in the endotoxemic rats treated with 1400W are similar to those in rats given saline (Fig. 4-2). On the other hand, L-NNA-treated rats, relative to the saline-treated or 1400W-treated endotoxemic rats, had reduced BF and conductance of the heart, brain, intestine and skeletal muscle, as well as reduced arterial conductance of the kidneys (Fig. 4-2).

Table 4-1 Time control baseline (MAP, HR, CO and TPR) in normal rats

	Time (h)	Vehicle	1400W	L-NNA
MAP	0	94 ± 2	96 ± 1	94 ± 3
(mmHg)	2.5	94 ± 2	95 ± 1	94 ± 2
	4	91 ± 3	91 ± 1	113 ± 3 ^a
Heart rate	0	343 ± 10	364 ± 13	348 ± 12
(beats/min)	2.5	339 ± 13	348 ± 13	354 ± 12
	4	340 ± 15	357 ± 13	350 ± 18
Cardiac output	0	109 ± 8	111 ± 3	106 ± 11
(ml/min)	2.5	105 ± 7	103 ± 3	104 ± 6
	4	101 ± 6	106 ± 5	46 ± 5 ^a
TPR	0	0.89 ± 0.07	0.87 ± 0.03	0.95 ± 0.13
(mmHg.min/ml)	2.5	0.91 ± 0.07	0.93 ± 0.03	0.92 ± 0.06
	4	0.92 ± 0.06	0.87 ± 0.05	2.6 ± 0.25 ^a

Mean arterial pressure (MAP), heart rate, cardiac output (CO) and total peripheral resistance (TPR) in normal thiobutabarbital-anesthetized rats treated with vehicle (0.9% NaCl), 1400W (3 mg/kg, 3 mg/kg h) or L-NNA (8 mg/kg, 3 mg/kg h). Values are mean ± S.E.M., n = 6. ^aDenotes significant difference ($P < 0.05$) relative to values at 0 h within the same group as well as values at the same time in rats treated with vehicle or 1400W. Vehicle, 1400W and L-NNA were given immediately after obtaining the second set of baseline readings at 2.5 h.

Table 4-2 Time control baseline (flow and conductance) in normal rats

Organs	Time (h)	Vehicle	1400W	L-NNA
Heart	0	4.1 ± 0.6	4.2 ± 0.5	4.4 ± 0.4 (4.7 ± 0.5)
	2.5	3.8 ± 0.7	4.0 ± 0.3	3.9 ± 0.6 (4.2 ± 0.6)
	4	3.7 ± 0.9	3.8 ± 0.3	2.1 ± 0.3 (1.9 ± 0.3) ^a
Brain	0	0.71 ± 0.09	0.72 ± 0.04	0.74 ± 0.09 (0.79 ± 0.10)
	2.5	0.69 ± 0.06	0.69 ± 0.11	0.71 ± 0.09 (0.76 ± 0.11)
	4	0.72 ± 0.07	0.75 ± 0.06	0.36 ± 0.06 (0.33 ± 0.06) ^a
Liver	0	0.31 ± 0.04	0.31 ± 0.05	0.31 ± 0.03 (0.33 ± 0.04)
	2.5	0.32 ± 0.05	0.30 ± 0.07	0.30 ± 0.05 (0.33 ± 0.06)
	4	0.32 ± 0.04	0.32 ± 0.05	0.21 ± 0.02 (0.19 ± 0.03) ^a
Kidneys	0	4.5 ± 0.4	4.4 ± 0.6	4.5 ± 0.7 (4.9 ± 0.8)
	2.5	4.4 ± 0.7	4.1 ± 0.6	4.4 ± 0.7 (4.7 ± 0.7)
	4	3.8 ± 0.8	3.8 ± 0.7	2.2 ± 0.4 (2.0 ± 0.4) ^a
Stomach	0	0.82 ± 0.15	0.86 ± 0.10	0.81 ± 0.17 (0.87 ± 0.20)
	2.5	0.69 ± 0.11	0.73 ± 0.10	0.69 ± 0.11 (0.72 ± 0.13)
	4	0.71 ± 0.13	0.72 ± 0.08	0.23 ± 0.03 (0.20 ± 0.04) ^a
Intestine	0	2.4 ± 0.2	2.3 ± 0.4	2.3 ± 0.4 (2.3 ± 0.3)
	2.5	2.2 ± 0.3	2.5 ± 0.4	2.2 ± 0.3 (2.2 ± 0.2)
	4	2.4 ± 0.2	2.4 ± 0.4	0.94 ± 0.2 (1.0 ± 0.2) ^a
Muscle ^b	0	5.8 ± 0.5	5.8 ± 0.3	6.0 ± 0.6 (6.4 ± 0.7)
	2.5	5.8 ± 0.3	6.2 ± 0.6	6.2 ± 0.2 (6.6 ± 0.3)
	4	6.1 ± 0.5	6.3 ± 0.4	2.6 ± 0.4 (2.3 ± 0.3) ^a
Skin ^b	0	12.9 ± 1.2	12.0 ± 1.2	12.7 ± 1.9 (13.6 ± 2.1)
	2.5	10.7 ± 1.8	11.2 ± 1.5	11.1 ± 1.6 (11.8 ± 1.6)
	4	10.7 ± 1.7	10.5 ± 1.0	2.3 ± 0.6 (2.1 ± 0.6) ^a

Flow and conductance (brackets) in normal thiobutabarbital-anesthetized rats given vehicle (0.9% NaCl), 1400W (3 mg/kg, 3 mg/kg h) or L-NNA (8 mg/kg, 3 mg/kg h). Vehicle, 1400W and L-NNA were given immediately after obtaining the second set of baseline values at 2.5 h. Values are mean ± S.E.M., n = 6. ^aSignificant ($P < 0.05$) difference from 0 and 2.5 h in the same group, and values at the same time in groups given vehicle or 1400W. ^bFlow (ml/min per 100 g of muscle and skin, and per g of other organs), and conductance (ml/min mmHg per 10⁴ g of muscle and skin, and ml/min mmHg per 100g of other organs).

Table 4-3 Effects of 1400W and L-NNA on LPS-treated rats

	Time (h)	LPS		
		Vehicle	1400W	L-NNA
MAP (mmHg)	0	95 ± 2	98 ± 3	94 ± 2
	2.5	83 ± 2 ^a	85 ± 4 ^a	84 ± 2 ^a
	4	59 ± 3 ^{a,b}	85 ± 4 ^{a,c}	57 ± 3 ^{a,b}
Heart rate (beats/min)	0	343 ± 12	350 ± 6	350 ± 10
	2.5	364 ± 18	368 ± 6	384 ± 15
	4	372 ± 26	365 ± 15	363 ± 19
Cardiac output (ml/min)	0	112 ± 7	107 ± 3	109 ± 5
	2.5	74 ± 7 ^a	73 ± 5 ^a	73 ± 8 ^a
	4	50 ± 7 ^{a,b}	49 ± 4 ^{a,b}	26 ± 2 ^{a,b,c}
TPR (mmHg.min ml)	0	0.85 ± 0.05	0.92 ± 0.03	0.87 ± 0.04
	2.5	1.2 ± 0.2 ^a	1.2 ± 0.1 ^a	1.3 ± 0.2 ^a
	4	1.3 ± 0.2 ^a	1.8 ± 0.2 ^{a,b}	2.3 ± 0.1 ^{a,b,c}

Mean arterial pressure (MAP), heart rate, cardiac output and total peripheral resistance (TPR) in thiobutabarbital-anesthetized rats pretreated with lipopolysaccharide (LPS, 10 mg/kg) followed by vehicle (0.9% NaCl), 1400W (3 mg/kg, 3 mg/kg h) or L-NNA (8 mg/kg, 3 mg/kg h). Vehicle, 1400W and L-NNA were given immediately after obtaining the second set of baseline readings at 2.5 h. Values are mean ± S.E.M., n = 6. ^aSignificant ($P < 0.05$) difference from values within the same group at 0 h. ^bSignificant difference from values within the same group at 2.5 h. ^cSignificant difference from rats treated with vehicle at the same time.

Table 4-4 Effects of Lipopolysaccharide (LPS) on blood flow in rats

Organs	Time (h)	LPS		
		Vehicle	1400W	L-NNA
Heart	0	4.4 ± 0.5	4.0 ± 0.4	4.3 ± 0.5
	2.5	4.0 ± 0.7	4.2 ± 0.4	3.9 ± 0.5
Brain	0	0.79 ± 0.09	0.71 ± 0.05	0.76 ± 0.06
	2.5	0.72 ± 0.11	0.78 ± 0.08	0.73 ± 0.13
Liver	0	0.30 ± 0.04	0.31 ± 0.03	0.30 ± 0.04
	2.5	0.34 ± 0.04	0.32 ± 0.03	0.32 ± 0.04
Kidneys	0	4.3 ± 0.2	4.3 ± 0.3	4.2 ± 0.5
	2.5	3.4 ± 0.5	3.7 ± 0.6	3.4 ± 0.2
Stomach	0	0.87 ± 0.13	0.80 ± 0.06	0.81 ± 0.08
	2.5 ^a	0.44 ± 0.07	0.47 ± 0.04	0.48 ± 0.04
Intestine	0	2.3 ± 0.1	2.4 ± 0.1	2.5 ± 0.3
	2.5	2.3 ± 0.2	2.3 ± 0.3	2.2 ± 0.2
Muscle ^b	0	5.8 ± 0.4	5.7 ± 0.5	6.1 ± 0.5
	2.5 ^a	3.5 ± 0.6	3.9 ± 0.3	3.8 ± 0.4
Skin ^b	0	12.9 ± 0.9	13.0 ± 0.9	11.5 ± 2.5
	2.5 ^a	4.8 ± 1.0	4.9 ± 1.0	4.4 ± 0.5

Blood flow in thiobutabarbital-anesthetized rats at 0 and 2.5 h after injection of LPS (10 mg/kg). Values are mean ± S.E.M., n = 6. ^aSignificant ($P < 0.05$) difference from pooled values of flow at 0 h. ^bFlow (ml/min per 100 g of muscle and skin, and ml/min per g of other organs).

Table 4-5 Effects of Lipopolysaccharide (LPS) on organ conductance in rats

Organs	Time (h)	LPS		
		Vehicle	1400W	L-NNA
Heart	0	4.7 ± 0.6	4.1 ± 0.3	4.7 ± 0.6
	2.5	4.7 ± 0.7	4.9 ± 0.5	4.7 ± 0.7
Brain	0	0.83 ± 0.09	0.73 ± 0.04	0.83 ± 0.09
	2.5	0.86 ± 0.14	0.91 ± 0.07	0.86 ± 0.13
Liver	0	0.31 ± 0.04	0.31 ± 0.03	0.31 ± 0.04
	2.5	0.42 ± 0.04	0.37 ± 0.03	0.42 ± 0.04
Kidneys	0	4.5 ± 0.3	4.5 ± 0.3	4.5 ± 0.2
	2.5	4.1 ± 0.5	4.2 ± 0.6	4.1 ± 0.5
Stomach	0	0.92 ± 0.13	0.83 ± 0.07	0.92 ± 0.15
	2.5 ^a	0.53 ± 0.08	0.56 ± 0.05	0.53 ± 0.08
Intestine	0	2.5 ± 0.2	2.5 ± 0.2	2.6 ± 0.3
	2.5	2.7 ± 0.3	2.7 ± 0.4	2.7 ± 0.3
Muscle ^b	0	6.1 ± 0.4	5.8 ± 0.4	6.1 ± 0.5
	2.5 ^a	4.1 ± 0.7	4.6 ± 0.5	4.1 ± 0.7
Skin ^b	0	14.8 ± 1.1	13.4 ± 0.9	14.5 ± 1.1
	2.5 ^a	5.8 ± 1.3	5.8 ± 1.1	5.8 ± 1.3

Organ conductance in thiobutabarbital-anesthetized rats at 0 and 2.5 h after injection of LPS (10 mg/kg). Values are mean ± S.E.M., n = 6. ^aSignificant ($P < 0.05$) difference from pooled values of conductance at 0 h. ^bConductance (ml/min/mmHg per 10⁴ g of muscle and skin, and ml/min/mmHg per 100 g of other organs).

Figure 4-1 Effects of 1400W and L-NNA on MAP in rats treated with vehicle or LPS

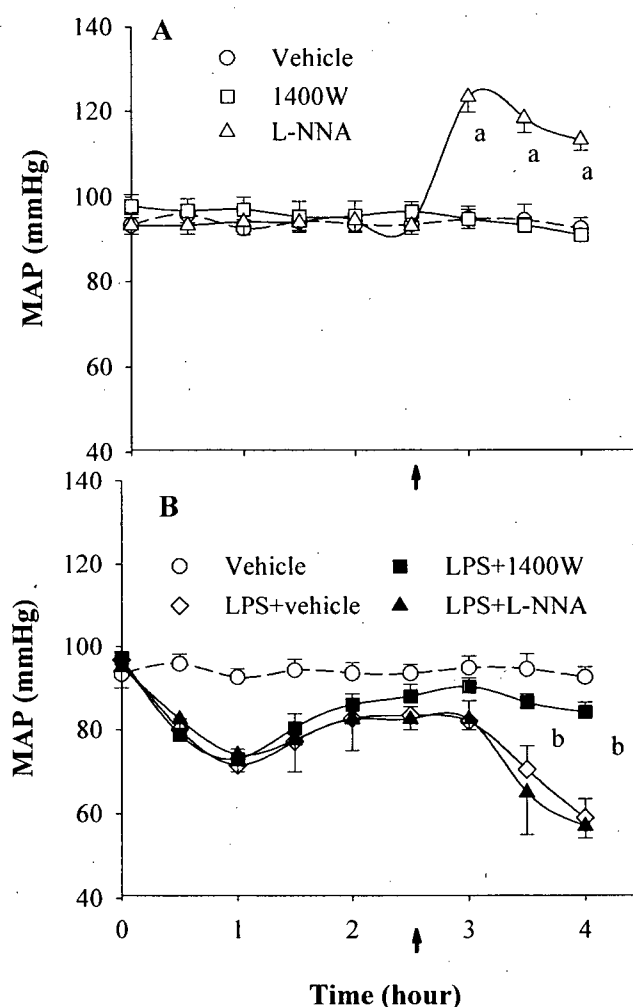


Fig. 4-1 Effects (mean \pm S.E.M.) of N-3-aminomethyl-benzyl-acetamidin \acute{e} (1400W, 3 mg/kg followed by 3 mg/kg h, i.v.), N^G-nitro-L-arginine (L-NNA, 8 mg/kg followed by 3 mg/kg h, i.v.) and vehicle (0.9% NaCl) given at 2.5 h (arrow) on mean arterial pressure (MAP) in thiobutabarbital-anesthetized (a) normal rats and (b) rats given lipopolysaccharide (LPS, 10 mg/kg) at 0 h. Values are mean \pm S.E.M., n = 6 per group. ^aSignificant ($P < 0.05$) difference from rats given vehicle or 1400W; ^bSignificant ($P < 0.05$) difference from endotoxemic rats given vehicle or L-NNA.

Figure 4-2 Effects of 1400W and L-NNA on blood flow and conductance in rats treated with vehicle or LPS

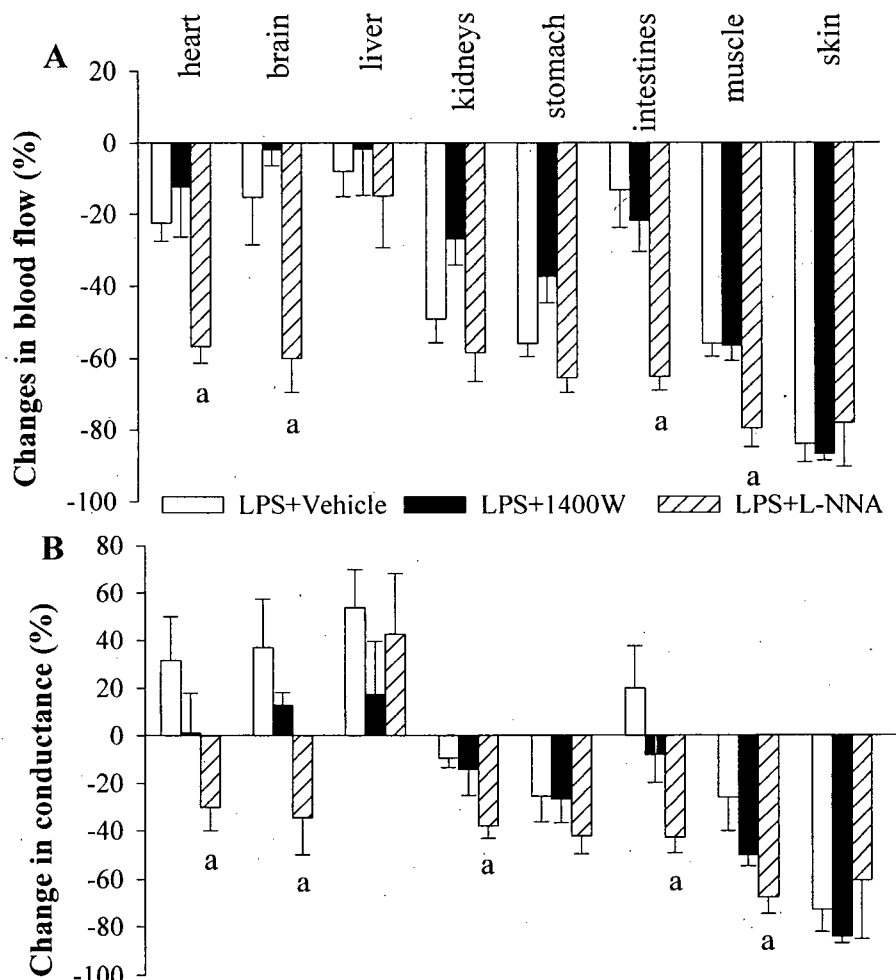


Fig. 4-2 Percentage change (mean \pm S.E.M.) in organ blood flow (a) and conductance (b) in three groups ($n = 6$ each) of thiobutabarbital-anesthetized rats at 4 h after injection of lipopolysaccharide (LPS, 10 mg/kg i.v.). The rats were given vehicle (0.9% NaCl), N-3-aminomethyl-benzyl-acetamidine (1400W, 3 mg/kg followed by 3 mg/kg h, i.v.) or N^G-nitro-L-arginine (L-NNA, 8 mg/kg followed by 3 mg/kg h, i.v.) at 2.5 h after injection of LPS. ^aSignificant ($P < 0.05$) difference from endotoxemic rats given vehicle.

5. RESULTS II (1400W-NAC-Diabetes)

5.1 Characteristics in rats with STZ-induced diabetes

Rats injected with STZ had higher blood glucose concentration at 48 h later compared to control rats, and this ranges from 21.3 ± 2.6 to 23.2 ± 3.8 mM ($n = 7-18$ per group) in STZ-induced diabetic rats versus from 5.6 ± 0.8 to 5.8 ± 0.3 mM in age-matched control rats ($n = 7-18$ per group). After two or three weeks of STZ injection, plasma glucose range from 23.5 ± 4.3 to 28.5 ± 1.2 mM in diabetic rats versus from 5.7 ± 1.4 to 6.1 ± 0.3 mM in age-matched control rats. In addition to hyperglycemia, the rats had other symptoms of diabetes, which include increased food and water intake, decreased body weight gain and heart rate, and no significantly decrease in MAP when compared with age-matched controls.

5.2 Study 2: 1400W-vascular constriction to NA and angiotensin II in conscious rats with STZ-induced diabetes

Arterial and venous constrictions to NA and angiotensin II as well as effects of 1400W on arterial and venous constrictions to NA at an early stage of STZ-induced diabetes in conscious rats

5.2.1 In conscious rats with STZ-induced diabetes for two weeks

Baseline values At two weeks after induction of STZ, the diabetic rats had lower body weight and higher plasma glucose, but similar MAP, HR, and MCFP relative to controls (Table 5.2-1). There are no significant differences in any of the above baseline values among the subgroups of diabetic rats (or control rats) to be infused with NA or angiotensin II.

Cardiovascular response to NA Propranolol did not significantly alter MAP and MCFP (results not shown), but caused a insignificantly smaller decrease in heart rate in control (-27 ± 4 beats/min) relative to diabetic (-46 ± 14 beats/min) rats at 10 min after the start of infusion.

NA caused dose-dependent increases in MAP and MCFP in both control and diabetic rats (Fig. 5.2-1A, C). The rats with diabetes, relative to controls, had significantly higher ED_{50} for the MAP (2.5-fold) as well as MCFP (4.3-fold) responses to NA, and significantly lower E_{max} for the MAP (77% of maximum), but similar E_{max} for the MCFP response to NA (Table 5.2-2). Heart rate was similarly decreased by NA in both groups (Fig. 5.2-1B).

Cardiovascular response to angiotensin II Angiotensin II also increased MAP and MCFP in a dose-dependent manner in control and diabetic rats (Fig. 5.2-2A, C). Relative to the control rats, the diabetic rats had greater ED_{50} but similar E_{max} for the MAP response to angiotensin II (Table 5.2-2). The control and diabetic rats had apparently similar dose MCFP response curves to angiotensin II (Fig. 5.2-2B). Maximum MCFP responses could not be determined for angiotensin II, however, since 3 of 7 controls and 5 of 7 diabetic rats died during inflation of the atrial balloon at the next higher dose of angiotensin II (than the highest dose shown on the graphs). Consequently, the $ED_{2.5 \text{ mmHg}}$ values (doses that increased MCFP by 2.5 mmHg) were obtained as the index of potency (Laurence and Carpenter, 1998). The diabetic rats had slightly, but insignificant, greater $ED_{2.5 \text{ mmHg}}$ for the MCFP response to angiotensin II relative to the readings in the control rats (0.40 ± 0.17 and 0.30 ± 0.11 nmol/kg/min, respectively).

Table 5.2-1 Baseline values of body weight, plasma glucose, MAP, HR and MCFP in conscious, diabetic and control rats

	Control (n = 13)	Diabetes (n = 13)
Body weight (g)	416 ± 15	361 ± 21 ^a
Plasma glucose (mM)	5.7 ± 1.4	23.5 ± 4.3 ^a
MAP (mmHg)	107 ± 3	102 ± 4
HR (beats/min)	375 ± 11	339 ± 15
MCFP (mmHg)	6.5 ± 0.2	6.2 ± 0.3

Baseline values of body weight, plasma glucose, mean arterial pressure (MAP), heart rate (HR) and mean circulatory filling pressure (MCFP) in conscious, diabetic and control rats. The measurements were made at two-weeks following injection of vehicle (0.9% NaCl, i.v., control) or streptozotocin (60 mg/kg, i.v., diabetes), respectively. All values are means ± S.E.M. ^aDenotes significant difference from controls ($P < 0.05$).

Table 5.2-2 Potency (ED_{50}) and efficacy (E_{max}) for MAP and MCFP response curves of noradrenaline & angiotensin II in conscious rats

	ED_{50} (nmol/kg)		E_{max} (mmHg)	
	Control	Diabetes	Control	Diabetes
Noradrenaline				
MAP	12 ± 4	30 ± 10 ^a	96 ± 5	74 ± 8 ^a
MCFP	12 ± 3	51 ± 16 ^a	6.5 ± 0.5	5.7 ± 0.6
Angiotensin II				
MAP	0.04 ± 0.01	0.20 ± 0.06 ^a	69 ± 5	65 ± 5

Potency (ED_{50}) and efficacy (E_{max}) for the mean arterial pressure (MAP) and mean circulatory filling pressure (MCFP) response curves of noradrenaline and angiotensin II in conscious rats. The rats were injected with streptozotocin (60 mg/kg, diabetes) or vehicle (0.9% NaCl, control) at two weeks prior to the study (n = 6 or 7 per group). All values are means ± S.E.M. All data of ED_{50} were log transformed prior to statistical analysis. ^aDenotes significant difference from control ($P < 0.05$).

Figure 5.2-1 Dose-response curves for the effects of NA on MAP, HR and MCFP.

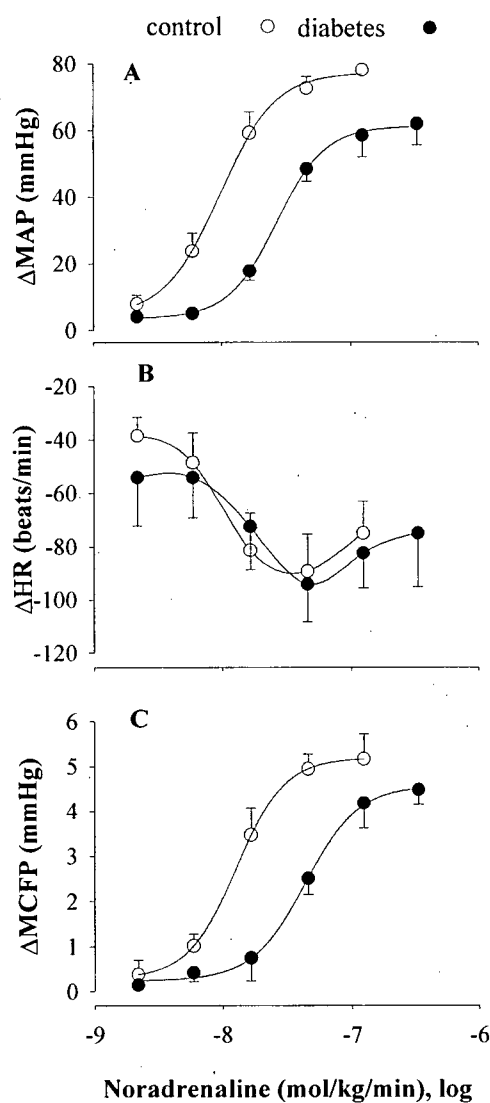


Fig. 5.2-1 Dose-response curves for the effects (mean \pm S.E.M, $n = 6$ per group) of i.v. infused noradrenaline (NA) on A) mean arterial pressure (MAP), B) heart rate (HR) and C) mean circulatory filling pressure (MCFP) in conscious rats injected with streptozotocin (60 mg/kg, closed circle, diabetic group) or vehicle (0.9% NaCl, open circle, control group) at two weeks prior to the study.

Figure 5.2-2 Dose-response curves for the effects of angiotensin II on MAP, HR and MCFP

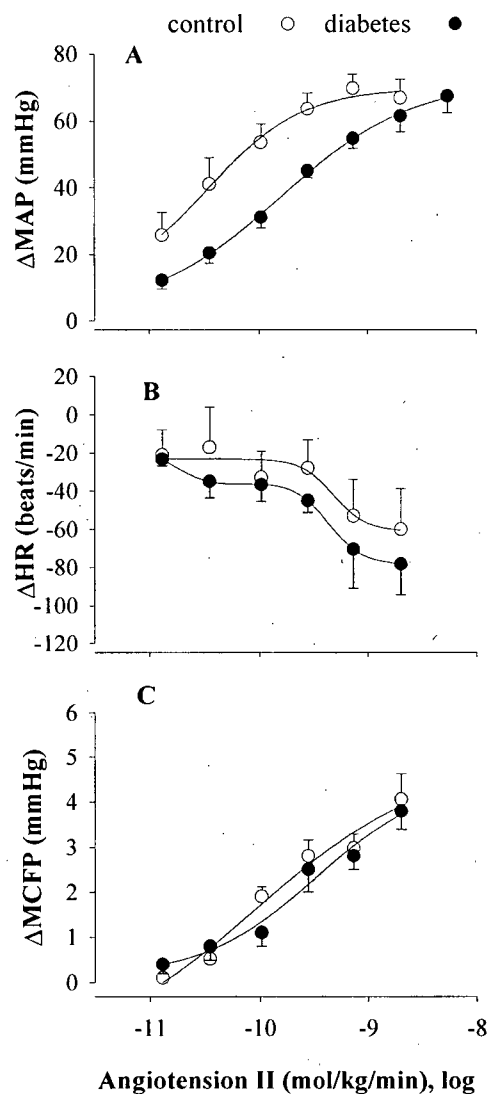


Fig. 5.2-2 Dose-response curves for the effects (mean \pm S.E.M, $n = 7$ per group) of i.v. infused angiotensin II on A) mean arterial pressure (MAP), B) heart rate (HR) and C) mean circulatory filling pressure (MCFP) in conscious rats injected with streptozotocin (60 mg/kg, closed circle, diabetic group) or vehicle (0.9% NaCl, open circle, control group) at two weeks prior to the study.

5.2.2 In conscious rats with STZ-induced diabetes for three weeks

Baseline values At three weeks after the induction, the diabetic rats had lower body weight and higher plasma glucose than the control rats (Table 5.2-3). At this time, the diabetic rats had slightly (insignificantly) lower baseline MAP, MCFP and HR (110 ± 2 mmHg, 6.3 ± 0.1 mmHg and 374 ± 13 beats/min, respectively) relative to the control rats (119 ± 5 mmHg, 6.5 ± 0.2 mmHg and 402 ± 7 beats/min).

Pretreatment with propranolol decreased HR in the diabetic (-35 ± 7 beats/min) as well as control (-33 ± 6 beats/min) rats at 10 min after the start of infusion, but did not significantly alter MAP and MCFP. After treatment with propranolol, heart rate was slightly lower in the diabetic rats relative to the control rats, but MAP and MCFP remained insignificantly lower in the diabetic rats relative to the controls (1st baseline, Table 5.2-3). The administration of 1400W did not significantly affect MAP, HR, and MCFP in either the diabetic or control rats (2nd baseline, Table 5.2-3)

Response to NA NA caused dose-dependent increases MAP and decreases in HR in the control and diabetic rats. Curve analyses show that the changes in MAP and decreases in HR in the diabetic rats were significantly smaller than those of the controls (Fig. 5.2-3A,B). The diabetic rats had lower potency (higher ED_{50}) and lower efficacy (E_{max}) of response to NA than the controls (Table 5.2-4). NA (16 and 45 nmol/kg/min) increased MCFP in the diabetic and control rats, and both changes were significantly and markedly less in the diabetic than the controls rats (Fig. 5.2-4A,B).

Effects of pre-treatment with 1400W on responses to NA Pretreatment (60 min) with 1400W did not significantly affect MAP, HR (Fig. 5.2-3) and MCFP (Fig. 5.2-4A) responses to NA in the control group. The 1400W treatment in the diabetic rats augmented NA-induced increases in MAP (Fig. 5.2-3A), such that maximum MAP was restored, and potency of response to NA was slightly (insignificantly) increased (Table 5.2-4). Pre-treatment with 1400W slightly (insignificantly) increased MCFP response to the low dose of NA but restored MCFP response to the high dose of NA relative to the corresponding response in the control rats (Fig. 5.2-4B).

Table 5.2-3 Baseline values of body weight, plasma glucose, MAP, HR and MCFP in conscious, diabetic and control rats

	Control	Diabetic
Body weight (g)	436 ± 21	332 ± 10*
Plasma glucose (mM)	6.1 ± 0.3	24.4 ± 2.4*
MAP (mmHg): 1 st baseline	118 ± 6	109 ± 2
2 nd baseline	113 ± 6	105 ± 2
HR (beats/min): 1 st baseline	369 ± 5	339 ± 10*
2 nd baseline	365 ± 4	336 ± 13*
MCFP (mmHg): 1 st baseline	7.3 ± 0.3	6.8 ± 0.4
2 nd baseline	6.7 ± 0.2	6.8 ± 0.5

Baseline values of body weight, plasma glucose, mean arterial pressure (MAP), heart rate (HR) and mean circulatory filling pressure (MCFP) in conscious, diabetic and control rats at three weeks following the administration of streptozotocin (60 mg/kg i.v.) or the vehicle (0.9% NaCl), respectively. MAP, HR and MCFP measurements were taken after treatment with propranolol. All values are means ± SEM (n = 6-7 per group). *Denotes significant difference from controls ($P < 0.05$). Baseline values of MAP, HR and MCFP were obtained at 5 min prior to the start of NA infusion, both before (1st baseline) and after (2nd baseline) the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.).

Table 5.2-4 ED_{50} and E_{max} for the dose-response curves in MAP to NA before and after the administration of 1400W

	ED_{50} (nmol/kg)		E_{max} (mmHg)	
	Control	Diabetic	Control	Diabetic
MAP before 1400W	6.8 ± 0.2	$23.6 \pm 3.7^*$	61 ± 3	$46 \pm 3^*$
MAP after 1400W	6.3 ± 0.6	$16.3 \pm 6.5^*$	62 ± 2	$63 \pm 5^\dagger$

ED_{50} and E_{max} for dose-response curves of the changes in mean arterial pressure (MAP) to noradrenaline (NA) before and after the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.) in diabetic rats and control rats ($n = 6-7$ per group) at 3 weeks following i.v. injection of streptozotocin (60 mg/kg) and the vehicle (0.9% NaCl), respectively. All values are means \pm SEM ($n = 6-7$ per group). All data of ED_{50} were log transformed prior to statistical analysis. *Denotes significant difference from control ($P < 0.05$). † Denotes significant difference from before the administration of 1400W ($P < 0.05$).

Figure 5.2-3 Dose-response curves for the effects of Noradrenaline on MAP and HR before and after administration of 1400W

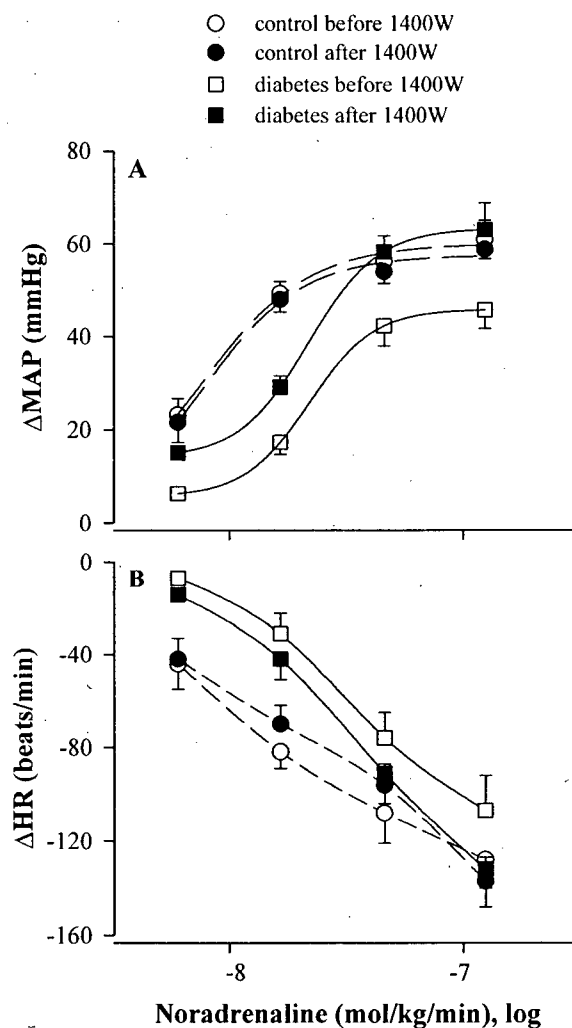


Fig. 5.2-3 Dose-response curves for the effects (mean \pm S.E.M, $n = 6-7$ per group) of noradrenaline on A) mean arterial pressure (Δ MAP), and B) heart rate (Δ HR) before (open circle and square) and after (closed circle and square) the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.) in conscious rats injected with streptozotocin (60 mg/kg, square) or the vehicle (0.9% NaCl, circle) at three weeks prior to these experiments.

Figure 5.2-4 Effects of Noradrenaline on MCFP before and after 1400W

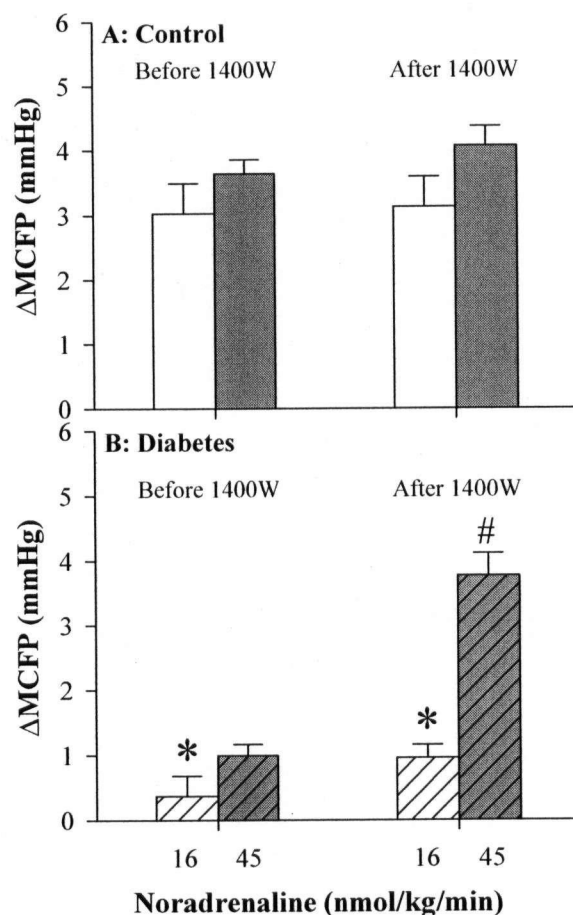


Fig. 5.2-4 Effects (mean \pm S.E.M, $n = 6-7$ per group) of noradrenaline (16 and 45 nmol/kg/min) on changes in mean circulatory filling pressure (Δ MCFP) before and after the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.) in conscious rats injected with vehicle (0.9% NaCl; A) or streptozotocin (60 mg/kg; B) at three weeks prior to these experiments. *Significantly different ($P < 0.05$) from corresponding responses (in A) in the control rats given the vehicle. #Significantly different from responses in the diabetic rats before the administration of 1400W.

5.3 Study 3: 1400W-hemodynamic response to NA in STZ-induced diabetic rats

The effect of 1400W on hemodynamic responses to NA at an early stage of anesthetized rats with STZ-induced diabetes

Baseline values and effects of 1400W At three weeks after injection of STZ, the body weight of the diabetic rats was lower whereas plasma glucose was higher than the corresponding values of the controls (Table 5.3-1). The diabetic rats, relative to the controls, had higher normalized organ weights (organ weight/body weight) of the kidneys, liver, intestine and colon/caecum (Table 5.3-1).

The diabetic and control rats (protocol 1) had similar baselines of MAP (100 ± 3 and 106 ± 2 mmHg, respectively, $n = 6-7$). Since neither propranolol nor 1400W altered MAP in either group, baselines of MAP in the control and diabetic rats remained similar (98 ± 3 versus 100 ± 4 mmHg) after both treatments. The diabetic rats had lower HR than the controls (312 ± 8 and 368 ± 10 beats/min, respectively, $P < 0.05$). Propranolol decreased HR more in the controls (-50 ± 7 beats/min) than the diabetic rats (-24 ± 2 beats/min), but 1400W did not alter HR in either group. Baseline HR of the control group after treatments with propranolol and 1400W remained higher than HR of the diabetic group (327 ± 7 versus 292 ± 9 beats/min, $P < 0.05$).

Baselines of CO and cardiac index (CI) were similar between the control and diabetic rats (CO: 100 ± 22 and 99 ± 13 ml/min; CI, 232 ± 58 and 280 ± 36 ml/min kg, respectively). TPR was also similar between the control and diabetic rats (0.99 ± 0.05 and 0.89 ± 0.05 mmHg min/ml). Relative to the controls, blood flow as well as flow conductance (normalized by tissue weights) in the diabetic rats were decreased in the kidneys, increased in the intestine and colon/caecum, but similar in other organs and tissues (Fig. 5.3-1A and D).

Effects of 1400W on NE-induced changes in MAP, CO, TPR and hemodynamics in rats pretreated with propranolol NA increased MAP and TPR in both groups but the increases were greater in the control than diabetic rats (Fig. 5.3-2A, C). NA did not affect CI of the control rats, but decreased CI in diabetic rats (Fig. 5.3-2B). MAP, TPR or CI responses to NA in the control rats were not affected by 1400W. In the diabetic rats, 1400W restored the TPR effect of NA (Fig. 5.3-2C), and augmented the effect of NA on MAP (Fig. 5.3-2A), but did not significantly affect NA effect on CI (Fig. 5.3-2B). NA did not significantly influence HR changes to NA in both groups of propranolol-pretreated rats (Fig. 5.3-2D).

In the control rats, NA reduced flows to the kidneys, increased flows to the brain, heart, stomach, intestine and muscle, but did not affect flows to other organs or tissues (significance not shown on the figures); none of these changes were significantly affected by pretreatment with 1400W (Fig. 5.3-1B). NA caused similar changes in regional flows in the diabetic rats relative to the controls; however, the increases in flows to the heart, stomach and intestine were less. Inhibition of iNOS by 1400W tended to reduce flows to all organs and tissue in the diabetic rats; significant decreases were obtained in the heart, stomach, intestine and colon/caecum (Fig. 5.3-1C).

When blood flow was normalized by MAP to reveal intrinsic constrictor tone, NA caused the following changes in the control rats: vasoconstriction (reduced conductance) of the kidneys, liver, colon/caecum, muscle and skin as well as vasodilatation (increased conductance) of the heart (significance not shown in figure). These changes were not affected by treatment with 1400W (Fig. 5.3-1E). In the diabetic rats, NA also caused vasoconstriction of the kidneys, liver and colon/caecum, and vasodilatation of the heart (significance not shown in figure). However, in contrast to the control rats, vasodilatation to NA also occurred in the skeletal muscle bed, and vasoconstriction in the skin was not statistically significant in the diabetic rats. Following treatment with 1400W, NA had greater vasoconstrictor influence in all organs and tissues, and significant changes were obtained in the heart, liver, stomach, intestine, colon/caecum and skin (Fig. 5.3-1F).

Table 5.3-1: Plasma glucose, body weight and organ weight of control and diabetic rats

		Control	Diabetic
Body weight (g)		440 ± 10	380 ± 10*
Plasma glucose (mM)		5.9 ± 0.4	27.3 ± 3.3*
Tissue/body Weights (g /kg)	Brain	2.7 ± 0.2	3.05 ± 0.12
	Heart	2.4 ± 0.1	2.6 ± 0.1
	Kidneys	6.2 ± 0.1	10.4 ± 0.4*
	Liver	34.3 ± 1.2	42.2 ± 0.8*
	Stomach	3.9 ± 0.1	4.3 ± 0.2
	Intestine	9.1 ± 0.4	15.76 ± 0.7*
Colon/caecum		5.7 ± 0.2	10.0 ± 0.4*

Plasma glucose, body weight and organ weight of control and diabetic rats (mean ± S.E.M; n = 6 or 7 per group) at 3 weeks following injection of streptozotocin (60 mg/ml/kg i.v.) and vehicle (0.9% NaCl), respectively. * $P < 0.05$ vs controls.

Figure 5.3-1 Baseline blood flow and conductance, effects of NA and 1400W on BF and Cond in STZ-induced diabetic rats

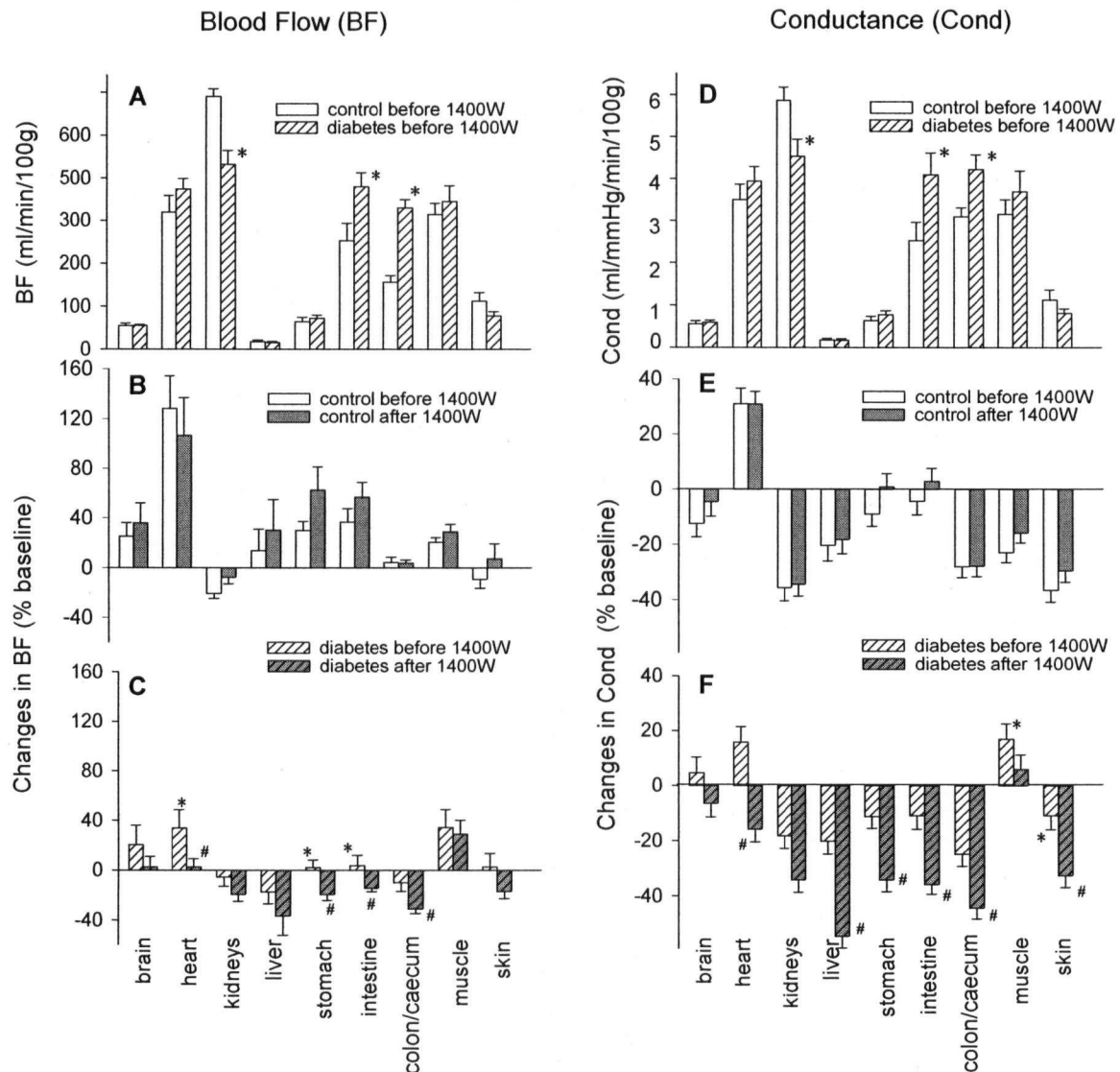


Fig. 5.3-1 Baseline blood flow (BF, A) and conductance (Cond, D), and effects of noradrenaline (NA, 16.5×10^{-9} mol/kg min) on BF and Cond prior to, and after, administration of 1400W (3 mg/kg followed by 3 mg/kg h, i.v.) in propranolol-pretreated control (B and E) and diabetic (C and F) pentobarbital-anesthetized rats at 3 weeks after i.v. injection of vehicle (0.9% NaCl) or streptozotocin, respectively. All values are mean \pm S.E.M. ($n = 6-7$ per group). Fig. 1A and D: *significantly different ($P < 0.05$) from controls; Fig. 1C and F: *significantly different ($P < 0.05$) from controls in B and E; #significantly different ($P < 0.05$) from responses before the administration of 1400W.

Figure 5.3-2 Responses to NA on MAP, HR, TPR and CI prior to, and after, treatment with 1400W

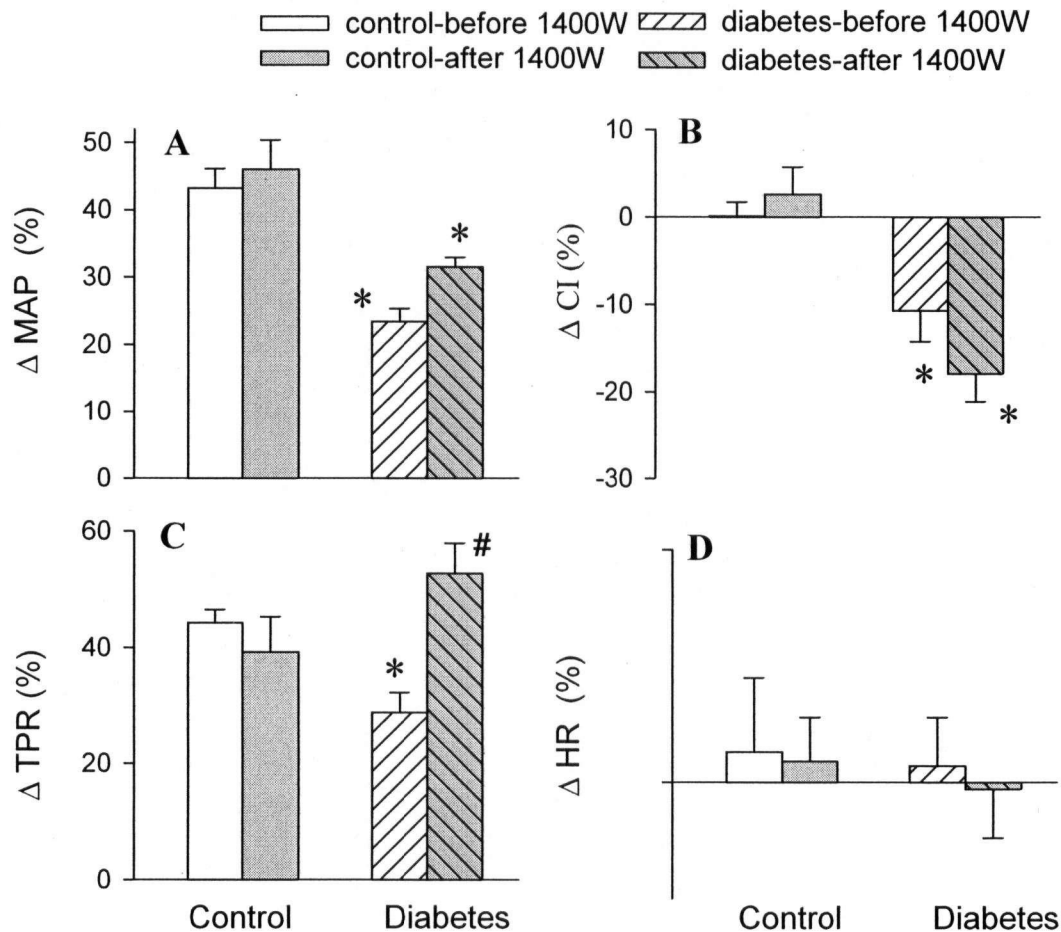


Fig. 5.3-2 Responses (%baseline, mean \pm S.E.M., $n = 6-7$) to noradrenaline (NA, 16.5×10^{-9} mol/kg min) on mean arterial pressure (Δ MAP), heart rate (Δ HR), total peripheral resistance (Δ TPR) and cardiac index (Δ CI) prior to, and after, treatment with 1400W (3 mg/kg followed by 3 mg/kg h, i.v.) in propranolol-pretreated control (clear bars) and diabetic (hatched bars) rats anesthetized with pentobarbital at 3 weeks after i.v. injection of vehicle (0.9% NaCl) or streptozotocin respectively. *Significantly different ($P < 0.05$) from responses in the control rats. #Significantly different from responses before the administration of 1400W.

5.4 **Study 4: 1400W-cardiac contractility in STZ-induced diabetic rats**

The effect of inhibition of iNOS by 1400W on cardiac contractility and contractile responses to NA at an early stage of STZ-induced diabetes in anesthetized rats

Baseline values and effects of 1400W Baseline of LVP, LV +dP/dt, LV -dP/dt and HR were significantly lower in the diabetic than control rats (Table 6.4-1). Treatment of 1400W did not affect all the baseline values in both diabetic and control groups (Table 5.4-1).

Effect of 1400W on cardiac contractile response to NA NA increased LVP, LV \pm dP/dt and HR in both the control and diabetic groups; however, the increases were markedly higher in the control than diabetic rats (Fig. 5.4-1). Treatment with 1400W did not alter responses to NA in control rats, but augmented HR and LV contractile responses to NA in the diabetic rats; however, significant increases were attained only for LVP and LV +dP/dt (Fig. 5.4-1A, B).

Activity of iNOS and eNOS and RT-PCR identification of iNOS and eNOS in the myocardium The activity of eNOS (Ca^{2+} -dependent) in the control and diabetic rats were similar in the absence or presence of 1400W (Fig. 5.4-2). In contrast, the activity of iNOS (Ca^{2+} -independent) was 3.2-fold greater in the diabetic rats relative to the controls. Treatment with 1400W did not affect the activity of iNOS in the controls but reduced iNOS activity in the diabetic rats (Fig. 5.4-2).

Fig. 5.4-3A shows representative ethidium bromide-stained gels with RT-PCR products for eNOS, iNOS and β -actin (positive control) derived from RNA, which were from the hearts of control and diabetic rats ($n = 3$ each). RT-PCR products of eNOS were identified in both groups and were of similar intensity. A product of iNOS was detected only in the diabetic rats. Both control and diabetic rats had similar ratio of intensity (optical density) of eNOS/ β -actin in the myocardium ($n = 3$; mean \pm range; Fig. 5.4-3B). However, the ratio of intensity of iNOS/ β -actin RT-PCR products in the hearts of diabetic rats were markedly higher than that of the controls ($n = 3$; mean \pm range; Fig. 5.4-3B).

Immunostaining of NT, iNOS and eNOS In the absence of the primary antibody, no immunostaining was apparent (Fig. 5.4-4, A4 and B4). Immunostainings for eNOS (dark red dots, indicated by arrows) were present in the control and diabetic groups, and were of similar intensity (Fig. 5.4-4, A1 and B1). In contrast, immunostaining for iNOS and NT were clearly identified in the diabetic rats (Fig. 5.3-4, A2 and A3), and not the control rats (Fig. 5.4-4, B2 and B3).

Table 5.4-1 Baselines values of LVP and \pm dP/dt before and after administration of 1400W

	+dP/dt (mmHg/sec)	-dP/dt (mmHg/sec)	LVSP (mmHg)	HR (beats/min)
Control rats				
Before 1400W	11400 \pm 575	9651 \pm 370	138 \pm 1	363 \pm 7
After 1400W	11244 \pm 517	9589 \pm 387	137 \pm 1	359 \pm 6
Diabetes rats				
Before 1400W	9526 \pm 243*	8074 \pm 210*	128 \pm 3*	313 \pm 9*
After 1400W	9660 \pm 329*	8230 \pm 276*	128 \pm 2*	308 \pm 9*

Baselines values (mean \pm S.E.M) of left ventricular pressure (LVP) and maximal rate of increase (+dP/dt) and decrease (-dP/dt) of LVP before and after administration of 1400W in pentobarbital-anesthetized control and diabetic rats. The rats were not pretreated with propranolol. n=6, *P < 0.05 vs controls.

Figure 5.4-1 Responses to NA on changes in LVP, LV +dP/dt, LV -dP/dt, and HR prior to and after treatment with 1400W

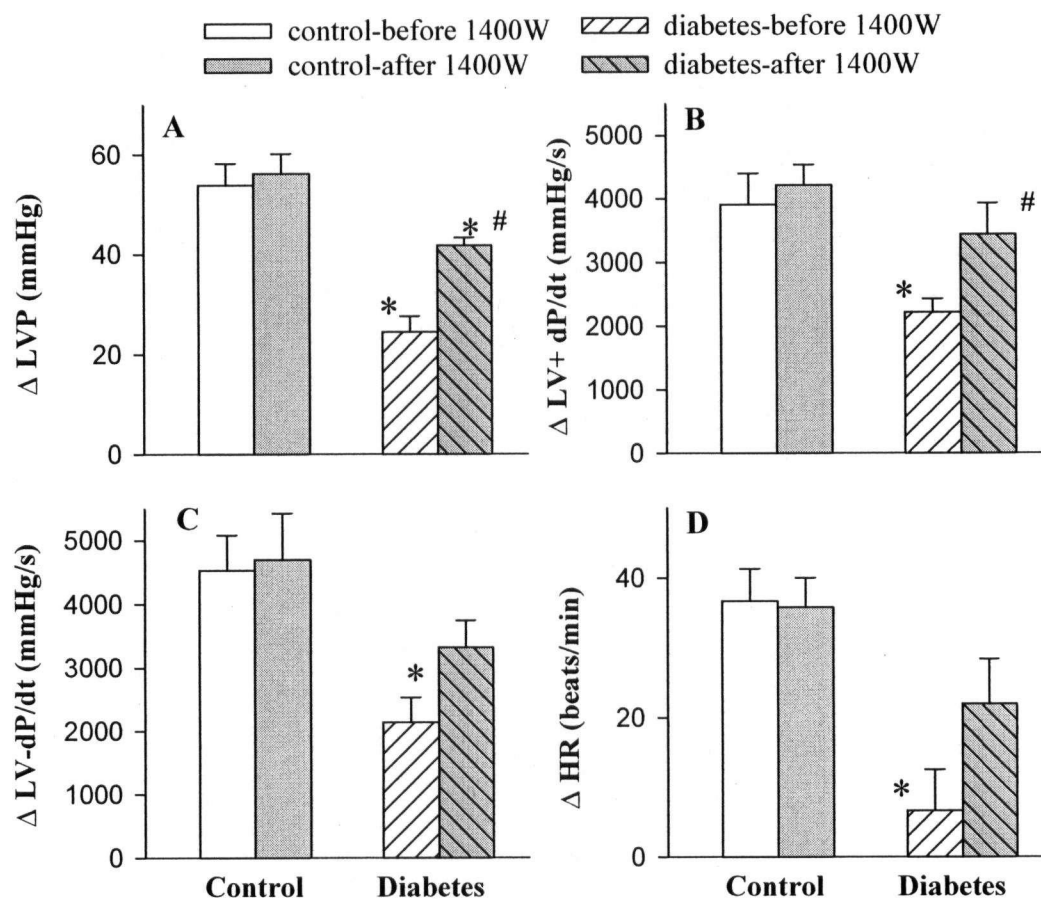


Fig. 5.4-1 Responses (mean \pm S.E.M, $n = 6-7$ per group) to noradrenaline (NA, 16.5×10^{-9} mol/kg min) on left ventricular (LV) systolic pressure (Δ LVP), maximal rate of rise (Δ LV +dP/dt) and fall (Δ LV -dP/dt) of LV pressure, and heart rate (Δ HR) prior to, and after, treatment with 1400W (3 mg/kg followed by 3 mg/kg h, i.v.) in control (clear bars) and diabetic (hatched bars) pentobarbital-anesthetized rats at 3 weeks after i.v. injection of vehicle (0.9% NaCl) or streptozotocin respectively. *Significantly different ($P < 0.05$) from responses in the control rats. #Significantly different from responses before the administration of 1400W.

Figure 5.4-2 Activities of iNOS and cNOS in the myocardium of control and diabetic rats prior to and after treatment with 1400W

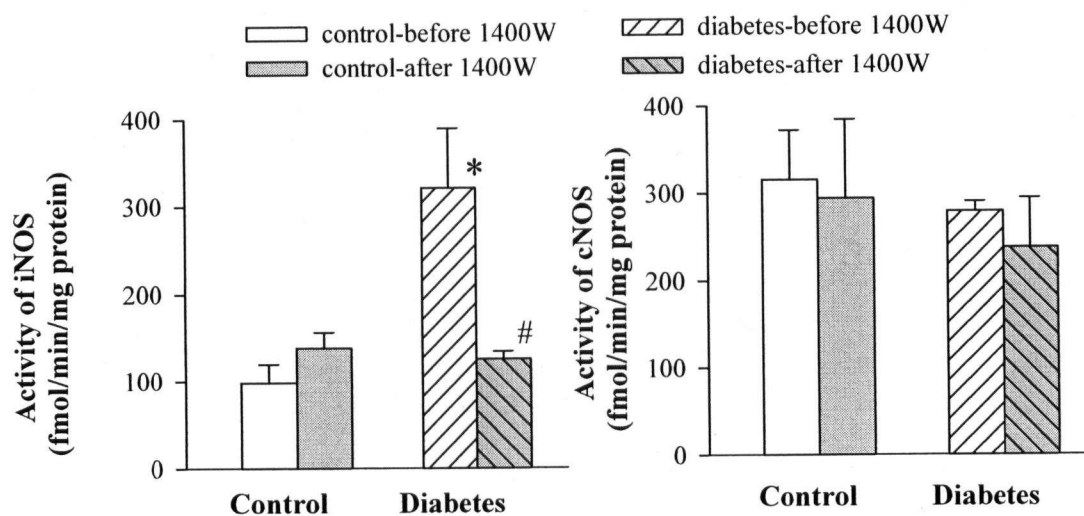


Fig. 5.4-2 Activities of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide (eNOS) in the myocardium of control and diabetic rats ($n = 6$ per group) before and after treatment with 1400W (3 mg/kg followed by 3 mg/kg h, i.v.). *Significantly different ($P < 0.05$) from the control. #Significantly different from value prior to the administration of 1400W.

Figure 5.4-3 RT-PCR products of iNOS, eNOS & intensity ratio of iNOS/ β -actin and eNOS/ β -actin RT-PCR products in the myocardium

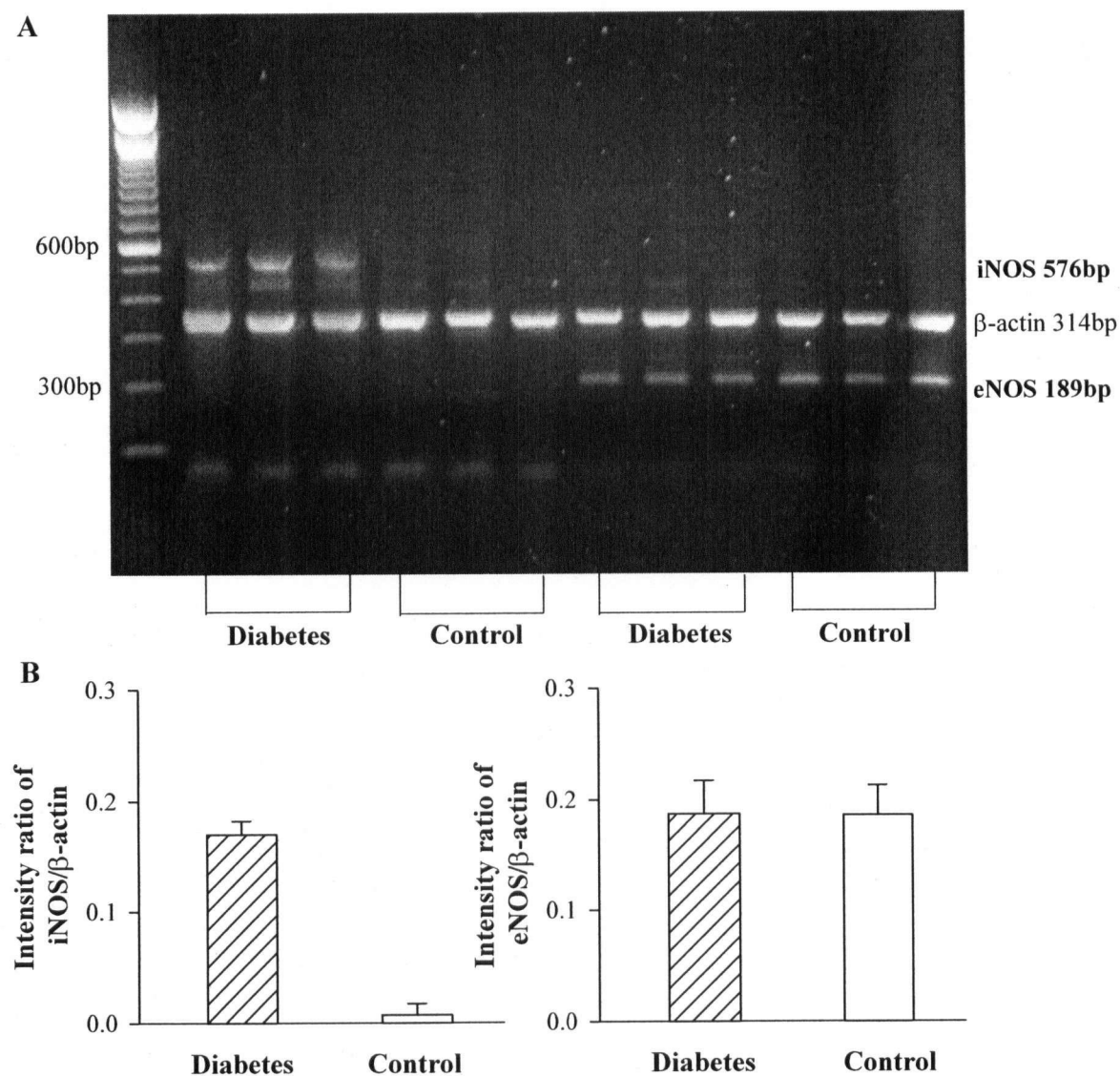


Fig. 5.4-3 **A:** An ethidium bromide-stained gel showing RT-PCR products of iNOS, eNOS and β -actin from RNA derived from the myocardium of three control and three diabetic rats; **B:** Intensity ratio of iNOS/ β -actin and eNOS/ β -actin RT-PCR products in the myocardium of control and diabetic rats at 3 weeks after i.v. injection of vehicle (0.9% NaCl) and streptozotocin respectively, $n=3$, mean \pm range.

Figure 5.4-4 Immunostaining of eNOS, iNOS and NT in the myocardium of diabetic and control rats

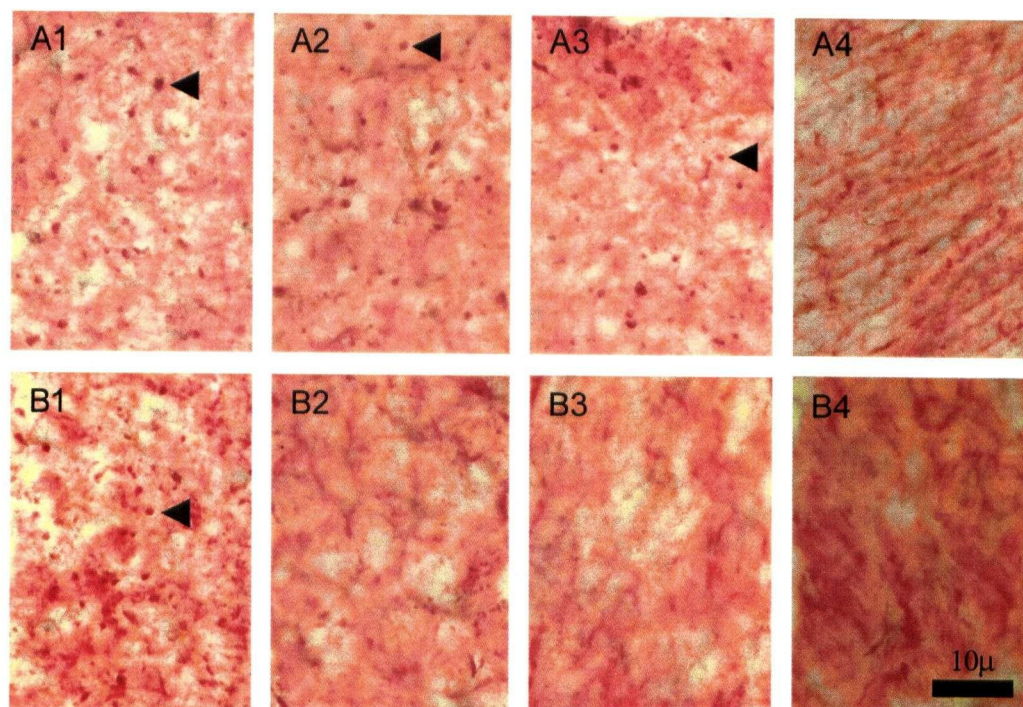


Fig. 5.4-4 Immunostaining (dark red dots, indicated by arrows) of endothelial nitric oxide synthase (eNOS, A1 and B1), nitrotyrosine (NT, A2 and B2), inducible nitric oxide synthase (iNOS, A3 and B3), and negative control (A4 and B4) in the myocardium of a representative diabetic rat (A1 to A4) and control rat (B1 to B4) at 3 weeks after i.v. injection of streptozotocin or vehicle (0.9% NaCl), respectively. The negative control involved staining with mouse nonspecific immunoglobulin G instead of the primary antibody. (Magnification: 100×)

5.5 Study 5: NAC-cardiac contractility in STZ-induced diabetic rats

The effect of the antioxidant NAC on cardiac contractility and contractile responses to dobutamine as well as on activation of iNOS, formation of peroxynitrite, and myocardium antioxidative capacity at an early stage of anesthetized rats with STZ-induced diabetes

Baseline values and effects of NAC in control and diabetic rats At 48 h after injection of STZ, the rats had a higher plasma concentration of glucose compared to that of control rats (23.5 ± 1.5 versus 5.7 ± 0.8 mM, $P < 0.05$, $n = 14-15$). At three weeks after injection of STZ, the body weight of the diabetic rats was lower while plasma glucose level was higher than those of the controls (Table 5.5-1). Baseline HR, LV +dP/dt and LV -dP/dt were significantly ($P < 0.05$) lower, but LVP was insignificantly lower in the diabetic relative to the control rats (Table 1).

Administration of NAC did not significantly alter any baseline readings in the control rats. NAC did not prevent changes in the body weight, plasma glucose and HR in diabetic rats, but it partially restored LV +dP/dt ($P > 0.05$) and LV -dP/dt ($P < 0.05$) in the diabetic rats (Table 5.5-1).

Cardiac contraction to dobutamine in control and diabetic rats Dobutamine caused dose-dependent increases in LVP, LV \pm dP/dt and HR in control and diabetic rats not treated with NAC; the increases were significantly greater in the control than diabetic rats (Fig. 5.5-1). Relative to the controls, the diabetic rats had significantly higher ED₅₀ for LVP (3.0-fold), +dP/dt (2.9-fold), -dP/dt (2.4-fold) and HR (3.9-fold) responses to dobutamine (Fig. 5.5-2), and significantly lower E_{max} for LVSP (60% of controls), +dP/dt (50%), -dP/dt (53%) and HR (69%) responses to dobutamine (Fig. 5.5-3)

Treatment with NAC did not significantly alter cardiac contractile responses to dobutamine in the control rats, but increased contractile and HR responses to dobutamine (Fig. 5.5-1) such that ED₅₀ of LVP, LV \pm dP/dt and HR responses to dobutamine were normalized relative to the corresponding readings in the control rats (Fig. 5.5-2). NAC also significantly augmented maximum LVP and LVP +dP/dt responses to dobutamine, and insignificantly increased maximum -dP/dt and HR responses to dobutamine in the diabetic rats (Fig. 5.5-3). The readings of -dP/dt and HR responses to dobutamine in the NAC-treated diabetic rats were not significantly different from those of the control rats.

Immunohistochemistry of NT, iNOS and eNOS in the heart. In the absence of primary antibodies, no immunostaining was apparent in any groups (Fig. 5.5-4, negative control).

Immunostainings of eNOS (dark red dots, indicated by arrows) were of similar intensity in all four groups (Fig. 5.5-4). In contrast, immunostainings for NT and iNOS were clearly identified in the diabetic but not control nor control + NAC groups, and were infrequently seen in the diabetes + NAC group (Fig. 5.5-4, NT and iNOS).

Myocardial level of 15-F_{2t}-isoprostane The level of 15-F_{2t}-isoprostane in the heart of the control rats was similar to that of the diabetic rats. The level of 15-F_{2t}-isoprostane was not altered by treatment with NAC in the control rats, but was markedly reduced to $\approx 50\%$ in the diabetic rats (Fig. 5.5-5A).

Myocardium antioxidant capacity Myocardial antioxidant capacity (TBARS formation following peroxidation challenge) was similar in the control and diabetic rats at the lowest (0.5 mM) and highest (5 mM) concentrations of t-BHP (Fig. 5.5-5B). Antioxidant capacity was lower (increased TBARS formation) in the diabetic than control group at 1 or 2 mM of t-BHP concentrations that provide a sensitive measure of rat tissue TBARS (Runzer et al, 2002). This optimal concentration of t-BHP is sufficiently high to allow the detection of adequate levels of TBARS, but is sufficiently low to avoid non-specific bleaching of the color produced by the thiobarbituric acid reaction which occurs at 5 mM of t-BHP (Xia et al., 2003).

Table 5.5-1 Plasma glucose, body weight and baseline values of MAP, HR, LVP and \pm dP/dt

	Control	Control+NAC	Diabetes	Diabetes+NAC
Body weight (g)	432 \pm 8	440 \pm 6	373 \pm 9*	362 \pm 15*
Plasma glucose (mM)	6.1 \pm 0.3	6.2 \pm 0.4	28.5 \pm 1.2*	27.0 \pm 2.9*
MAP (mmHg)	102 \pm 4	102 \pm 2	99 \pm 3	106 \pm 5
HR (beats/min)	347 \pm 8	348 \pm 6	316 \pm 9*	315 \pm 7*
LVP (mmHg)	135 \pm 3	138 \pm 2	128 \pm 3	134 \pm 4
+dP/dt (mmHg/sec)	11655 \pm 421	11978 \pm 388	9729 \pm 371*	11096 \pm 202
-dP/dt (mmHg/sec)	10638 \pm 378	11054 \pm 339	8335 \pm 210*	9935 \pm 141 [#]

Plasma glucose, body weight and baseline values of mean arterial pressure (MAP), heart rate (HR), maximal left ventricular systolic pressure (LVP), maximal rate of increase (+dP/dt) and decrease (-dP/dt) of LVP in four groups of pentobarbital-anesthetized rats at 3 weeks following injection of streptozotocin (60 mg/ml/kg i.v., diabetic) or vehicle (0.9% NaCl, control), either with or without administration of N-acetylcysteine (NAC, 1.2 \pm 0.1 g/day) in the drinking water.

n = 6-7 per group. * P < 0.05 vs corresponding values in the untreated control group; [#] P < 0.05 vs corresponding values in the diabetic untreated group.

Figure 5.5-1 Effects of chronic treatment of N-acetylcysteine on actions of dobutamine on LV $\pm dP/dt$, LVP and HR

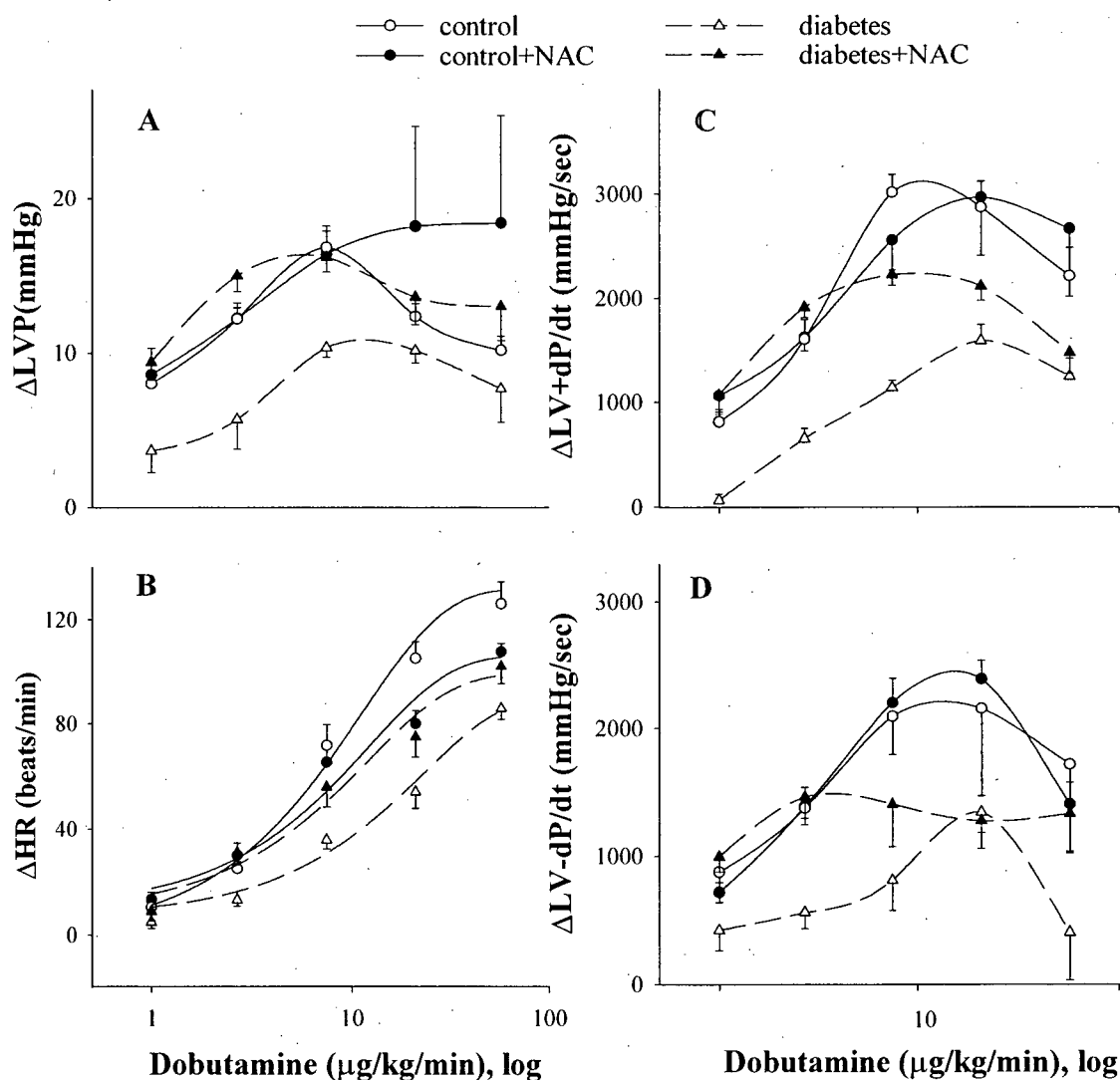


Fig. 5.5-1 Effects (mean \pm SEM, $n = 6-7$ each) of chronic treatment of N-acetylcysteine (NAC, 1.23 g/kg per day) on actions of dobutamine (1-57 $\mu\text{g/kg/min}$) on left ventricular (LV) pressure (LVP, A), heart rate (ΔHR , B), LV maximal rate of rise ($+dP/dt$, C) and fall ($-dP/dt$, D) of pressure in pentobarbital-anesthetized control rats and rats with streptozotocin (60 mg/kg, i.v.)-induced diabetes.

Figure 5.5-2 Effects of chronic treatment of N-acetylcysteine on potency (ED_{50}) of response to dobutamine on $LV \pm dP/dt$, LVP and HR

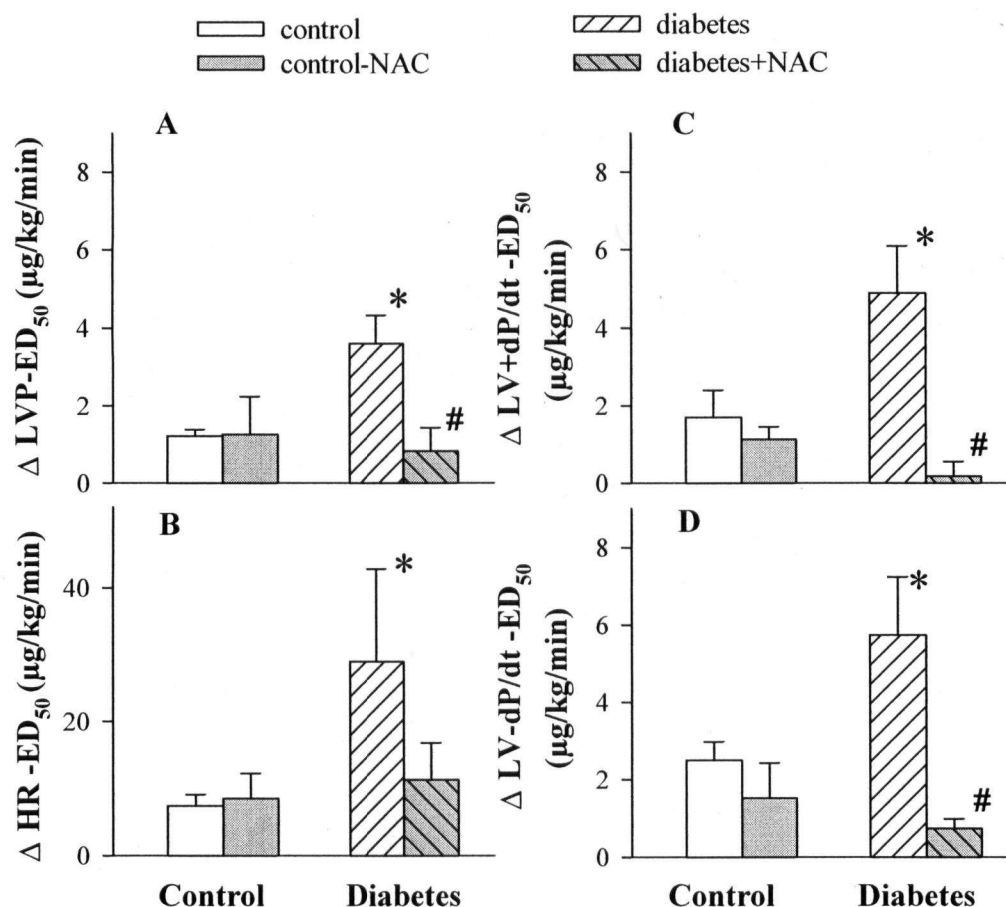


Fig. 5.5-2 Effects (mean \pm SEM, $n = 6-7$ each) of chronic treatment of N-acetylcysteine (NAC, 1.2 g/kg per day) on potency (ED_{50}) of response to dobutamine (1-57 $\mu g/kg/min$) on left ventricular (LV) pressure (LVP, A), heart rate (ΔHR , B), LV maximal rate of rise (+ dP/dt , C) and fall ($-dP/dt$, D) of pressure in pentobarbital-anesthetized control rats and rats with streptozotocin (60 mg/kg, i.v.)-induced diabetes. *Significantly different ($P < 0.05$) from untreated control rats. #Significantly different from untreated diabetic rats.

Figure 5.5-3 Effects of chronic treatment of N-acetylcysteine on maximum effects (E_{\max}) of response to dobutamine on $LV \pm dP/dt$, LVP and HR

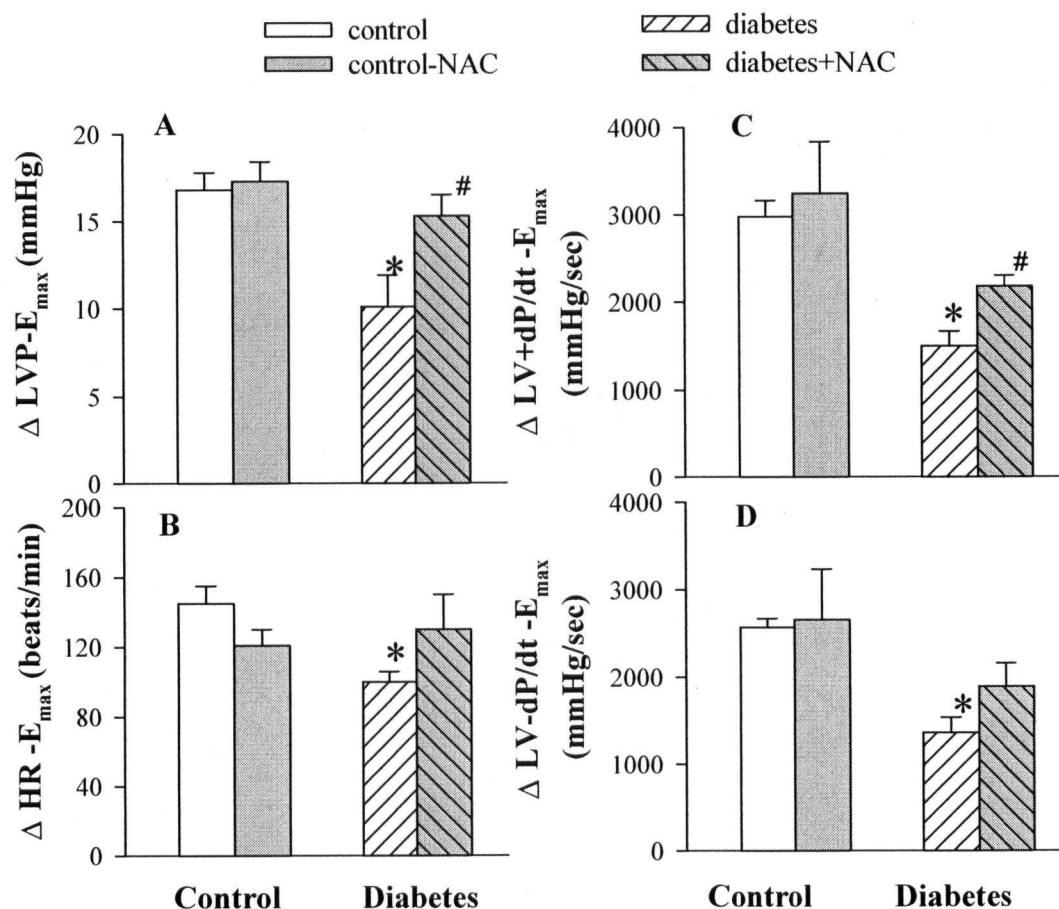


Fig. 5.5-3 Effects (mean \pm SEM, $n = 6-7$ each) of chronic treatment of N-acetylcysteine (NAC, 1.23 g/kg per day) on maximum effects (E_{\max}) of dobutamine (1-57 μ g/kg/min) on left ventricular (LV) pressure (LVP, A), heart rate (ΔHR , B), LV maximal rate of rise (+dP/dt, C) and fall (-dP/dt, D) of pressure in pentobarbital-anesthetized control rats and rats with streptozotocin (60 mg/kg, i.v.)-induced diabetes. *Significantly different ($P < 0.05$) from untreated control rats. #Significantly different from untreated diabetic rats.

Figure 5.5-4 Effects of chronic treatment of N-acetylcysteine on immunohistochemistry of NT, iNOS, and eNOS in myocardial tissues

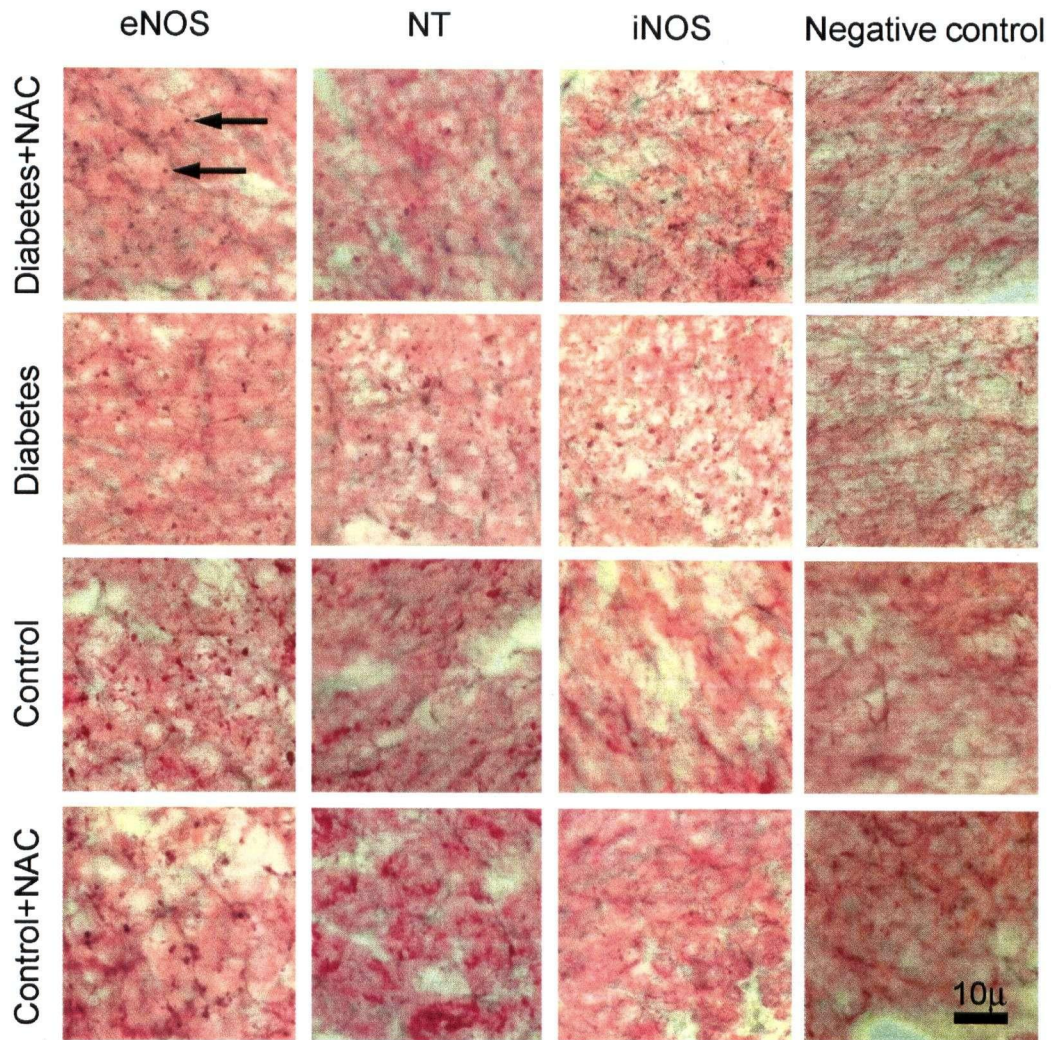


Fig. 5.5-4 Effect of chronic treatment with N-acetylcysteine (NAC, 1.2 g/kg per day) on immunohistochemistry of nitrotyrosine (NT), inducible (iNOS), endothelial (eNOS) nitric oxide synthase in myocardial tissues from control and diabetic rats i.v. injected with saline (0.9% NaCl) or streptozotocin (STZ, 60 mg/kg), respectively. Immunostaining was performed using anti-NT, anti-iNOS, or anti-eNOS antibodies at 3 weeks after i.v. injection of STZ or saline (n = 3). The negative control involved staining with mouse nonspecific immunoglobulin G instead of the primary antibody. Magnification: 100×

Figure 5.5-5 Effects of chronic treatment of N-acetylcysteine myocardial 15-F_{2t}-isoprostane level and TBARS formation

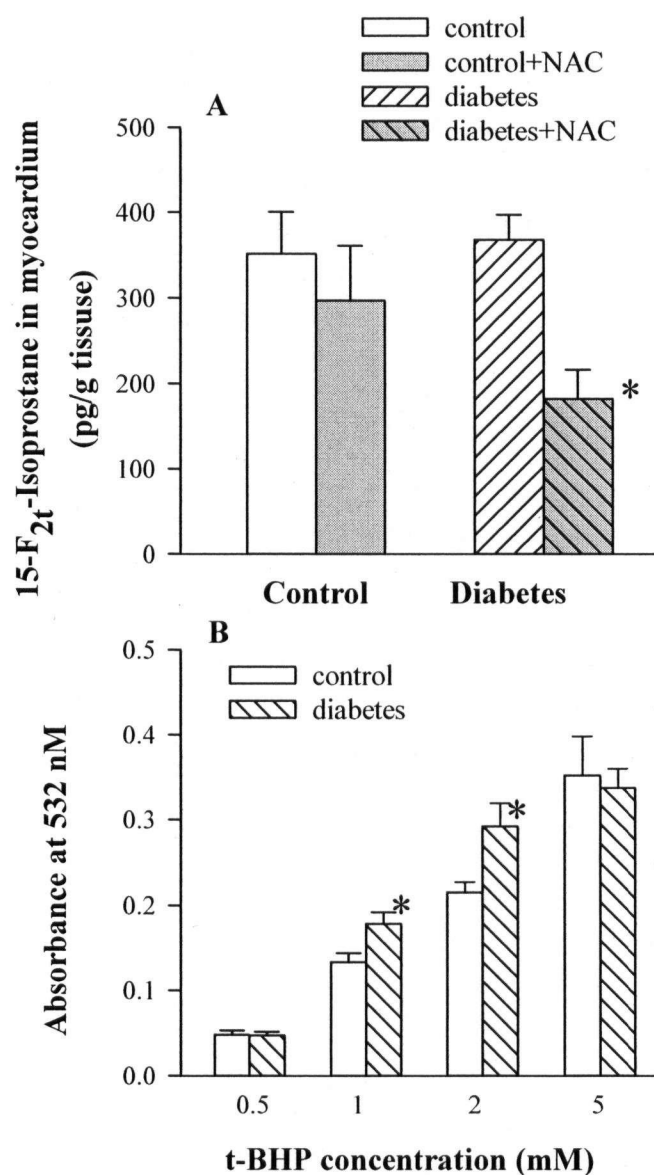


Fig. 5.5-5 **A)** Effects of chronic treatment with N-acetylcysteine (NAC, 1.23 g/kg/day) on myocardial 15-F_{2t}-isoprostane level in control rats and STZ (60 mg/kg, i.v.)-diabetic rats. **B)** Thiobarbituric acid-reactive substance (TBARS) formation (as reflected by absorbance at 532 nm) as a function of *t*-butylhydroperoxide (*t*-BHP) concentration in myocardium of control and diabetic rats. *Significantly different ($P < 0.05$) between diabetes vs control group, (n = 6-8).

6. DISCUSSION I: Selective inhibition of iNOS by 1400W in endotoxemic rats

6.1 Discussion

The first study examined the effects of 1400W, relative to those of L-NNA, on cardiac output and regional BF in normal as well as endotoxemic rats.

The results show that the dose of 1400W used appeared to be functionally selective for iNOS, as it prevented the further decrease in mean arterial pressure in the endotoxemic rats, but did not alter mean arterial pressure, cardiac output, total peripheral resistance and regional blood flow and conductance in normal rats. In contrast to 1400W, L-NNA increased mean arterial pressure and total peripheral resistance, reduced cardiac output, and decreased flow and conductance of the heart, brain, liver, kidneys, stomach, intestine, skeletal muscle and skin in normal rats. The cardiovascular effects of L-NNA in this study are similar to those of L-NAME (Wang et al., 1995).

To examine the ability of 1400W and L-NNA to restore mean arterial pressure and cardiac output in endotoxemic shock, the inhibitors were administered at 2.5 h after injection of LPS. It has been shown that hypotension and vascular hypo-reactivity occurring at the early phase (1-2 h) of endotoxemia are associated with the activation of eNOS, whereas those at the late phase (after 2-3 h) are related to the activation of iNOS (Bateson et al., 1996; Boughton-Smith et al., 1993; Szabo et al., 1993). Wray *et al.* (1998) reported that the administration of a high dose of 1400W (10 mg/kg, i.v. bolus followed by 10 mg/kg/h) into anesthetized rats, initiated at 30 min before LPS-challenge, attenuated the delayed (3-6 h after LPS) but not early (<2 h after LPS) phase of hypotension. In consideration of the involvement of iNOS at the late phase of endotoxemia, the NOS inhibitors were administered at 2.5 h after injection of LPS, just prior to the second phase decline of mean arterial pressure.

The injection of LPS caused progressive decreases in mean arterial pressure as well as cardiac output, and an increase in total peripheral resistance at 2.5 and 4 h. This condition resembles the late stage of human septic shock when a hyperdynamic (low total peripheral resistance and high cardiac output) turns into a hypodynamic (high total peripheral resistance and low cardiac output) state (Colardyn et al., 1989; Schumer, 1984). In this study, we did not investigate the mechanism by which LPS increased total peripheral resistance. There is, however, substantial evidence that septic shock is associated with marked increases in the plasma concentrations of vasoconstrictor agents such as angiotensin II, vasopressin as well as endothelin-1, which function together to help maintain vascular resistance in an attempt to

oppose hypotension (e.g., Brackett, 1985; Schaller et al., 1985; Gardner et al., 1995, 1996; Sharma et al., 1997; Mitaka et al., 1999).

The reductions in mean arterial pressure at 2.5 and 4 h after injection of LPS in the present study were due to an increase in total peripheral resistance, which was primarily a result of increased vasoconstriction and reduced flows in the stomach, skeletal muscle and skin. Flow and conductance to the kidneys were insignificantly decreased, while flow and conductance of the heart, brain and intestine were not affected at this time. Reduced flows to the skin, skeletal muscle, intestine and stomach have been reported in anesthetized rats at 1 h after injection of endotoxin (Mulder et al., 1996). LPS did not alter hepatic arterial flow at 2.5 h in this study; however, it slightly (insignificantly) increased hepatic arterial conductance. Increased hepatic arterial flow in endotoxemia has been shown (Mulder et al., 1996). Winslow and Dorinsky (Winslow and Dorinsky, 1994) reported that flows to the brain and kidneys were unaffected by endotoxin, whereas flows to the splanchnic organs and skeletal muscle were markedly reduced in dogs at 3 h after the onset of endotoxemia. Lambalgen *et al.* (1993) reported that endotoxin challenge for 2 h in rats did not affect flows to the heart and brain, but decreased flows to the kidneys, skin and skeletal muscle. At 4 h after the injection of LPS in this study, flows to the heart, kidneys, stomach, skeletal muscle and skin were decreased relative to the corresponding baseline readings at 0 h. When blood flow was normalized by mean arterial pressure to reveal intrinsic vascular tone, LPS was found to increase arterial conductance of the heart, brain and liver, and to decrease conductance of the stomach, skeletal muscle and skin.

The administration of 1400W at 2.5 h after injection of LPS prevented the second phase decrease in mean arterial pressure, which is consistent with the results of Wray and Hamilton (Hamilton and Warner, 1998; Wray et al., 1998). Compound 1400W, however, did not restore cardiac output, and further increased total peripheral resistance. Although arterial conductance in several beds (e.g., heart, brain, liver, intestine and skeletal muscle) was slightly reduced by 1400W, relative to the values obtained in saline-treated endotoxemic rats, none of the readings reached statistical significance.

Several groups have examined the effect of 1400W on LPS-induced organ injury. Garvey *et al.* (1997) found that 1400W (1 mg/kg), given concurrently with LPS (3 mg/kg i.v.), inhibited vascular [125 I]albumin leakage into the ileum, colon, lungs, liver and heart completely and the kidneys by $\approx 50\%$ after 5 h. Moreover, when injected at 3 h after LPS, 1400W abolished vascular leakage in the ileum. Compound 1400W (0.2 and 1 mg/kg, s.c.) injected

concurrently with LPS (3 mg/kg i.v.) was also shown to dose-dependently prevent albumin leakage in the heart, kidneys, liver, lungs (Whittle et al., 2001) and the stomach and duodenum (Kiss et al., 2001) at 4 h. Furthermore, pretreatment of rats with 1400W (5 mg/kg, s.c.) reduced cellular damage to the colon at 2 h after injection of LPS (3 mg/kg, i.p.) (Tepperman et al., 2000). These results show that 1400W has a protective effect against vascular injury. In contrast, Wray (1998) found that 1400W (10 mg/kg i.v.), administered either before, or at 2 h after the administration of LPS (6 mg/kg, i.v.), did not attenuate injury to the liver, kidneys or pancreas.

In contrast to 1400 W, L-NNA did not prevent the secondary phase of decrease in mean arterial pressure, and it caused a marked increase in total peripheral resistance, and this further reduced cardiac output to approximately half that of the vehicle-treated endotoxemic rats. Moreover, L-NNA increased vasoconstriction in the heart, brain, kidneys, intestine and skeletal muscle significantly, and stomach insignificantly, at the late phase of endotoxemia. Thus, non-selective inhibition of NOS drastically compromised blood flow by increasing vasoconstriction in almost all organs and tissues (except the liver), and this critically reduced cardiac output. The lack of effect of L-NNA on hepatic arterial conductance suggests that NO is not an important regulator of hepatic arterial tone. Indeed, (Lautt et al., 1985) has shown that hepatic arterial flow is largely regulated by the concentration of adenosine within the space of Mall in the liver through a mechanism termed "the hepatic arterial buffer response", in which a decrease in portal venous flow leads to reduced washout of local adenosine, and the subsequent accumulation of adenosine in the liver dilates the hepatic artery.

Therefore, 1400W prevented LPS-induced decrease in blood pressure, and this was via an increase in vascular resistance which decreased cardiac output by 33%. The further reduction of cardiac output is undesirable, however, the maintenance of mean arterial pressure in shock is also of importance, since glomerular filtration ceases below a critical renal perfusion pressure of 45 mmHg. Although L-NNA, by blocking the activities of inducible as well as constitutive iNOS, was also able to increase vascular resistance and mean arterial pressure, its vasoconstrictor action was too drastic, since cardiac output was decreased by 64%. Our results have shown that it might be more rational to administer 1400W than L-NNA in septic shock, because the blockade of the activities of iNOS, eNOS and nNOS by L-NNA exacerbated vasoconstriction and further reduced organ perfusion and cardiac output. Indeed, cardiac output in the 1400W-treated rats with sepsis was similar to that of the vehicle-treated rats with

sepsis (despite the increase in total peripheral resistance), and almost twice that of the rats treated with L-NNA.

6.2 Summary and conclusion

To summarize, our results show that selective inhibition of NOS by 1400W restored mean arterial pressure without compromising cardiac output and tissue perfusion, whereas non-selective inhibition of NOS by L-NNA did not restore mean arterial pressure and exacerbated the decrease in cardiac output and tissue perfusion. Therefore, highly selective inhibitors of NOS are useful in the restoration of mean arterial pressure at the late stage of septic shock, whereas non-selective NOS inhibitors are detrimental at this time.

7. DISCUSSION II: Effects of 1400W and N-acetylcysteine in STZ-induced diabetic rats

7.1 Effects of STZ-induced diabetes on arterial and venous constrictions as well as hemodynamic response to noradrenaline in conscio mean arterial pressure us and anesthetized rats

The results showed that rats at an early phase (two or three weeks) of STZ (60 mg/kg)-induced Type 1 diabetes and the age-matched control rats had similar mean arterial pressure and mean circulatory filling pressure. The systolic and diastolic blood pressures of the diabetic and control rats were also similar (results not shown). Heart rate decreased insignificantly at two weeks in conscious rats and significantly at three weeks after injection of STZ in both the conscious and anesthetized rats. Both the diabetic and control rats had dose-dependent bradycardic response to noradrenaline, which was likely due to reflex parasympathetic activation and not sympathetic withdrawal, since the rats were pretreated with propranolol to block the chronotropic and inotropic (β -adrenoceptor mediated) effects of noradrenaline. It is of interest that rats at the early phase of STZ (60 mg/kg)-induced diabetes have been shown to have similar, slightly reduced, or slightly increased blood pressure relative to the age-matched controls. For example, mean arterial pressure of conscious rats with STZ-induced diabetes for four to six weeks had mean arterial pressure similar to (Yu and McNeill, 1992) or lower (Jackson and Carrier, 1983) than the pressure of controls. Mean arterial pressure was also reported to decrease significantly from 106 ± 3 mmHg to 89 ± 2 mmHg in one week STZ-diabetic rats, along with deficient aortic Ca uptake and contraction to noradrenaline ex vivo (Rebolledo et al., 2001). The systolic blood pressure and heart rate of conscious rats with STZ-induced diabetes for three to six weeks were however, slightly lower than those of age-matched controls (Hebden et al., 1987; Yu and McNeill, 1992). In contrast, the conscious rats with STZ-induced diabetes for four weeks had higher mean arterial pressure relative to that of the controls (Hayashi et al., 1982). Litwin et al. (1991) had shown that thiobutabarbitione-anesthetised rats (220-250 g) with STZ-induced diabetes for four weeks had similar mean arterial pressure, heart rate and mean circulatory filling pressure relative to those of age-matched controls. Recently Tatchum-Talom et al. reported that BP was decreased in four weeks STZ-induced diabetic rats when pressure was recorded with radiotelemetry, and this may be related to a nonspecific decrease in responsiveness to vasoconstrictor stimuli, such as noradrenaline and phenylephrine (Tatchum-Talom et al., 2000). The small differences in

baseline mean arterial pressure between controls and diabetic rats among the various studies are likely due to the use of different strain or age of rats and/or varying experimental conditions.

The diabetic rats in the present study had reduced potency (higher ED_{50}) of mean arterial pressure and mean circulatory filling pressure responses to infused noradrenaline, as well as reduced efficacy (E_{max}) of mean arterial pressure response to noradrenaline. These results reflect reduced α -adrenoceptor mediated vasoconstriction, since the rats were pretreated with propranolol to block the chronotropic and inotropic effects of β -adrenoceptor activation by noradrenaline. Indeed, baseline heart rate in both the diabetic and control rats were decreased, to a similar extent, by pre-treatment with propranolol. Furthermore, both groups had similar dose-dependent bradycardic responses to noradrenaline, which were likely due to pressor response-induced parasympathetic activation (and not sympathetic withdrawal, since the rats were pretreated with propranolol). Reduced pressor response to noradrenaline had been reported in pithed rats at two weeks following induction with STZ (Foy and Lucas, 1976; Lucas, 1985), in conscious rats at four to five weeks (Jackson and Carrier, 1983), and six weeks (Yu and McNeill, 1992) following induction with STZ; however, unlike the present study, these rats were not pretreated with a β -adrenoceptor antagonist. Our results further showed that along with reduced potency as well as efficacy of the pressor effect of noradrenaline, there was reduced potency of mean circulatory filling pressure response to noradrenaline. The latter suggested reduced sensitivity of venoconstriction to noradrenaline, since mean circulatory filling pressure reflects total body venous tone (Pang, 2000). Furthermore, mean circulatory filling pressure has been shown to be proportional to venous return, and is an important determinant of cardiac output (Guyton et al., 1954). Our results therefore suggested that not only was constriction of arterial resistance vessels reduced, but constriction of capacitance vessels to α -adrenoceptor activation was also impaired as early as two weeks after the onset of diabetes. The mechanism responsible for reduced α -adrenoceptor response is unclear since no measurement of neuronal noradrenaline uptake or release was made. It is of interest that autonomic neuropathy was not yet detected in rats at 3.5 months, but existed at six months, after injection of STZ (Schmidt and Scharp, 1982).

Diabetic status did not significantly affect the cardiac output (or cardiac index if body weight was taken into account), which agreed with the results of Lucas and Hill obtained in pithed or anesthetized rats with STZ-induced diabetes (Hill and Larkins, 1989). These results (Lucas, 1985) disagreed with those of Brands et al (2001) in conscious rats, which showed that

cardiac output was decreased to 85% of the baseline by day seven after the injection of STZ (Brands et al., 2000). The Brands et al (2000) study was conducted soon after injecting STZ; however it takes several weeks after STZ treatment before fluid intake and output reach a new steady state. Herden et al. had reported that there were no signs of hypovolemia, and that fluid and electrolyte balance was reached at three weeks after injection of STZ (Hebden et al., 1986). Also, total peripheral resistance has a tendency to decrease (0.99 vs 0.89 mmHg.min/ml, $P > 0.05$, in controls and diabetes) in the diabetic rats of the present study. Therefore different results of cardiac output among the various studies could be a consequence of duration of diabetes.

The diabetic rats in the present study had generalized depression of contractile function in the cardiovascular system, which was evident upon exogenous challenge with noradrenaline and angiotensin II. Specifically, noradrenaline caused less increases in mean arterial pressure and total peripheral resistance in the diabetic rats relative to the control rats. This was associated with the attenuations of α -adrenoceptor-mediated increase in total peripheral resistance. Noradrenaline decreased cardiac index in the diabetic but not control rats. Reduced cardiac index response to noradrenaline in the diabetic rats was due to decreased stroke volume since heart rate was unchanged. Reduced stroke volume in the diabetic rats, on the other hand, was likely due to attenuated venoconstriction to noradrenaline (since reduced arteriolar resistance would lead to an increase in cardiac index). Indeed, we had shown that rats with STZ-induced diabetes, relative to the controls, had reduced *in vivo* venoconstrictor response to noradrenaline. Depressed pressor response to noradrenaline has been reported in rats treated with STZ for two weeks (Cheng et al., 2003b) (Foy and Lucas, 1976; Lucas, 1985; Yu and McNeill, 1992) or four to six weeks (Jackson and Carrier, 1983). The increases of blood flow and conductance in the intestine and colon of the diabetic rats were in general agreement with the results of Lukas (1985) and Hill and Larkins (1989) at two and four weeks after the injection of STZ. Although the conductance in kidneys when normalized by weight was decreased, the conductance to the whole kidney did not change (diabetes, 0.17 ± 0.01 vs controls: 0.16 ± 0.01 ml/mmHg/min/rat) since the kidneys were enlarged in diabetics.

Noradrenaline caused vasoconstriction (reduced arterial conductance) in the kidneys, liver and colon/caecum, and vasodilatation (increased conductance) in the heart in both the control and diabetic rats. Relative to the controls, noradrenaline-mediated increases of blood flow and conductance were significantly lower in the heart of the diabetic rats. Conductance in

the skeletal muscle bed was, however, reduced by noradrenaline in the control rats but increased by noradrenaline in the diabetic rats, and was reduced significantly more in the skin of the control rats. The mechanism of varying responses to noradrenaline in the heart and skeletal muscle beds is elusive, and is unlikely due to the activation of β -adrenoceptors in the diabetic rats, since the rats were pretreated with propranolol.

The mass of kidneys, liver, intestine and colon increased significantly in diabetes compared to controls which is consistent with those of another report (Hill and Larkins, 1989). These differences can be explained by the fact that diabetic rats eat and drink substantially more than normal rats, and therefore have marked intestinal hyperplasia and enlarged kidneys and liver to adapt to the metabolic disorder.

To summarize, the propranolol-pretreated rats with STZ-induced diabetes had attenuated potency and efficacy of pressor responses to noradrenaline, as well as reduced potency of mean circulatory filling pressure response to noradrenaline. Furthermore, the diabetic rats had reduced potency of mean arterial pressure response to angiotensin II. The increase in vasoconstrictor influence was less in diabetic rats than the controls. Variable influence of diabetes on blood flow to different beds may have been due to differing extents of neuropathy and/or local release of dilator or constrictor mediators. The results suggest that functional impairment of arterial and venous constrictions occur at the early phase of Type 1 diabetes.

7.2 Effects of 1400W on arterial and venous constrictions as well as hemodynamic response to noradrenaline in conscious and anesthetized rats with STZ-induced diabetes

It is unclear what is the primary cause of attenuated vasoconstriction and hemodynamic changes. There is evidence that iNOS is activated at the acute phase of diabetes (see Chapter 1 Introduction). Furthermore, iNOS induction in diabetes may be a consequence of hyperglycemia. The exposure of aortic endothelial cells to high glucose for seven to ten days was reported to increase the release of peroxynitrite, a highly reactive and cytotoxic oxidant derived from the interaction of NO with the free radical superoxide (Zou et al., 2002). As well, two hours perfusion of hearts with a solution containing a high concentration of glucose *in vitro* was associated with an increase in the expression of the iNOS (but not eNOS) gene, the generation of NO and superoxide, and apoptosis of cardiac cells (Ceriello et al., 2002). The expression of eNOS, in contrast to that of iNOS, was decreased in retinal vascular endothelial

cells incubated with high glucose and glycated proteins (Chakravarthy et al, 1998). There is also indication that in addition to causing cellular damage, an overproduction of NO through iNOS induction can cause cardiovascular depression. Indeed, hypotension in LPS-induced septicemia was associated with iNOS induction, and the blockade of iNOS by 1400W in this condition reversed hypotension (Wray et al., 1998) and augmented total peripheral resistance and cardiac output (Cheng et al., 2003b). It is therefore imperative to determine if the blockade of iNOS restores vasoconstriction in diabetes.

In the present studies, 1400W treatment restored the efficacy (maximum response) and insignificantly increased the potency (reduced ED₅₀) of mean arterial pressure response to noradrenaline, and it also significantly increased mean circulatory filling pressure response to a high dose of noradrenaline. Furthermore, 1400W did not affect any measured variables in the control rats. This ability of 1400W to selectively restore vascular response to noradrenaline in the diabetic rats showed that overproduction of NO by iNOS played an important role in depressing α -adrenoceptor mediated arterial and venous constriction at the acute phase of diabetes. This interpretation is in line with well-known clinical and experimental observations that NO is a potent and efficacious dilator of arterial resistance as well as capacitance vessels. Furthermore, the exogenous supply of NO through the administration of nitrovasodilators, such as acetylpenicillamine or sodium nitroprusside, has been shown to reduce mean arterial pressure and mean circulatory filling pressure of anaesthetised rats (Ng and Pang, 1998). An important mechanism by which NO mediates vasodilatation is through the inhibition of noradrenaline-mediated vasoconstriction (Zanzinger et al., 1994).

Compound 1400W significantly decreased the response of blood flow to noradrenaline in the heart and digestive system and increased vasoconstriction response to noradrenaline in most organs, including the heart in the diabetic but not control rats. These can not be explained by changes in cardiac output because 1400W did not caused significant decrease of cardiac output. It may reflect tissue and organ adaptation to the metabolic disorders in order to maintain important organ perfusion (Hill and Larkins, 1989). Since 1400W altered the distribution of blood flow, it is suggested that iNOS, and most likely NO produced by iNOS, plays a role in vascular system adaptation.

In summary, the present study demonstrates that the selective inhibition of iNOS by 1400W increases α -adrenoceptor-mediated constriction in arterial resistance as well as

capacitance vessels at the acute phase of diabetes. Our findings suggest that overproduced NO by iNOS may be involved in vascular abnormalities in diabetes.

7.3 Effects of STZ-induced diabetes on cardiac contractile response to adrenoceptor agonists in rats with STZ-induced diabetes

The diabetic rats in the present study had reduced heart rate, and left ventricular contractile function at three weeks after injection of STZ. These results are generally consistent with the results of other studies: in STZ-induced (26 day) conscious diabetic rats (Litwin et al., 1990), in STZ-induced (four and eight weeks) anesthetized diabetic rat (Banyasz et al., 1996; Dowell et al., 1986; Paulson et al., 1986), and in isolated hearts from rats with STZ-induced (five and eight weeks) diabetes (Okayama et al., 1994; Smith et al., 1997). Although some negative results have been reported, such as unchanged or partially changed cardiac function in young adult patients with diabetes mellitus (Borow et al., 1990) and in rat ventricular myocytes after eight weeks of STZ-induced diabetes (Wold et al., 2001), data from most *in vivo* and *in vitro* studies clearly support that STZ administration is associated with depressed cardiac function (McNeill, 1999). The controversy may be caused by the routes, methods, and duration of diabetes; or the varying strain of animals used for the studies. For example, the Sprague-Dawley rats have a slower rate of development of diabetes-induced myocardial dysfunction compared with other strains (Dai and McNeill, 1992; Dowell et al., 1986; Rodrigues et al., 1999). To further examine cardiac contractile function in diabetes, the rats were challenged with α and β_1 -adrenoceptor agonists noradrenaline and dobutamine. The results show that the diabetic rats had reduced inotropic and chronotropic responses to noradrenaline and dobutamine, exemplified by decreases in the efficacy (reduced E_{\max}) and potency (increased EC_{50}) of heart rate, and left ventricular developed tension as well as contractility ($\pm dP/dt$) responses to dobutamine. The depressed cardiac inotropic and chronotropic responsiveness to noradrenaline or dobutamine were observed in diabetic rats relative to controls in our study, which is consistent with both studies in isolated hearts (Smith et al., 1997), *in vivo* rats with STZ-induced diabetes (Paulson et al., 1986 and Heller et al., 1988), and spontaneously BB diabetic rats (Broderick et al., 1994). The maximal response (efficacy, E_{\max}) and potency (EC_{50}) of LVP to dobutamine decreased significantly, which partly agrees with Beenen's results. Beenen et al. reported (1997). reported the decreased E_{\max} and unchanged EC_{50} (potency) in isolated hearts of rats with STZ-induced diabetes for eight weeks. Our results are

also consistent with the observation that the number of β -adrenoceptors is decreased in cardiac membranes from diabetic rats at one to ten weeks after injection of STZ (Atkins et al., 1985; Beenen et al., 1997; Nishio et al., 1988; Ramanadham and Tenner, 1987).

In diabetic cardiomyopathy, the mechanism related to decreased myocardial contraction may be a consequence of 1) mechanical defects in the hearts (intrinsic contractility) and 2) alterations in β -adrenoceptor function and post receptor defects (Cai and Kang, 2001; Malhotra and Sanghi, 1997a; Ramanadham and Tenner, 1987). Any direct or indirect factors affecting these pathways may depress myocardial function, e.g. abnormalities in the structure of contractile and/or regulatory proteins, a reduction in the number of β -adrenoceptors, and abnormality in intracellular Ca^{2+} handling in the myocardium (see Chapter 1.3.3).

7.4 The activities, gene and protein expression of iNOS and cNOS, the formation of nitrotyrosine, the level of 15-F2t-isoprostane and the antioxidative capacity in myocardium of rats with STZ-induced diabetes rats

It is unclear what mechanism causes the decrease in vascular and cardiac contractile function at an early phase of diabetes, since the condition is associated with multiple neurohumoral and metabolic abnormalities. More recent studies have suggested that the overproduction of NO by iNOS and subsequent formation of cytotoxic products of NO (e.g., superoxide, peroxynitrite and nitrotyrosine may be responsible for cardiovascular abnormality at the acute phase of diabetes (see Chapter 1 Introduction).

Biochemical studies were performed to confirm that iNOS was activated and NT was formed in diabetes. Firstly, RT-PCR products (RNA) of iNOS was detected in the hearts of the diabetic but not control rats. Secondly, the activity of iNOS was three-fold in the hearts of diabetic rats relative to the controls, and the increase was inhibited by 1400W. Thirdly, immunostaining (proteins) of iNOS and nitrotyrosine (*in vivo* marker of peroxynitrite, an oxidant and cytotoxic mediator) were clearly identified in the hearts of the diabetic rats, but not the controls. In contrast, the RT-PCR products of eNOS, activity of cNOS (including eNOS and nNOS) as well as intensity of immunostaining eNOS were of similar in the hearts of the control and diabetes rats. Our results of biochemical studies are supportive of the hypothesis that the activation of iNOS at the acute phase of Type 1 diabetes causes the suppression of cardiac and vascular contractile functions. Activation of iNOS in our study was also confirmed by the fact that selective *in vivo* inhibition of iNOS activity by 1400W abolished elevated

activity of iNOS without affecting the activity of eNOS in the myocardium. In contrast to our results, increase in mRNA encoding eNOS (but not iNOS) occurred in the hearts of rats injected with STZ for four to six weeks (Stockklauser-Farber et al., 2000).

An initiating factor for the induction of iNOS may be high glucose. Indeed exposure of human aortic endothelial cells to high glucose was associated with the formation of the highly reactive oxidant peroxynitrite and nitration of tyrosine (Zou et al., 2002a). High glucose, however, suppressed the activity of eNOS in retinal vascular endothelial cells (Chakravarthy et al., 1998). There is also evidence that nitrotyrosine and iNOS-dependent peroxynitrite were detected in the blood of Type 2 diabetic patients (Ceriello et al., 2001; Ceriello et al., 2002b; Zou et al., 2002a), and the platelets of patients with Type 1 and 2 diabetes (Tannous et al., 1999). The biological actions of reactive nitrogen oxide species such as peroxynitrite (ONOO^-) and NO_x (NO_2 and N_2O_3) have received considerable attention since it causes the oxidation and nitration of amino acids, residues of proteins, and guanine of DNA; lipid peroxidation; DNA cleavage; and depletion of antioxidants, such as reduced glutathione (Akaike et al., 1998; Ferdinandy and Schulz, 2003; Gordge, 1998; Ischiropoulos, 1998; Keller and Mattson, 1998). All these changes ultimately lead to cytotoxicity and myocardial contractile failure (Ferdinandy et al., 2000).

It is of interest that there was no change in baseline myocardial 15-F₂-isoprostane, an *in vivo* indicator of oxidative stress, at three weeks after the induction of diabetes rats. However, myocardium antioxidant capacity was lowered as evidenced by increased formation of thiobarbituric acid reactive substance following *ex vivo* peroxidation challenge. Our results are in agreement with observations of similar levels of F₂-isoprostane in the kidneys of control and diabetes rats at three weeks after induction of diabetes, but significant reduction in the level of 15-F₂-isoprostane in the kidneys of the diabetic rats after treatment with the antioxidant DL- α -lipoic acid (Obrosova et al., 2003). As well, although baseline plasma levels of 15-F₂-isoprostane were similar in the control and diabetic subjects, plasma 15-F₂-isoprostane increased significantly during myocardial ischemia following heart surgery in high-risk patients with Type 1 and 2 diabetes, relative to the levels in low-risk patients (Ansley et al., 2002). It has been suggested that increased plasma levels of 15-F₂-isoprostane is inversely correlated with cardiac function post-operatively (Ansley et al., 2003).

Collectively, our results are supportive of the involvement of iNOS, nitrotyrosine and lowered antioxidative capacity in causing cardiovascular abnormalities in diabetes.

7.5 Effects of 1400W on cardiac contractile response to noradrenaline in rats with STZ-induced diabetes

One of the main focuses of our investigation was to find out if iNOS could be detected in the hearts of rats with STZ-induced diabetes for three weeks, and if selective *in vivo* inhibition of iNOS activity by 1400W could restore cardiovascular function. The selectivity of 1400W in inhibiting iNOS over eNOS has been demonstrated *in vitro* (Garvey et al., 1997; Young et al., 2000) as well as *in vivo* (Garvey et al., 1997), and verified in the first part of this investigation.

Acute treatment with 1400W did not alter baseline hemodynamic variables or responses to noradrenaline in the control rats. Treatment with 1400W however, enhanced the influence of noradrenaline on cardiac contractility (LVP, $LV \pm dP/dt$) in the diabetic rats. Furthermore, 1400W reduced the activity of iNOS in the diabetic myocardium. These results further confirmed that iNOS is activated at three weeks after injection of STZ, and this activation attenuates α -adrenoceptor mediated vasoconstriction as well as increase in cardiac contractility. Collectively, these results show that reduced cardiovascular responses to noradrenaline in STZ-induced diabetes is partially due to the activation of iNOS and formation of nitrotyrosine.

The link between ONOO⁻ or/and NO and depressed cardiac function has been reported in cardiac dysfunctional states: Increased iNOS activity and peroxynitrite generation/enhanced NT formation were immunohistochemically detected in the myocardium of rats with experimental autoimmune myocarditis (Ishiyama et al., 1997), and were associated with depression of baseline cardiac contractility and contractile response to β -adrenoceptor agonists. Increased iNOS activity and enhanced dityrosine formation, an indicator of peroxynitrite generation in myocardium were detected chemically in the perfusate of isolated rat hearts during prolonged perfusion at a time when cardiac work was decreased spontaneously (Ferdinandy et al., 1999). Correlation analyses revealed an inverse relationship between cardiac nitrotyrosine level and left ventricular fractional shortening *in vivo* following doxorubicin-induced cardiotoxicity in mice (Weinstein et al., 2000). The infusion of L-NAME (an inhibitor of NOS) potentiated cardiac contractility response to dobutamine by 51% in patients with heart failure (Hare et al., 1998). It seems reasonable to postulate that there exists a similar link between the levels of ONOO⁻ and/or NO and depressed myocardial contractility in the diabetic rat.

7.6 Effects of N-acetylcysteine on cardiac contractility, contractile responses to dobutamine, activation of iNOS, formation of peroxynitrite, and myocardium antioxidative capacity in rats with STZ-induced diabetes

In the present study, reduced cardiac contractile function in the diabetic rats was associated with increased immunostaining of iNOS and nitrotyrosine but not eNOS, and increased formation of thiobarbituric acid reactive substance upon peroxidation challenge, although baseline myocardial level of 15-F₂-isoprostane was not significantly changed. These results suggested that iNOS, but not eNOS, was activated, and formation of peroxynitrite was increased at the acute phase of diabetes. At this time there was similar baseline lipid peroxidation (formation of 15-F₂-isoprostane), but decreased tissue antioxidation capacity (increased thiobarbituric acid reactive substance formation upon peroxidation challenge).

Chronic treatment with N-acetylcysteine did not affect any baseline measurements or responses to dobutamine in the control rats. In the diabetic rats, N-acetylcysteine did not alter body weight, plasma glucose and heart rate, but partially restored baseline of left ventricular contractility (LV \pm dP/dt). N-acetylcysteine markedly restored heart rate and contractile responses to dobutamine, such that the potency of LVP, LV \pm dP/dt and heart rate responses to dobutamine were similar in the N-acetylcysteine -treated diabetic rats relative to the control rats. Moreover, maximum LVP and LV +dP/dt response to dobutamine were also increased significantly, whereas maximum heart rate and LV -dP/dt response to dobutamine were increased insignificantly. These results show that chronic antioxidant therapy with N-acetylcysteine improved cardiovascular function at the acute phase of diabetes.

There is evidence that antioxidant therapy offers protection against cellular damage in various animal models of diabetes and in diabetic patients. N-acetylcysteine, an antioxidative agent, has a wide spectrum of biological activities, either related to its ability to increase intracellular thiols or its direct action as an antioxidant. It also inhibits the production of NO by iNOS in rats treated with endotoxin (Bergamini et al., 2001). Chronic treatment of alloxan-induced diabetic mice with N-acetylcysteine reduced the activation of NF κ B in the pancreas, and this has the potential of reducing the production of proinflammatory cytokines such as interleukins and iNOS (Ho et al., 1999). Acute administration of N-acetylcysteine also improved vasodilatation response to electrical stimulation or to substance P in rats with STZ-induced diabetes (Bassirat and Khalil, 2000). Chronic treatment with N-acetylcysteine

prevented the development of peripheral neuropathy in rats with STZ-induced diabetes (Sagara et al., 1996) as well as ultrastructure changes in the erythrocytes of patients with Type 2 diabetes (Marfella et al., 2000; Sagara et al., 1996; Straface et al., 2002). It was of interest that chronic treatment with FP15, a peroxynitrite decomposer catalyst, inhibited tyrosine nitration in the pancreas of a mouse given multiple low-doses of STZ (Szabo et al., 2002). Oral administration of FP15 also reduced LV end-diastolic pressure and increased LV $-dP/dt$ in mice given a single high dose treatment of STZ (Szabo et al., 2002).

Further experiments were undertaken to find out if chronic treatment with N-acetylcysteine alters the antioxidative status in the rats. N-acetylcysteine did not alter eNOS immunostaining in the control or diabetic rats, but reduced the staining of iNOS and nitrotyrosine in the diabetic rats. F_2 -isoprostanes is currently the most reliable marker of lipid peroxidation *in vivo* for the quantification of oxidative stress (Mezzetti et al., 2000). The quantification of 15- F_{2t} -isoprostane is also a useful way of evaluating the effects of antioxidant interventions (See Chapter 1.5.2). The present results show that N-acetylcysteine treatment decreased the total amount of 15- F_{2t} -isoprostane in the myocardium to ~50% in the diabetic rats but not in the controls although there was no change in baseline myocardial 15- F_{2t} -isoprostane in the diabetic rats relative to the controls. These suggest that N-acetylcysteine apparently enhanced the decreased capacity of endogenous antioxidant, which was revealed as increased formation of thiobarbituric acid reactive substance following peroxidation challenge in the diabetic rat hearts.

Taken together, it is logical to speculate that improved cardiac performance by N-acetylcysteine in the diabetic rats is due to the reduction of oxidative stress which results in the preservation of proteins, such as receptors, enzymes, and transport and structural proteins, as well as reduction of cellular damage through lipid peroxidation. Our findings suggest that overproduced NO by iNOS may be involved in cardiovascular abnormalities in diabetes.

8. SUMMARY AND CONCLUSIONS FOR DISCUSSION II

1. STZ-induced diabetic status (two or three weeks) did not significantly affect baseline measurements of mean arterial pressure and mean circulatory filling pressure but altered the regional distribution of blood flow. STZ-induced diabetic rats had attenuated potency and efficacy of pressor responses to noradrenaline, as well as reduced potency of mean circulatory filling pressure response to noradrenaline. Furthermore, the diabetic rats had reduced potency of pressor response to angiotensin II. These results suggest that functional impairment of arterial and venous constrictions occur at an early phase of Type 1 diabetes. The selective inhibition of iNOS by 1400W increased α -adrenoceptor-mediated constriction in arterial resistance as well as capacitance vessels at the acute phase of diabetes. Our findings suggest that overproduction of NO by iNOS may cause vascular abnormalities in diabetes.
2. Rats with STZ-induced diabetes for three weeks had depressed cardiac contractile function, and this was associated with the activation of iNOS and formation of nitrotyrosine, a footprint of peroxynitrite *in vivo*. Selective inhibition of iNOS by 1400W reduced the activity of iNOS and partially restored cardiac response to noradrenaline. To our knowledge, these cardiac and vascular (see above) results are the first that link cellular activation of iNOS in diabetes to abnormal adrenoceptor-mediated responses in the heart and vasculature *in vivo*, and the first that demonstrate the restoration of cardiovascular function with selective inhibition of the activity of iNOS.
3. Direct evidence was provided to link depressed cardiac contractility to the production of peroxynitrite as well as peroxynitrite-mediated nitration of tyrosine and reduced antioxidative capacity (increased thiobarbituric acid reactive substance formation upon peroxidation challenge). At this time, baseline lipid peroxidation (formation of 15-F_{2t}-isoprostane) in the myocardium was not yet altered. Chronic treatment of N-acetylcysteine, an antioxidant and inhibitor of iNOS, reduced the activation of iNOS, decreased peroxynitrite-mediated nitration of tyrosine and lipid peroxidation, and augmented cardiac contractility to dobutamine. To our best knowledge, this is the first evidence that the antioxidant N-acetylcysteine improved cardiac function and concurrently reduced the level of myocardium 15-F_{2t}-isoprostane at three weeks after the induction of diabetes. Our results may highlight the necessity for antioxidant

supplementation in the management of cardiac contractile dysfunction in diabetes mellitus even when baseline levels of lipid peroxidation products are not yet elevated.

9. REFERENCES

1. Akaike T, Suga M, Maeda H. Free radicals in viral pathogenesis: molecular mechanisms involving superoxide and NO. *Proc Soc Exp Biol Med*. 1998;217:64-73.
2. Akella AB, Ding XL, Cheng R, Gulati J. Diminished Ca²⁺ sensitivity of skinned cardiac muscle contractility coincident with troponin T-band shifts in the diabetic rat. *Circ Res*. 1995;76:600-6.
3. Alberti KG and Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998;15:539-53.
4. American Diabetes Association . Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*. 1997;20:1183-97.
5. American Diabetes Association. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. American Diabetes Association Clinical Practice Recommendations 2001. *Diabetes Care*. 2001;24 Suppl 1:S5-S20.
6. Amos AF, McCarty DJ, Zimmet P. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabet Med*. 1997;14 Suppl 5:S1-85.
7. Ansley DM, Dhaliwal BS, Xia Z. F₂-isoprostane formation in high risk patients during ACBP surgery. . *Can J Anaesth*. 2002;49:43A.
8. Ansley DM, Xia Z, Dhaliwal BS. The relationship between plasma free 15-F_{2t}-isoprostane concentration and early postoperative cardiac depression following warm heart surgery. *J Thorac Cardiovasc Surg*. 2003;126:1222-3.
9. Atkins FL, Dowell RT, Love S. beta-Adrenergic receptors, adenylate cyclase activity, and cardiac dysfunction in the diabetic rat. *J Cardiovasc Pharmacol* . 1985;7:66-70.
10. Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc Natl Acad Sci U S A*. 1993a;90:347-51.
11. Balligand JL, Kobzik L and others. Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial (type III) nitric oxide synthase in cardiac myocytes. *J Biol Chem*. 1995;270:14582-6.
12. Balligand JL, Ungureanu D and others. Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. *J Clin Invest*. 1993b;91:2314-9.
13. Banyasz T, Kalapos I, Kelemen SZ, Kovacs T. Changes in cardiac contractility in IDDM and NIDDM diabetic rats. *Gen Physiol Biophys*. 1996;15:357-69.

14. Bardell AL and MacLeod KM. Evidence for inducible nitric-oxide synthase expression and activity in vascular smooth muscle of streptozotocin-diabetic rats. *J Pharmacol Exp Ther.* 2001;296:252-9.
15. Barden A, Beilin LJ and others. Plasma and urinary 8-iso-prostane as an indicator of lipid peroxidation in pre-eclampsia and normal pregnancy. *Clin Sci (Lond).* 1996;91:711-8.
16. Bassirat M and Khalil Z. Endothelin and free radicals modulate microvascular responses in streptozotocin-induced diabetic rats. *Microvasc Res.* 2000;59:88-98.
17. Bateson AN, Jakiwczyk OM, Schulz R. Rapid increase in inducible nitric oxide synthase gene expression in the heart during endotoxemia. *Eur J Pharmacol.* 1996;303:141-4.
18. Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol.* 1996a;9:836-44.
19. Beckman JS. Parsing the effects of nitric oxide, S-nitrosothiols, and peroxynitrite on inducible nitric oxide synthase-dependent cardiac myocyte apoptosis. *Circ Res.* 1999;85:870-1.
20. Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. *Methods Enzymol.* 1994;233:229-40.
21. Beckman JS and Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol.* 1996b;271:C1424-37.
22. Beenen OH, Batink HD, Pfaffendorf M, van Zwieten PA. Beta-adrenoceptors in the hearts of diabetic-hypertensive rats: radioligand binding and functional experiments. *Blood Press.* 1997;6:44-51.
23. Bergamini S, Rota C and others. N-acetylcysteine inhibits in vivo nitric oxide production by inducible nitric oxide synthase. *Nitric Oxide.* 2001;5:349-60.
24. Bone RC. Gram-positive organisms and sepsis. *Arch Intern Med.* 1994;154:26-34.
25. Borow KM, Jaspan JB and others. Myocardial mechanics in young adult patients with diabetes mellitus: effects of altered load, inotropic state and dynamic exercise. *J Am Coll Cardiol.* 1990;15:1508-17.
26. Boughton-Smith NK, Evans SM, Laszlo F, Whittle BJ, Moncada S. The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. *Br J Pharmacol.* 1993;110:1189-95.
27. Brady AJ, Poole-Wilson PA, Harding SE, Warren JB. Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia. *Am J Physiol.* 1992;263:H1963-6.

28. Brands MW and Fitzgerald SM. Acute endothelium-mediated vasodilation is not impaired at the onset of diabetes. *Hypertension*. 1998;32:541-7.
29. Brands MW, Fitzgerald SM, Hewitt WH, Hailman AE. Decreased cardiac output at the onset of diabetes: renal mechanisms and peripheral vasoconstriction. *Am J Physiol Endocrinol Metab*. 2000;278:E917-24.
30. Bravi MC, Pietrangeli P and others. Polyol pathway activation and glutathione redox status in non-insulin- dependent diabetic patients. *Metabolism*. 1997;46:1194-8.
31. Broderick TL, Kopp SJ, Daar JT, Romano FD, Paulson DJ. Relation of glycosylated hemoglobin to in vivo cardiac function in response to dobutamine in spontaneously diabetic BB Wor rats. *Can J Physiol Pharmacol*. 1994;72:722-37.
32. Brown GC. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta*. 2001;1504:46-57.
33. Bunn HF. Nonenzymatic glycosylation of protein: relevance to diabetes. *Am J Med*. 1981;70:325-30.
34. Cai L and Kang YJ. Oxidative stress and diabetic cardiomyopathy: a brief review. *Cardiovasc Toxicol*. 2001;1:181-93.
35. Cai M, Sakamoto A, Ogawa R. Inhibition of nitric oxide formation with L-canavanine attenuates endotoxin-induced vascular hyporeactivity in the rat. *Eur J Pharmacol*. 1996;295: 215-20.
36. Cameron NE and Cotter MA. Rapid reversal by aminoguanidine of the neurovascular effects of diabetes in rats: modulation by nitric oxide synthase inhibition. *Metabolism*. 1996;45:1147-52.
37. Caprio S, Wong S, Alberti KG, King G. Cardiovascular complications of diabetes. *Diabetologia*. 1997;40 Suppl 3:B78-82.
38. Ceppi ED, Smith FS, Titheradge MA. Nitric oxide, sepsis and liver metabolism. *Biochem Soc Trans*. 1997;25:929-34.
39. Ceriello A, Mercuri F and others. Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. *Diabetologia*. 2001;44:834-8.
40. Ceriello A, Quagliaro L and others. Role of hyperglycemia in nitrotyrosine postprandial generation. *Diabetes Care*. 2002b;25:1439-43.
41. Ceriello A, Quagliaro L and others. Acute hyperglycemia induces nitrotyrosine formation and apoptosis in perfused heart from rat. *Diabetes*. 2002a;51:1076-82.
42. Chakravarthy U, Hayes RG, Stitt AW, McAuley E, Archer DB. Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes*. 1998;47:945-52.

43. Chan NN, Vallance P, Colhoun HM. Nitric oxide and vascular responses in Type I diabetes. *Diabetologia*. 2000;43:137-47.
44. Chappey O, Dosquet C, Wautier MP, Wautier JL. Advanced glycation end products, oxidant stress and vascular lesions. *Eur J Clin Invest*. 1997;27:97-108.
45. Cheng X, Leung SW, Lim SL, Pang CC. Attenuated arterial and venous constriction in conscious rats with streptozotocin-induced diabetes. *Eur J Pharmacol*. 2003b;458:299-304.
46. Cheng X, Leung SW, Lo LS, Pang CC. Selective versus non-selective suppression of nitric oxide synthase on regional hemodynamics in rats with or without LPS-induced endotoxemia. *Naunyn Schmiedebergs Arch Pharmacol*. 2003a;367:372-9.
47. Cheng X and Pang CC. Pressor and vasoconstrictor effects of methylene blue in endotoxaemic rats. *Naunyn Schmiedebergs Arch Pharmacol*. 1998;357:648-53.
48. Chin R and Rubin M. Diabetic neuropathy. In: *Principles of diabetes mellitus*. Ed by Proretsky, L. Kluwer Academic Publishers. 2002: pp367-384.
49. Colardyn FC, Vandenbogaerde JF, Vogelaers DP, Verbeke JH. Use of dopexamine hydrochloride in patients with septic shock. *Crit Care Med*. 1989;17:999-1003.
50. Colasanti M and Suzuki H. The dual personality of NO. *Trends Pharmacol Sci*. 2000;21:249-52.
51. Cooke CL and Davidge ST. Peroxynitrite increases iNOS through NF-kappaB and decreases prostacyclin synthase in endothelial cells. *Am J Physiol Cell Physiol*. 2002;282:C395-402.
52. Corbett JA and McDaniel ML. The Use of Aminoguanidine, a Selective iNOS Inhibitor, to Evaluate the Role of Nitric Oxide in the Development of Autoimmune Diabetes. *Methods*. 1996;10:21-30.
53. Cosenzi A, Bernobich E and others. Role of nitric oxide in the early renal changes induced by high fructose diet in rats. *Kidney Blood Press Res*. 2002b;25:363-9.
54. Cosenzi A, Bernobich E and others. Early effects of diabetes on inducible nitric oxide synthase in the kidney. *Acta Diabetol*. 2002a;39:91-6.
55. Dai S and McNeill JH. Myocardial performance of STZ-diabetic DOCA-hypertensive rats. *Am J Physiol*. 1992;263:H1798-805.
56. Davi G, Ciabattoni G and others. In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. *Circulation*. 1999;99:224-9.
57. Davies MJ. The cardiomyopathies: an overview. *Heart*. 2000;83:469-74.

58. Dawson KG, Gomes D, Gerstein H, Blanchard JF, Kahler KH. The economic cost of diabetes in Canada, 1998. *Diabetes Care*. 2002;25:1303-7.
59. DCCT Research Group. Diabetes Control and Complications Trial (DCCT): results of feasibility study. The DCCT Research Group. *Diabetes Care*. 1987;10:1-19.
60. DCCT Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med*. 1993;329:977-86.
61. DCCT Research Group. The relationship of glycemic exposure (HbA1c) to the risk of development and progression of retinopathy in the diabetes control and complications trial. *Diabetes*. 1995;44:968-83.
62. De Mattia G, Bravi MC and others. Reduction of oxidative stress by oral N-acetyl-L-cysteine treatment decreases plasma soluble vascular cell adhesion molecule-1 concentrations in non-obese, non-dyslipidaemic, normotensive, patients with non-insulin-dependent diabetes. *Diabetologia*. 1998;41:1392-6.
63. De Rosa SC, Zaretsky MD and others. N-acetylcysteine replenishes glutathione in HIV infection. *Eur J Clin Invest*. 2000;30:915-29.
64. Dincer UD, Bidasee KR and others. The effect of diabetes on expression of beta1-, beta2-, and beta3- adrenoreceptors in rat hearts. *Diabetes*. 2001;50:455-61.
65. Doi K, Sawada F and others. Alteration of antioxidants during the progression of heart disease in streptozotocin-induced diabetic rats. *Free Radic Res*. 2001;34:251-61.
66. Dowell RT, Atkins FL, Love S. Integrative nature and time course of cardiovascular alterations in the diabetic rat. *J Cardiovasc Pharmacol*. 1986;8:406-13.
67. Du XL, Edelstein D and others. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A*. 2000;97:12222-6.
68. Du Y, Smith MA, Miller CM, Kern TS. Diabetes-induced nitrate stress in the retina, and correction by aminoguanidine. *J Neurochem*. 2002;80:771-9.
69. El-Omar MM, Lord R, Draper NJ, Shah AM. Role of nitric oxide in posthypoxic contractile dysfunction of diabetic cardiomyopathy. *European journal of heart failure*. 2003;5:229-239.
70. Elhadd TA, Kennedy G and others. Abnormal markers of endothelial cell activation and oxidative stress in children, adolescents and young adults with type 1 diabetes with no clinical vascular disease. *Diabetes Metab Res Rev*. 1999;15:405-11.
71. Ellis EA, Guberski DL, Hutson B, Grant MB. Time course of NADH oxidase,

- inducible nitric oxide synthase and peroxynitrite in diabetic retinopathy in the BBZ/WOR rat. *Nitric Oxide*. 2002;6:295-304.
72. Farkas K, Sarman B, Jermendy G, Somogyi A. Endothelial nitric oxide in diabetes mellitus: too much or not enough? *Diabetes Nutr Metab*. 2000;13:287-97.
 73. Feillet-Coudray C, Rock E and others. Lipid peroxidation and antioxidant status in experimental diabetes. *Clin Chim Acta*. 1999a;284:31-43.
 74. Feng Q, Lu X, Jones DL, Shen J, Arnold JM. Increased inducible nitric oxide synthase expression contributes to myocardial dysfunction and higher mortality after myocardial infarction in mice. *Circulation*. 2001;104:700-4.
 75. Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R. Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res*. 2000;87:241-7.
 76. Ferdinandy P, Panas D, Schulz R. Peroxynitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts. *Am J Physiol*. 1999;276:H1861-7.
 77. Ferdinandy P and Schulz R. Inhibition of peroxynitrite-induced dityrosine formation with oxidized and reduced thiols, nitric oxide donors, and purine derivatives. *Antioxid Redox Signal*. 2001a;3:165-71.
 78. Ferdinandy P and Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia- reperfusion injury and preconditioning. *Br J Pharmacol*. 2003;138:532-43.
 79. Finotti P, Pagetta A, Ashton T. The oxidative mechanism of heparin interferes with radical production by glucose and reduces the degree of glycooxidative modifications on human serum albumin. *Eur J Biochem*. 2001;268:2193-200.
 80. Fischer LG, Horstman DJ, Hahnenkamp K, Kechner NE, Rich GE. Selective iNOS inhibition attenuates acetylcholine- and bradykinin-induced vasoconstriction in lipopolysaccharide-exposed rat lungs. *Anesthesiology*. 1999;91:1724-32.
 81. Flaim SF, Morris ZQ, Kennedy TJ. Dextran as a radioactive microsphere suspending agent: severe hypotensive effect in rat. *Am J Physiol*. 1978;235:H587-91.
 82. Fleming I, Gray GA, Schott C, Stoclet JC. Inducible but not constitutive production of nitric oxide by vascular smooth muscle cells. *IMP: Eur J Pharmacol*. 1991b;200:375-6.
 83. Fleming I, Julou-Schaeffer G, Gray GA, Parratt JR, Stoclet JC. Evidence that an L-arginine/nitric oxide dependent elevation of tissue cyclic GMP content is involved in depression of vascular reactivity by endotoxin. *Br J Pharmacol*. 1991a;103:1047-52.
 84. Foy JM and Lucas PD. Effect of experimental diabetes, food deprivation and genetic obesity on the sensitivity of pithed rats to autonomic agents. *Br J Pharmacol*. 1976;57:229-34.
 85. Frustaci A, Kajstura J and others . Myocardial cell death in human diabetes. *Circ Res*.

2000;87:1123-32.

86. Furchgott RF and Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288:373-6.
87. Garg UC and Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest*. 1989;83:1774-7.
88. Garvey EP, Oplinger JA and others . 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J Biol Chem*. 1997;272:4959-63.
89. Gealekman O , Abassi Z, Rubinstein I, Winaver J, Binah O. Role of myocardial inducible nitric oxide synthase in contractile dysfunction and beta-adrenergic hyporesponsiveness in rats with experimental volume-overload heart failure. *Circulation*. 2002;105: 236-43.
90. George WJ, Polson JB, O'Toole AG, Goldberg ND. Elevation of guanosine 3',5'-cyclic phosphate in rat heart after perfusion with acetylcholine. *Proc Natl Acad Sci U S A*. 1970;66:398-403.
91. Giugliano D , Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. *Diabetes Care*. 1996;19:257-67.
92. Gnudi L and Gruden G. Pathogenesis of diabetic nephropathy. in *Textbook of Diabetes*, Ed by Pickup, J.C. and Williams, G. Blackwell Science Ltd., Berline, Germany. 2003;pp52.1-52.22.
93. Gomez-Jimenez J, Salgado A and others. L-arginine: nitric oxide pathway in endotoxemia and human septic shock. *Crit Care Med*. 1995;23:253-8.
94. Gordge MP. How cytotoxic is nitric oxide? *Exp Nephrol*. 1998;6:12-6 .
95. Griffen SC, Wang J, German MS. A genetic defect in beta-cell gene expression segregates independently from the fa locus in the ZDF rat. *Diabetes*. 2001;50:63-8.
96. Guest TM and Sperling LS. Cardiovascular complications of diabetes mellitus . In: *Clinical diabetes mellitus*. 3rd ed. Ed by Davidson, J.K. Thieme, New York. 2000;pp539-547.
97. Guillon JM, Thiry C, Roach AG, Caverio I. Preferential reduction in vascular responses to endothelin-1 in rats with streptozotocin-induced diabetes. *J Cardiovasc Pharmacol*. 1998;31 Suppl 1:S133-7.
98. Guyton AC, POLIZO D, ARMSTRONG GG. Mean circulatory filling pressure measured immediately after cessation of heart pumping. *Am J Physiol*. 1954;179:261-7.
99. Halliwell B. Antioxidant characterization. Methodology and mechanism. *Biochem*

Pharmacol. 1995;49:1341-8.

100. Halliwell B. What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? *FEBS Lett.* 1997;411:157-60.
101. Hamilton LC and Warner TD. Interactions between inducible isoforms of nitric oxide synthase and cyclo-oxygenase in vivo: investigations using the selective inhibitors, 1400W and celecoxib. *Br J Pharmacol.* 1998;125:335-40.
102. Hare JM, Givertz MM, Creager MA, Colucci WS. Increased sensitivity to nitric oxide synthase inhibition in patients with heart failure: potentiation of beta-adrenergic inotropic responsiveness. *Circulation.* 1998;97:161-6.
103. Hare JM, Loh E, Creager MA, Colucci WS. Nitric oxide inhibits the positive inotropic response to beta- adrenergic stimulation in humans with left ventricular dysfunction. *Circulation.* 1995c;92:2198-203.
104. Hattori Y, Banba N, Gross SS, Kasai K. Glycated serum albumin-induced nitric oxide production in vascular smooth muscle cells by nuclear factor kappaB-dependent transcriptional activation of inducible nitric oxide synthase. *Biochem Biophys Res Commun.* 1999;259:128-32.
105. Hattori Y, Hattori S, Sato N, Kasai K. High-glucose-induced nuclear factor kappaB activation in vascular smooth muscle cells. *Cardiovasc Res.* 2000a;46:188-97.
106. Hattori Y, Matsuda N and others. Diminished function and expression of the cardiac Na⁺-Ca²⁺ exchanger in diabetic rats: implication in Ca²⁺ overload. *J Physiol.* 2000b;527 Pt 1:85-94.
107. Hebden RA, Bennett T, Gardiner SM. Pressor sensitivities to vasopressin, angiotensin II, or methoxamine in diabetic rats. *Am J Physiol.* 1987;253:R726-34.
108. Hebden RA, Gardiner SM, Bennett T, MacDonald IA. The influence of streptozotocin-induced diabetes mellitus on fluid and electrolyte handling in rats. *Clin Sci (Lond).* 1986;70:111-7.
109. Heller BA, Paulson DJ, Kopp SJ, Peace DG, Tow JP. Depressed in vivo myocardial reactivity to dobutamine in streptozotocin diabetic rats: influence of exercise training. *Cardiovasc Res.* 1988 ;22:417-24.
110. Hewett JA and Roth RA. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol Rev.* 1993;45:382-411.
111. Hill MA and Larkins RG. Alterations in distribution of cardiac output in experimental diabetes in rats. *Am J Physiol.* 1989;257: H571-80.
112. Ho E, Chen G, Bray TM. Supplementation of N-acetylcysteine inhibits NFkappaB activation and protects against alloxan-induced diabetes in CD-1 mice. *FASEB J.* 1999;13:1845-54.

113. Hoeldtke RD, Bryner KD and others. Nitrosative stress, uric Acid, and peripheral nerve function in early type 1 diabetes. *Diabetes*. 2002;51:2817-25.
114. Hoftiezer V and Carpenter AM. Comparison of streptozotocin and alloxan-induced diabetes in the rat, including volumetric quantitation of the pancreatic islets. *Diabetologia*. 1973;9:178-84.
115. Inoguchi T , Battan R and others. Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A*. 1992;89:11059-63.
116. Inoguchi T , Li P and others. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes*. 2000;49:1939-45.
117. Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys*. 1998;356:1-11.
118. Ishii H, Koya D, King GL. Protein kinase C activation and its role in the development of vascular complications in diabetes mellitus. *J Mol Med*. 1998;76:21-31.
119. Ishise S, Pegram BL, Yamamoto J, Kitamura Y, Frohlich ED. Reference sample microsphere method: cardiac output and blood flows in conscious rat. *Am J Physiol*. 1980;239:H443-H449.
120. Ishiyama S , Hiroe M and others. Nitric oxide contributes to the progression of myocardial damage in experimental autoimmune myocarditis in rats. *Circulation*. 1997;95:489-96.
121. Jackson CV and Carrier GO. Influence of short-term experimental diabetes on blood pressure and heart rate in response to norepinephrine and angiotensin II in the conscious rat. *J Cardiovasc Pharmacol*. 1983;5:260-5.
122. Jain SK and McVie R. Hyperketonemia can increase lipid peroxidation and lower glutathione levels in human erythrocytes in vitro and in type 1 diabetic patients. *Diabetes*. 1999;48:1850-5.
123. Jarrett RJ. Cardiovascular disease and hypertension in diabetes mellitus. *Diabetes Metab Rev*. 1989;5:547-58.
124. Joe EK, Schussheim AE and others . Regulation of cardiac myocyte contractile function by inducible nitric oxide synthase (iNOS): mechanisms of contractile depression by nitric oxide. *J Mol Cell Cardiol*. 1998;30:303-15.
125. Junod A, Lambert AE and others. Studies of the diabetogenic action of streptozotocin. *Proc Soc Exp Biol Med*. 1967;126:201-5.
126. Junod A, Lambert AE, Stauffacher W, Renold AE. Diabetogenic action of

- streptozotocin: relationship of dose to metabolic response. *J Clin Invest.* 1969;48:2129-39.
127. Kajstura J, Fiordaliso F and others. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. *Diabetes.* 2001;50:1414-24.
 128. Kaneto H, Kajimoto Y and others. Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. *Diabetes.* 1999;48:2398-406.
 129. Kannel WB, Hjortland M, Castelli WP. Role of diabetes in congestive heart failure: the Framingham study. *Am J Cardiol.* 1974;34:29-34.
 130. Kannel WB and McGee DL. Diabetes and cardiovascular disease. The Framingham study. *JAMA.* 1979;241:2035-8.
 131. Kashiwagi A, Nishio Y and others. Plasma membrane-specific deficiency in cardiac beta-adrenergic receptor in streptozocin-diabetic rats. *Am J Physiol.* 1989;257:E127-32.
 132. Kawai M, Bandiera SM and others. Modulation of hepatic CYP2A1, CYP2C11, and CYP3A9 expression in adult rats by neonatal administration of tamoxifen. *Drug Metab Dispos.* 1999; 27:1392-8.
 133. Keaney JF Jr, Hare JM and others. Inhibition of nitric oxide synthase augments myocardial contractile responses to beta-adrenergic stimulation. *Am J Physiol.* 1996;271: H2646-52.
 134. Keller JN and Mattson MP. Roles of lipid peroxidation in modulation of cellular signaling pathways, cell dysfunction, and death in the nervous system. *Rev Neurosci.* 1998;9:105-16.
 135. Kihara M, Schmelzer JD and others. Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. *Proc Natl Acad Sci USA.* 1991;88:6107-11.
 136. Kilbourn RG, Traber DL, Szabo C. Nitric oxide and shock. *Dis Mon.* 1997;43:277-348.
 137. Kim HW, Ch YS, Lee HR, Park SY, Kim YH. Diabetic alterations in cardiac sarcoplasmic reticulum Ca²⁺-ATPase and phospholamban protein expression. *Life Sci.* 2001;70:367-79.
 138. King GL, Kunisaki M and others. Biochemical and molecular mechanisms in the development of diabetic vascular complications. *Diabetes.* 1996;45 Suppl 3:S105-8.
 139. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care.* 1998;21:1414-31.
 140. Kiss J, Lamarque D and others. Helicobacter pylori lipopolysaccharide-provoked injury

- to rat gastroduodenal microvasculature involves inducible nitric oxide synthase. *Eur J Pharmacol.* 2001;420:175-9.
141. Klemm K and Moody FG. Regional intestinal blood flow and nitric oxide synthase inhibition during sepsis in the rat. *Ann Surg.* 1998;227:126-33.
 142. Knowles RG and Moncada S. Nitric oxide as a signal in blood vessels. *Trends Biochem Sci.* 1992;17:399-402.
 143. Knowles RG, Salter M, Brooks SL, Moncada S. Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem Biophys Res Commun.* 1990;172: 1042-8.
 144. Kooy NW, Lewis SJ and others. Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite. *Crit Care Med.* 1997;25:812-9.
 145. Koya D and King GL. Protein kinase C activation and the development of diabetic complications. *Diabetes.* 1998;47:859-66.
 146. Lagadic-Gossmann D, Buckler KJ, Le Prigent K, Feuvray D. Altered Ca²⁺ handling in ventricular myocytes isolated from diabetic rats. *Am J Physiol.* 1996;270:H1529-37.
 147. Lamas S, Marsden PA, Li GK, Tempst P, Michel T. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Second: *Proc Natl Acad Sci U S A.* 1992;89:6348-52.
 148. Lapolla A, Traldi P, Fedele D. AGE in micro- and macroangiopathy. *Contrib Nephrol.* 2001;10-21.
 149. Laszlo F and Whittle BJ. Actions of isoform-selective and non-selective nitric oxide synthase inhibitors on endotoxin-induced vascular leakage in rat colon. *Eur J Pharmacol.* 1997;334:99-102.
 150. Lautt WW, Legare DJ, d'Almeida MS. Adenosine as putative regulator of hepatic arterial flow (the buffer response). *Am J Physiol.* 1985;248:H331-8.
 151. Leiter LA, Barr A and others. Diabetes Screening in Canada (DIASCAN) Study: prevalence of undiagnosed diabetes and glucose intolerance in family physician offices. *Diabetes Care.* 2001;24:1038-43.
 152. Liang Q, Carlson EC and others. Overexpression of metallothionein reduces diabetic cardiomyopathy. *Diabetes.* 2002;51:174-81.
 153. Litwin SE, Raya TE, Anderson PG, Daugherty S, Goldman S. Abnormal cardiac function in the streptozotocin-diabetic rat. Changes in active and passive properties of the left ventricle. *J Clin Invest.* 1990;86:481-8.
 154. Litwin SE, Raya TE, Daugherty S, Goldman S. Peripheral circulatory control of

- cardiac output in diabetic rats. *Am J Physiol.* 1991;261: H836-42.
155. Liu X, Takeda N, Dhalla NS. Troponin I phosphorylation in heart homogenate from diabetic rat. *Biochim Biophys Acta.* 1996;1316:78-84.
 156. Lopez BL, Liu GL, Christopher TA, Ma XL. Peroxynitrite, the product of nitric oxide and superoxide, causes myocardial injury in the isolated perfused rat heart. *Coron Artery Dis.* 1997;8:149-53.
 157. Lucas PD. Effects of streptozotocin-induced diabetes and noradrenaline infusion on cardiac output and its regional distribution in pithed rats. *Diabetologia.* 1985;28:108-12.
 158. Lyall F, Gibson JL and others. Increased nitrotyrosine in the diabetic placenta: evidence for oxidative stress. *Diabetes Care.* 1998;21:1753-8.
 159. Ma XL, Gao F, Lopez BL, Christopher TA, Vinten-Johansen J. Peroxynitrite, a two-edged sword in post-ischemic myocardial injury- dichotomy of action in crystalloid-versus blood-perfused hearts. *J Pharmacol Exp Ther.* 2000;292:912-20.
 160. Ma XL, Lopez BL, Liu GL, Christopher TA, Ischiropoulos H. Peroxynitrite aggravates myocardial reperfusion injury in the isolated perfused rat heart. *Cardiovasc Res.* 1997;36:195-204.
 161. MacMicking JD, Nathan C and others. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell.* 1995;81:641-50.
 162. Makino A and Kamata K. Elevated plasma endothelin-1 level in streptozotocin-induced diabetic rats and responsiveness of the mesenteric arterial bed to endothelin-1. *Br J Pharmacol.* 1998a;123:1065-72.
 163. Makino N, Dhalla KS, Elimban V, Dhalla NS. Sarcolemmal Ca^{2+} transport in streptozotocin-induced diabetic cardiomyopathy in rats. *Am J Physiol.* 1987;253:E202-7.
 164. Malhotra A , Lopez MC, Nakouzi A. Troponin subunits contribute to altered myosin ATPase activity in diabetic cardiomyopathy. *Mol Cell Biochem.* 1995;151:165-72.
 165. Malhotra A , Mordes JP, McDermott L, Schaible TF. Abnormal cardiac biochemistry in spontaneously diabetic Bio- Breeding/Worcester rat. *Am J Physiol.* 1985;249:H1051-5.
 166. Malhotra A , Penpargkul S, Fein FS, Sonnenblick EH, Scheuer J. The effect of streptozotocin-induced diabetes in rats on cardiac contractile proteins. *Circ Res.* 1981;49:1243-50.
 167. Malhotra A and Sanghi V. Regulation of contractile proteins in diabetic heart. *Cardiovasc Res.* 1997a;34:34-40.
 168. Marfella R , Nappo F and others. Hemodynamic effects of acute hyperglycemia in type

- 2 diabetic patients. *Diabetes Care*. 2000;23:658-63.
169. Marfella R , Quagliaro L, Nappo F, Ceriello A, Giugliano D. Acute hyperglycemia induces an oxidative stress in healthy subjects. *J Clin Invest*. 2001;108:635-6.
 170. Marfella R , Verrazzo G and others. Glutathione reverses systemic hemodynamic changes induced by acute hyperglycemia in healthy subjects. *Am J Physiol*. 1995;268:E1167-73.
 171. Matkovics B, Kotorman M, Varga IS, Hai DQ, Varga C. Oxidative stress in experimental diabetes induced by streptozotocin. *Acta Physiol Hung*. 1997-1998;85:29-38.
 172. Matsuda N, Hattori Y and others. Diabetes-induced down-regulation of beta1-adrenoceptor mRNA expression in rat heart. *Biochem Pharmacol*. 1999;58:881-5.
 173. Mayer B and Andrew P. Nitric oxide synthases: catalytic function and progress towards selective inhibition. *Naunyn Schmiedebergs Arch Pharmacol*. 1998;358:127-33.
 174. McKendry JB. Direct costs of diabetes care: a survey in Ottawa, Ontario 1986. *Can J Public Health*. 1989;80:124-8.
 175. McNeill JH. Role of elevated lipids in diabetic cardiomyopathy. *Diabetes Res Clin Pract*. 1996;31 Suppl:S67-71.
 176. McNeill JH. Experimental models of diabetes. *C.R.C. Press L.L.C., Florida*. 1999.
 177. Meier M and King GL. Protein kinase C activation and its pharmacological inhibition in vascular disease. *Vasc Med*. 2000;5:173-85.
 178. Meltzer S, Leiter L and others. 1998 clinical practice guidelines for the management of diabetes in Canada. Canadian Diabetes Association. *CMAJ*. 1998;159 Suppl 8:S1-29.
 179. Mezzetti A , Cipollone F, Cuccurullo F. Oxidative stress and cardiovascular complications in diabetes: isoprostanes as new markers on an old paradigm. *Cardiovasc Res*. 2000;47:475-88.
 180. Millar CG and Thiernemann C. Intrarenal haemodynamics and renal dysfunction in endotoxaemia: effects of nitric oxide synthase inhibition. *Br J Pharmacol*. 1997;121:1824-30.
 181. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991a;43:109-42.
 182. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991b;43:109-42.
 183. Monnier VM. Aminoguanidine and diabetic neuropathy. *Eur J Endocrinol*. 1996;134:398-400.

184. Morris SM Jr and Billiar TR. New insights into the regulation of inducible nitric oxide synthesis. *Am J Physiol*. 1994;266: E829-39.
185. Morrish NJ , Wang SL, Stevens LK, Fuller JH, Keen H. Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. *Diabetologia*. 2001;44 Suppl 2:S14-21.
186. Morrow JD, Chen Y and others. The isoprostanes: unique prostaglandin-like products of free-radical- initiated lipid peroxidation. *Drug Metab Rev*. 1999;31:117-39.
187. Morrow JD and Roberts LJ. The isoprostanes: unique bioactive products of lipid peroxidation. *Prog Lipid Res*. 1997;36:1-21.
188. Mulder MF, van Lambalgen AA and others. Protective role of NO in the regional hemodynamic changes during acute endotoxemia in rats. *Am J Physiol*. 1994;266:H1558-64.
189. Mulder MF, van Lambalgen AA, van den Bos GC, Thijs LG. The fall of cardiac output in endotoxemic rats cannot explain all changes in organ blood flow: a comparison between endotoxin and low venous return shock. *Shock*. 1996;5:135-40.
190. Muller JM, Rupec RA, Baeuerle PA. Study of gene regulation by NF-kappa B and AP-1 in response to reactive oxygen intermediates. *Methods*. 1997;11:301-12.
191. Nakamura M , Thourani VH and others. Glutathione reverses endothelial damage from peroxynitrite, the byproduct of nitric oxide degradation, in crystalloid cardioplegia. *Circulation*. 2000;102:III332-8.
192. Ng SS and Pang CC. Venous versus arterial actions of diethylamine/nitric oxide (DEA/NO) complex and S-nitroso-N-acetylpenicillamine (SNAP) in vivo. *Br J Pharmacol*. 1998;125:1247-51.
193. Nishikawa T, Edelstein D, Brownlee M. The missing link: a single unifying mechanism for diabetic complications. *Kidney Int Suppl*. 2000a;77:S26-30.
194. Nishio Y, Kashiwagi A and others . Deficiency of cardiac beta-adrenergic receptor in streptozocin-induced diabetic rats. *Diabetes*. 1988;37:1181-7.
195. Nourooz-Zadeh J, Rahimi A and others. Relationships between plasma measures of oxidative stress and metabolic control in NIDDM. *Diabetologia*. 1997;40:647-53.
196. Obrosova IG, Fathallah L, Greene DA. Early changes in lipid peroxidation and antioxidative defense in diabetic rat retina: effect of DL-alpha-lipoic acid. ANTO: *Eur J Pharmacol*. 2000;398:139-46.
197. Obrosova IG, Fathallah L, Liu E, Nourooz-Zadeh J. Early oxidative stress in the diabetic kidney: effect of DL-alpha- lipoic acid. ANTO: *Free Radic Biol Med*. 2003;34:186-95.

198. Odetti P, Pesce C and others. Comparative trial of N-acetyl-cysteine, taurine, and oxerutin on skin and kidney damage in long-term experimental diabetes. *Diabetes*. 2003;52:499-505.
199. Okayama H, Hamada M, Hiwada K. Contractile dysfunction in the diabetic-rat heart is an intrinsic abnormality of the cardiac myocyte. *Clin Sci (Lond)*. 1994;86:257-62.
200. Oyama J, Shimokawa H and others. Role of nitric oxide and peroxynitrite in the cytokine-induced sustained myocardial dysfunction in dogs in vivo. *J Clin Invest*. 1998;101: 2207-14.
201. Packer L and Tritschler HJ. Alpha-lipoic acid: the metabolic antioxidant. *Free Radic Biol Med*. 1996;20:625-6.
202. Palmer HJ and Paulson KE. Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutr Rev*. 1997;55:353-61.
203. Pang CC. Measurement of body venous tone. *J Pharmacol Toxicol Methods*. 2000;44:341-60.
204. Pang CC. Autonomic control of the venous system in health and disease: effects of drugs. *Pharmacol Ther*. 2001;90:179-230.
205. Patz A and Smith RE. The ETDRS and Diabetes 2000. *Ophthalmology*. 1991;98:739-40.
206. Paulson DJ, Kopp SJ, Tow JP, Feliksik JM, Peace DG. Impaired in vivo myocardial reactivity to norepinephrine in diabetic rats. *Proc Soc Exp Biol Med*. 1986;183:186-92.
207. Penpargkul S, Fein F, Sonnenblick EH, Scheuer J. Depressed cardiac sarcoplasmic reticular function from diabetic rats. *J Mol Cell Cardiol*. 1981;13:303-9.
208. Pickup JC and Williams G. Textbook of Diabetes. *Blackwell Science Ltd., Berline, Germany*. 2003.
209. Pieper GM. Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. *Diabetologia*. 1999;42:204-13.
210. Pierce GN and Russell JC. Regulation of intracellular Ca^{2+} in the heart during diabetes. *Cardiovasc Res*. 1997b;34:41-7.
211. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys*. 1991;288:481-7.
212. Rahbar S, Kumar Yernini K, Scott S, Gonzales N, Lalezari I. Novel inhibitors of advanced glycation endproducts. *Biochem Biophys Res Commun*. 1999;262:651-6.

213. Rakieten N , Rakieten ML, Nadkarni MV. Studies on the diabetogenic action of streptozotocin (NSC-37917). *Cancer Chemother Rep.* 1963;29:91-8.
214. Ramanadham S and Tenner TE Jr. Alterations in the myocardial beta-adrenoceptor system of streptozotocin-diabetic rats. *Eur J Pharmacol.* 1987;136:377-89.
215. Rebolledo A, Ayala-Paredes F, Milesi V, Grassi AO , Rinaldi GJ. Short-term streptozotocin-induced diabetes induces blood pressure decrease associated with reduced aortic (45)Ca(2+) uptake and selective depression of the sustained noradrenergic contraction. *Diabetes Metab.* 2001;27:40-8.
216. Rees DD, Celtek S, Palmer RM, Moncada S. Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem Biophys Res Commun.* 1990;173:541-7.
217. Regan TJ, Lyons MM and others. Evidence for cardiomyopathy in familial diabetes mellitus. *J Clin Invest.* 1977;60:884-99.
218. Ren J and Davidoff AJ. Diabetes rapidly induces contractile dysfunctions in isolated ventricular myocytes. *Am J Physiol.* 1997a;272:H148-58.
219. Richardson P, McKenna W and others. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. *Circulation.* 1996;93:841-2.
220. Robbins MJ , Sharp RA, Slonim AE, Burr IM. Protection against streptozotocin-induced diabetes by superoxide dismutase. *Diabetologia.* 1980;18:55-8.
221. Roberts AB , Roche NS, Winokur TS, Burmester JK, Sporn MB. Role of transforming growth factor-beta in maintenance of function of cultured neonatal cardiac myocytes. Autocrine action and reversal of damaging effects of interleukin-1. *J Clin Invest.* 1992a;90: 2056-62.
222. Roberts AB , Vodovotz Y, Roche NS, Sporn MB, Nathan CF. Role of nitric oxide in antagonistic effects of transforming growth factor-beta and interleukin-1 beta on the beating rate of cultured cardiac myocytes. *Mol Endocrinol.* 1992b;6:1921-30.
223. Rocic B, Vucic M and others. Total plasma antioxidants in first-degree relatives of patients with insulin-dependent diabetes. *Exp Clin Endocrinol Diabetes.* 1997;105:213-7.
224. Rodrigues B, Goyal RK, McNeill JH. Effects of hydralazine on streptozotocin-induced diabetic rats: prevention of hyperlipidemia and improvement in cardiac function. *J Pharmacol Exp Ther.* 1986;237:292-9.
225. Rodrigues B, Poucheret P, Battell M, McNeill JH. STZ-induced diabetes: induction, mechanism(s), and dose dependency in Experimental models of diabetes. *Edited by McNeill, J.H., C.R.C. Press L.L.C., Florida.* 1999: pp 3-17.

226. Rose P, Widder S and others. Inhibition of peroxynitrite-mediated cellular toxicity, tyrosine nitration, and alpha1-antiproteinase inactivation by 3-mercapto-2-methylpentan-1-ol, a novel compound isolated from *Allium cepa*. *ANTO: Biochem Biophys Res Commun*. 2003;302:397-402.
227. Rosen P, Du X, Tschope D. Role of oxygen derived radicals for vascular dysfunction in the diabetic heart: prevention by alpha-tocopherol? *Mol Cell Biochem*. 1998;188:103-11.
228. Rosen P, Nawroth PP and others. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev*. 2001;17:189-212.
229. Rosenstock JL and Appel GB. Diabetic nephropathy. In: *Principles of diabetes mellitus*. Ed by Proretsky, L. Kluwer Academic Publishers. 2002;pp353-366.
230. Rosselet A, Feihl F and others. Selective iNOS inhibition is superior to norepinephrine in the treatment of rat endotoxic shock. *Am J Respir Crit Care Med*. 1998;157:162-70.
231. Rota C, Bergamini S and others. N-Acetylcysteine negatively modulates nitric oxide production in endotoxin-treated rats through inhibition of NF-kappaB activation. *NO: Antioxid Redox Signal*. 2002;4:221-6.
232. Rothe CF. Mean circulatory filling pressure: its meaning and measurement. *J Appl Physiol*. 1993;74:499-509.
233. Roufail E, Soulis T, Boel E, Cooper ME, Rees S. Depletion of nitric oxide synthase-containing neurons in the diabetic retina: reversal by aminoguanidine. *Diabetologia*. 1998;41:1419-25.
234. Rubler S, Dlugash J and others. New type of cardiomyopathy associated with diabetic glomerulosclerosis. *Am J Cardiol*. 1972;30:595-602.
235. Runzer TD, Ansley DM, Godin DV, Chambers GK. Tissue antioxidant capacity during anesthesia: propofol enhances in vivo red cell and tissue antioxidant capacity in a rat model. *Anesth Analg*. 2002;94:89-93, table of contents.
236. Sagara M, Satoh J and others. Inhibition of development of peripheral neuropathy in streptozotocin- induced diabetic rats with N-acetylcysteine. *Diabetologia*. 1996;39:263-9.
237. Salgo MG, Bermudez E, Squadrito GL, Pryor WA. Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes. *Arch Biochem Biophys*. 1995a;322:500-5.
238. Salgo MG, Squadrito GL, Pryor WA. Peroxynitrite causes apoptosis in rat thymocytes. *Biochem Biophys Res Commun*. 1995b;215:1111-8.
239. Salter M, Knowles RG, Moncada S. Widespread tissue distribution, species distribution

- and changes in activity of Ca(2+)-dependent and Ca(2+)-independent nitric oxide synthases. *FEBS Lett.* 1991 ;291:145-9.
240. Sambandam N, Abrahani MA and others. Metabolism of VLDL is increased in streptozotocin-induced diabetic rat hearts. *Am J Physiol Heart Circ Physiol.* 2000;278: H1874-82.
 241. Sampson MJ , Gopaul N, Davies IR, Hughes DA, Carrier MJ. Plasma F2 isoprostanes: direct evidence of increased free radical damage during acute hyperglycemia in type 2 diabetes. *Diabetes Care.* 2002;25:537-41.
 242. Sano T, Umeda F, Hashimoto T, Nawata H, Utsumi H. Oxidative stress measurement by in vivo electron spin resonance spectroscopy in rats with streptozotocin-induced diabetes. *Diabetologia.* 1998;41:1355-60.
 243. Schaper NC , Houben AJ and others . Venous compliance and the venodilatory effect of nitroglycerin in insulin-dependent diabetic patients with and without (incipient) nephropathy. *Eur J Clin Invest.* 1994;24:382-7.
 244. Schmidt RE and Scharp DW. Axonal dystrophy in experimental diabetic autonomic neuropathy. *Diabetes.* 1982;31:761-70.
 245. Schnedl WJ , Ferber S, Johnson JH, Newgard CB. STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. *Diabetes.* 1994;43:1326-33.
 246. Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *Br J Pharmacol.* 1992a;105:575-80.
 247. Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *Br J Pharmacol.* 1992b;105:575-80.
 248. Schumer W. Pathophysiology and treatment of septic shock. *Am J Emerg Med.* 1984;2:74-7.
 249. Sen CK and Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J.* 1996;10:709-20.
 250. Sharma K, Danoff TM, DePiero A, Ziyadeh FN. Enhanced expression of inducible nitric oxide synthase in murine macrophages and glomerular mesangial cells by elevated glucose levels: possible mediation via protein kinase C. *Biochem Biophys Res Commun.* 1995;207:80-8.
 251. Shaw KM. Macrovascular disease in diabetes. In: *Diabetic complications.* Ed Shaw, K.M. John Wiley & Sons Ltd., Baffins Lane, England. 1996:pp179-205.
 252. Sima AA and Sugimoto K. Experimental diabetic neuropathy: an update. *Diabetologia.* 1999;42:773-88.

253. Smith JM, Paulson DJ, Romano FD. Inhibition of nitric oxide synthase by L-NAME improves ventricular performance in streptozotocin-diabetic rats. *J Mol Cell Cardiol.* 1997;29:2393-402.
254. Southan GJ and Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem Pharmacol.* 1996a;51:383-94.
255. Southan GJ and Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem Pharmacol.* 1996b;51:383-94.
256. Stanek KA, Coleman TG, Smith TL, Murphy WR. Two hemodynamic problems commonly associated with the microsphere technique for measuring regional blood flow in rats. *J Pharmacol Methods.* 1985;13:117-24.
257. Stansberry KB, Hill MA and others. Impairment of peripheral blood flow responses in diabetes resembles an enhanced aging effect. *Diabetes Care.* 1997;20:1711-6.
258. Statistics Canada. Health Indicators. National Population Health Survey. 1998-1999.
259. Stevens RB , Sutherland DE and others. Insulin down-regulates the inducible nitric oxide synthase pathway: nitric oxide as cause and effect of diabetes? *J Immunol.* 1997;159: 5329-35.
260. Stockklauser-Farber K, Ballhausen T, Laufer A, Rosen P. Influence of diabetes on cardiac nitric oxide synthase expression and activity. IMP: *Biochim Biophys Acta.* 2000;1535:10-20.
261. Straface E , Rivabene R and others. Structural changes of the erythrocyte as a marker of non-insulin- dependent diabetes: protective effects of N-acetylcysteine. *Biochem Biophys Res Commun.* 2002;290:1393-8.
262. Strunk V, Hahnenkamp K, Schneuing M, Fischer LG, Rich GF. Selective iNOS inhibition prevents hypotension in septic rats while preserving endothelium-dependent vasodilation. *Anesth Analg.* 2001;92:681-7.
263. Szabo C, Mabley JG and others. Part I: Pathogenetic Role of Peroxynitrite in the Development of Diabetes and Diabetic Vascular Complications: Studies With FP15, A Novel Potent Peroxynitrite Decomposition Catalyst. *Mol Med.* 2002;8:571-80.
264. Szabo C, Mitchell JA, Thiernemann C, Vane JR. Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. *Br J Pharmacol.* 1993;108:786-92.
265. Szabo C, Salzman AL, Ischiropoulos H. Endotoxin triggers the expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in the rat aorta in vivo. *FEBS Lett.* 1995;363:235-8.
266. Takeda N, Nakamura I, Hatanaka T, Ohkubo T, Nagano M. Myocardial mechanical and myosin isoenzyme alterations in streptozotocin-diabetic rats. *Jpn Heart J.*

- 1988;29:455-63.
267. Tamada A, Hattori Y and others. Effects of beta-adrenoceptor stimulation on contractility. *Am J Physiol.* 1998;274:H1849-57.
 268. Tamborlane WV and Ahern J. Implications and results of the Diabetes Control and Complications Trial. *Pediatr Clin North Am.* 1997;44:285-300.
 269. Tanaka Y, Shimizu H, Sato N, Mori M, Shimomura Y. Involvement of spontaneous nitric oxide production in the diabetogenic action of streptozotocin. *Pharmacology.* 1995;50:69-73.
 270. Tannous M, Rabini RA and others. Evidence for iNOS-dependent peroxynitrite production in diabetic platelets. *Diabetologia.* 1999;42:539-44.
 271. Tatchum-Talom R, Gopalakrishnan V, McNeill JR. Radiotelemetric monitoring of blood pressure and mesenteric arterial bed responsiveness in rats with streptozotocin-induced diabetes. *Can J Physiol Pharmacol.* 2000;78:721-8.
 272. Tepperman BL, Chang Q, Soper BD. Protein kinase C mediates lipopolysaccharide- and phorbol-induced nitric-oxide synthase activity and cellular injury in the rat colon. *J Pharmacol Exp Ther.* 2000;295:1249-57.
 273. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med.* 1993 ;329:977-986.
 274. Thiernemann C. Nitric oxide and septic shock. *Gen Pharmacol.* 1997;29:159-66.
 275. Thiernemann C, Ruetten H, Wu CC, Vane JR. The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase. *Br J Pharmacol.* 1995;116: 2845-51.
 276. Titheradge MA. Nitric oxide in septic shock. *Biochim Biophys Acta.* 1999;1411:437-55.
 277. Tomlinson KC, Gardiner SM, Bennett T. Diabetes mellitus in Brattleboro rats: cardiovascular, fluid, and electrolyte status. *Am J Physiol.* 1989;256:R1279-85.
 278. Tomlinson KC, Gardiner SM, Hebden RA, Bennett T. Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacol Rev.* 1992;44:103-50.
 279. Tooke JE. Diabetic angiopathy. *Oxford University Press Inc., New York.* 1999.
 280. Trost SU, Belke DD and others. Overexpression of the sarcoplasmic reticulum Ca(2+)-ATPase improves myocardial contractility in diabetic cardiomyopathy. *Diabetes.* 2002;51:1166-71.

281. Tsuchiya M , Walsh GM, Frohlich ED. Systemic hemodynamic effects of microspheres in conscious rats. *Am J Physiol*. 1977;233:H617-21.
282. Turko IV, Marcondes S, Murad F. Diabetes-associated nitration of tyrosine and inactivation of succinyl- CoA:3-oxoacid CoA-transferase. *Am J Physiol Heart Circ Physiol*. 2001;281:H2289-94.
283. Turko ILLAKSDCJMF. Protein tyrosine nitration in the mitochondria from diabetic mouse heart. Implications to dysfunctional mitochondria in diabetes. 2003.
284. Turlapaty PD, Lum G, Altura BM. Vascular responsiveness and serum biochemical parameters in alloxan diabetes mellitus. *Am J Physiol*. 1980;239:E412-21.
285. Uchigata Y , Yamamoto H, Kawamura A, Okamoto H. Protection by superoxide dismutase, catalase, and poly(ADP-ribose) synthetase inhibitors against alloxan- and streptozotocin-induced islet DNA strand breaks and against the inhibition of proinsulin synthesis. *J Biol Chem*. 1982;257:6084-8.
286. Ungureanu-Longrois D, Balligand JL, Kelly RA, Smith TW. Myocardial contractile dysfunction in the systemic inflammatory response syndrome: role of a cytokine-inducible nitric oxide synthase in cardiac myocytes. *J Mol Cell Cardiol*. 1995a;27:155-67.
287. Utkan T, Sarioglu Y, Yildirim S. Impaired contraction and relaxation in the aorta of streptozotocin- diabetic rats. *Pharmacology*. 1998;56:207-15.
288. van Dam PS. Oxidative stress and diabetic neuropathy: pathophysiological mechanisms and treatment perspectives. *Diabetes Metab Res Rev*. 2002;18:176-84.
289. van Lambalgen AA, van Kraats AA and others. Organ blood flow and distribution of cardiac output in dopexamine- or dobutamine-treated endotoxemic rats. *J Crit Care*. 1993;8:117-27.
290. Varvarovska J, Racek J and others. Parameters of oxidative stress in-children with Type 1 diabetes mellitus and their relatives. *J Diabetes Complications*. 2003;17:7-10.
291. Venkat Narayan KM, Gregg EW, Fagot-Campagna A, Engelgau MM, Vnncor. Diabetes — a common, growing, serious, costly, and potentially preventable public health problem. *Diabetes research and clinical practice* . 2000;50:S77-84.
292. Verma S and McNeill JH. Metformin improves cardiac function in isolated streptozotocin-diabetic rat hearts. *Am J Physiol*. 1994;266: H714-9.
293. Vinicor F. Is diabetes a public-health disorder? *Diabetes Care*. 1994;17 Suppl 1:22-7.
294. Vinicor F. The public health burden of diabetes and the reality of limits. *Diabetes Care*. 1998;21 Suppl 3:C15-8.

295. Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. *Diabetes Care*. 2003;26:1553-79.
296. Vinik AI, Park TS, Stansberry KB, Pittenger GL. Diabetic neuropathies. *Diabetologia*. 2000;43:957-73.
297. Vinten-Johansen J. Physiological effects of peroxynitrite: potential products of the environment. *Circ Res*. 2000;87:170-2.
298. Volkovova K, Chorvathova V, Jurcovicova M, Koszeghyova L, Bobek P. Antioxidative state of the myocardium and kidneys in acute diabetic rats. *Physiol Res*. 1993;42:251-5.
299. Wakasaki H, Koya D and others. Targeted overexpression of protein kinase C beta2 isoform in myocardium causes cardiomyopathy. *Proc Natl Acad Sci U S A*. 1997;94:9320-5.
300. Wang YX, Lim SL, Pang CC. Increase by NG-nitro-L-arginine methyl ester (L-NAME) of resistance to venous return in rats. *Br J Pharmacol*. 1995;114:1454-8.
301. Wang YX, Zhou T, Pang CC. Pressor effects of L and D enantiomers of NG-nitro-arginine in conscious rats are antagonized by L- but not D-arginine. *Eur J Pharmacol*. 1991;200: 77-81.
302. Watkins PJ. Cardiovascular disease, hypertension, and lipids. *BMJ*. 2003;326:874-6.
303. Watkins PJ and Thomas PK. Diabetes mellitus and the nervous system. *J Neurol Neurosurg Psychiatry*. 1998;65:620-32.
304. Wei XQ, Charles IG and others. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature*. 1995;375:408-11.
305. Weinstein DM, Mihm MJ, Bauer JA. Cardiac peroxynitrite formation and left ventricular dysfunction following doxorubicin treatment in mice. *J Pharmacol Exp Ther*. 2000;294: 396-401.
306. Wentzel P, Welsh N, Eriksson UJ. Developmental damage, increased lipid peroxidation, diminished cyclooxygenase-2 gene expression, and lowered prostaglandin E2 levels in rat embryos exposed to a diabetic environment. *Diabetes*. 1999;48:813-20.
307. Whittle BJ, Morschl E, Pozsar J, Moran AP, Laszlo F. Helicobacter pylori lipopolysaccharide provokes iNOS-mediated acute systemic microvascular inflammatory responses in rat cardiac, hepatic, renal and pulmonary tissues. *J Physiol Paris*. 2001;95:257-9.
308. Williams R and Airey M. Epidemiology of diabetic angiopathy. In: *Diabetic angiopathy*. Ed by Tooke, JE. Oxford University Press Inc., New York. 1999:pp7-18.
309. Winslow C and Dorinsky PM. Regional blood flow distribution in endotoxin-treated

- dogs: modification by ibuprofen. *J Crit Care*. 1994;9:159-68.
310. Wold LE, Relling DP and others. Characterization of contractile function in diabetic hypertensive cardiomyopathy in adult rat ventricular myocytes. *J Mol Cell Cardiol*. 2001;33:1719-26.
 311. Wolfard A, Kaszaki J and others. Effects of selective nitric oxide synthase inhibition in hyperdynamic endotoxemia in dogs. *Eur Surg Res*. 1999;31:314-23.
 312. Wolff SP and Dean RT. Glucose autooxidation and protein modification. The potential role of 'autooxidative glycosylation' in diabetes. *Biochem J*. 1987 ;245:243-50.
 313. World Health Organization. Diabetes Mellitus: report of a WHO Study Group. *Technical Report Series* . 1985;727:Geneva:WHO.
 314. Wray GM, Millar CG, Hinds CJ, Thiemermann C. Selective inhibition of the activity of inducible nitric oxide synthase prevents the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin. *Shock*. 1998;9:329-35.
 315. Wu F, Cepinskas G, Wilson JX, Tyml K. Nitric oxide attenuates but superoxide enhances iNOS expression in endotoxin- and IFNgamma-stimulated skeletal muscle endothelial cells. *Microcirculation* . 2001;8:415-25.
 316. Wu M, Pritchard KA Jr and others. Involvement of nitric oxide and nitrosothiols in relaxation of pulmonary arteries to peroxynitrite. *Am J Physiol*. 1994;266:H2108-13.
 317. Xia Y, Roman LJ, Masters BS, Zweier JL. Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J Biol Chem*. 1998;273:22635-9.
 318. Xia Y and Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Natl Acad Sci U S A*. 1997;94:6954-8.
 319. Xia Z, Godin DV, Ansley DM. Propofol enhances ischemic tolerance of middle-aged rat hearts: effects on 15-F(2t)-isoprostane formation and tissue antioxidant capacity. *Cardiovasc Res*. 2003;59:113-21.
 320. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem*. 1994;269:4705-8.
 321. Xie YW and Wolin MS. Role of nitric oxide and its interaction with superoxide in the suppression of cardiac muscle mitochondrial respiration. Involvement in response to hypoxia/reoxygenation. *Circulation*. 1996;94:2580-6.
 322. Xu R, Morales JA and others. Interleukin-1beta-induced nitric oxide production in rat aortic endothelial cells: inhibition by estradiol in normal and high glucose cultures. *Life Sci*. 1999;64:2451-62.
 323. Yamamoto J , Trippodo NC, Ishise S, Frohlich ED. Total vascular pressure-volume relationship in the conscious rat. *Am J Physiol*. 1980;238:H823-8.

324. Yan SD, Schmidt AM and others. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem.* 1994;269:9889-97.
325. Ye G, Metreveli NS, Ren J, Epstein PN. Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. *Diabetes.* 2003;52:777-83.
326. Yokoyama M, Yagyu H and others. Apolipoprotein B production reduces lipotoxic cardiomyopathy: Studies in heart specific lipoprotein lipase transgenic mouse. *J Biol Chem.* 2003.
327. Young RJ, Beams RM and others. Inhibition of inducible nitric oxide synthase by acetamidine derivatives of hetero-substituted lysine and homolysine. *Bioorg Med Chem Lett.* 2000;10:597-600.
328. Yu Z and McNeill JH. Blood pressure and heart rate response to vasoactive agents in conscious diabetic rats. *Can J Physiol Pharmacol.* 1992;70:1542-8.
329. Yu Z, Quamme GA, McNeill JH. Depressed $[Ca^{2+}]_i$ responses to isoproterenol and cAMP in isolated cardiomyocytes from experimental diabetic rats. *Am J Physiol.* 1994a;266:H2334-42.
330. Yu Z, Tibbits GF, McNeill JH. Cellular functions of diabetic cardiomyocytes: contractility, rapid-cooling contracture, and ryanodine binding. *Am J Physiol.* 1994b;266:H2082-9.
331. Zajaz J, Shrestha A, Poretsky L. The main events in the history of diabetes mellitus. In: *Principles of diabetes mellitus.* Ed by Poretsky, L., Kluwer Academic Publishers, Norwell. 2002:pp18-38.
332. Zaltzberg H, Kanter Y, Aviram M, Levy Y. Increased plasma oxidizability and decreased erythrocyte and plasma antioxidative capacity in patients with NIDDM. *Isr Med Assoc J.* 1999;1:228-31.
333. Zanzinger J, Czachurski J, Saller H. Inhibition of sympathetic vasoconstriction is a major principle of vasodilation by nitric oxide in vivo. *Circ Res.* 1994;75:1073-7.
334. Zou MH, Shi C, Cohen RA. High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes.* 2002a;51:198-203.
335. Laurence, D., Carpenter, J., 1998. A Dictionary of Pharmacology and Allied Topics. 2nd Ed, Elsevier Science B.V.