CARDIOVASCULAR DEPRESSION IN STREPTOZOTOCIN-DIABETIC RATS:
ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE AND EFFECTS OF
ANTIOXIDANT THERAPY

by

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to the required standard

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ABSTRACT

Diabetes mellitus is associated with an increased incidence of cardiovascular complications. Among these, the development of cardiovascular depression is quite common, particularly in animal models of Type 1 diabetes such as streptozotocin (STZ) diabetic rats. These rats exhibit depressed mean arterial blood pressure (MABP) and heart rate (HR). In addition, they also demonstrate attenuated pressor responses in vivo yet enhanced contractile responses to various vasoconstrictor agents in vitro, despite exhibiting impaired endothelial function. Since endothelium derived nitric oxide (NO) is the major vasodilator involved in, it is unclear as to how endothelial dysfunction and subsequent changes in NO bioavailability contribute to the development of cardiovascular depression.

Chronic hyperglycemia is associated with increased oxidative stress and expression of inducible nitric oxide synthase (iNOS) in various cardiovascular tissues. It is possible that the interaction of reactive oxygen species with NO result in the formation of reactive nitrogen species (nitrosative stress) such as peroxynitrite that are detrimental to cardiovascular tissue. Thus, we hypothesized that diabetes causes the induction of iNOS resulting in the elevation of nitrosative stress that may cause cardiovascular depression. Treatment with an antioxidant and/or iNOS inhibitor may therefore improve the cardiovascular depression.

Using the STZ diabetic rat model, we measured the MABP and HR in freely moving conscious rats at different time points in the progression of diabetes (1, 3, 9 and 12 week duration). Using 1400W, a specific inhibitor of iNOS, we investigated the effects of angiotensin-2 (AT II) and methoxamine on changes in MABP and HR. In addition, using immunohistochemistry, we investigated the localization of iNOS, endothelial nitric oxide synthase (eNOS) and nitrotyrosine (NT) proteins in different cardiovascular tissues. We also studied the acute and chronic effects of antioxidant therapy using N-acetylcysteine (NAC), a strong antioxidant on iNOS mediated cardiovascular abnormalities in STZ diabetic rats. Further, we investigated the effects of NAC treatment on cardiovascular depression as measured by the MABP and HR in 3 and 9 week diabetic rats. In addition, using immunohistochemical techniques, the expression of eNOS, iNOS and NT were examined in different cardiovascular tissues. Also, the effect of NAC treatment on plasma nitrite and nitrate levels (NOx) and antioxidant defense mechanisms were studied.
There are several observations from our studies. STZ diabetic rats had depressed MABP and HR by three weeks of diabetes. Treatment with NAC significantly improved the MABP and HR. In addition, STZ diabetic rats demonstrated impaired endothelial function as shown by attenuated pressor response to a non-selective nitric oxide synthase inhibitor, N\textsubscript{o}-nitro-L-arginine methyl ester hydrochloride (L-NAME). Pressor responses to bolus doses of methoxamine and AT II were attenuated by 3 weeks of diabetes. Methoxamine produced attenuated pressor responses in 12-week diabetic rats compared to 1-week diabetic rats. Similarly, the pressor responses to AT II were also significantly reduced in 9 and 12-week diabetic rats compared to 1-week diabetic rats. These results suggest that the duration of diabetes significantly affects the pressor responses of both methoxamine and AT II.

Pretreatment with 1400W did not affect the MABP and HR responses of either methoxamine or AT II in control rats. However, selective inhibition of iNOS by 1400W completely restored the pressor responses to both AT II and methoxamine in all diabetic groups except the 12-week diabetic group, where methoxamine increased the pressor response but failed to normalize it. These data suggest that iNOS plays a significant role in modulating the pressor responses to vasoactive agents in diabetes.

Diabetic rats exhibited a differential regulation of iNOS and eNOS wherein prolonged diabetic state led to a downregulation of eNOS with a concomitant upregulation of iNOS protein expression. iNOS protein was first detected in the myocardial tissue of 3-week diabetic rats and the intensity increased with the duration of diabetes. This was associated inversely with the expression of eNOS, wherein prolonged diabetes led to decreased expression of eNOS in diabetic rat heart, aorta and superior mesenteric arteries. Increased expression of iNOS in concert with increased oxidative stress results in the formation of reactive nitrogen species such as peroxynitrite. Immunohistochemical localization of NT, an indirect marker of peroxynitrite reveals an increased formation of reactive nitrogen species in the heart and arteries of diabetic rats.

A triphasic response of unchanged, decreased and elevated levels of plasma nitrite/nitrate (NO\textsubscript{x}) concentrations was observed in 3, 9 and 12-week diabetic rats respectively. This observation is difficult to explain with the data available but it may be possible that a simultaneous increase of reactive oxygen species (ROS) and NO in the beginning (unchanged), followed by increased scavenging of NO by ROS (decreased) by 9
weeks and finally leading to an uncontrollable production of NO from iNOS (increased) in 12 weeks may affect the plasma NOx levels. However, further studies are required to validate the conclusions.

NAC treatment normalized the expression of iNOS, eNOS and NT in these tissues. Our results indicate that chronic hyperglycemia may facilitate the downregulation of eNOS by producing large quantities of NO and/or reactive nitrogen species from iNOS. This was supported by the reduced levels of nitrosative stress (NT) observed in diabetic rats treated with NAC. In addition, a significant reduction in plasma 15-F_{2t} isoprostanes, increased plasma NOx concentration, enhanced myocardial antioxidant capacity and total antioxidant concentrations were observed in NAC treated diabetic rats.

Results presented in this thesis therefore suggest that depressed MABP and HR in STZ diabetic rats may be due to increased levels of nitrosative stress. The activation of iNOS, which depends on the duration of diabetes, may assume a critical role under oxidative conditions in modulating pressor responses to vasoactive agents. The interactions between iNOS and oxidative stress in diabetes could thus influence the subsequent hemodynamic outcomes.
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C. Nitrosative stress in diabetes

- Inducible nitric oxide synthase in diabetes
- iNOS - a possible link between diabetes and cardiovascular depression

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2.1 Specific research objectives and rationale

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- **Study-2a**: The effect of selective iNOS inhibition (1400W) on the hemodynamic effects of vasoactive agents in unrestrained conscious STZ diabetic rats: Effect of the duration of diabetes.

- **Study-2b**: The effect of duration of diabetes on the differential regulation of eNOS and iNOS in STZ diabetic rats.

- **Study-3**: The effect of N-acetylcysteine, an antioxidant on iNOS mediated cardiovascular changes in acute and chronic STZ diabetic rats.

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<td>--------------</td>
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<tr>
<td>+dP/dT</td>
<td>Rate of contraction</td>
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<td>N-[3(Aminomethyl) Benzyl] acetamidine, dihydrochloride</td>
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<td>2hPG</td>
<td>Two-hour postprandial glucose</td>
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<td>8-iso PGF 2α</td>
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<tr>
<td>Ach</td>
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<td>AChE</td>
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<td>American Diabetes Association</td>
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<tr>
<td>ADMA</td>
<td>Dimethylarginine</td>
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</tr>
<tr>
<td>AG</td>
<td>Aminoguanidine</td>
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<tr>
<td>AGE</td>
<td>Advanced glycosylation end product</td>
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<td>ANOVA</td>
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<td>ANS</td>
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<td>AR</td>
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<td>BB rat</td>
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<td>BH4</td>
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<td>CHG</td>
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<td>cGMP</td>
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<td>-dP/dT</td>
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<td>FMN</td>
<td>Flavin adenine mononucleotide</td>
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<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
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<td>GTP</td>
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<tr>
<td>H₂O₂</td>
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<td>--------------</td>
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<tr>
<td>HLA-D</td>
<td>Histocompatibility complex (Class II)</td>
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<td>HPLC</td>
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<tr>
<td>HR</td>
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<tr>
<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<tr>
<td>IF-γ</td>
<td>Interferon- γ</td>
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<tr>
<td>IGF-1</td>
<td>Insulin like growth factor</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
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<td>Interleukin-1 β</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
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</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
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<tr>
<td>LCMS</td>
<td>Liquid chromatography-Mass spectrometry</td>
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<tr>
<td>L-NAME</td>
<td>Nω-nitro-L-arginine methyl ester hydrochloride</td>
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<td>L-NMMA</td>
<td>NG-monomethyl-L-arginine monoacetate</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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</tr>
<tr>
<td>LVP</td>
<td>left ventricular developed pressure</td>
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</tr>
<tr>
<td>MABP</td>
<td>Mean arterial blood pressure</td>
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</tr>
<tr>
<td>MAD</td>
<td>Malonyldialdehyde</td>
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</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
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</tr>
<tr>
<td>mNOS</td>
<td>Mitochondrial nitric oxide synthase</td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>Methoxamine</td>
<td></td>
</tr>
<tr>
<td>NA/NE</td>
<td>Noradrenaline or norepinephrine</td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
<td></td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide dinucleotide reduced</td>
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<td>Nicotinamide adenine dinucleotide phosphate reduced</td>
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<td>NBF</td>
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<td>nNOS</td>
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<td>Nitrogen dioxide</td>
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<td>Nitric Oxide synthase</td>
<td></td>
</tr>
<tr>
<td>NOx</td>
<td>Nitrite and nitrate level</td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>Nitrotyrosine</td>
<td></td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide anion</td>
<td></td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral Glucose tolerance test</td>
<td></td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxyl ion</td>
<td></td>
</tr>
<tr>
<td>ONO 1714</td>
<td>7-chloro-3-imino-5-methyl-2-azabicycloheptane hydrochloride</td>
<td></td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
<td></td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>PE-50</td>
<td>Polyethylene-50</td>
<td></td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol biphosphate</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
<td></td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
<td></td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
<td></td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
<td></td>
</tr>
<tr>
<td>RAS</td>
<td>Renin aldosterone angiotensin system</td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay assay</td>
<td></td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
<td></td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
<td></td>
</tr>
<tr>
<td>SH</td>
<td>Sulphahydryl group</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
<td></td>
</tr>
<tr>
<td>SMA</td>
<td>Superior mesenteric artery</td>
<td></td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
<td></td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
<td></td>
</tr>
<tr>
<td>t-BHP</td>
<td>t-butylhydroperoxide</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor</td>
<td></td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
<td></td>
</tr>
<tr>
<td>VAM-1</td>
<td>Vascular adhesion molecule-1</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
<td></td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
<td></td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto rat</td>
<td></td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty rats</td>
<td></td>
</tr>
</tbody>
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LIST OF PUBLICATIONS

Publications related to the present thesis


Other publications


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1. INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a major chronic metabolic disorder characterized by elevated levels of blood glucose. Diabetes mellitus was recognized as early as 1500 BC by Egyptian physicians, who described this as a disease associated with “passage of more urine”. The term diabetes (the Greek word for siphon) mellitus (meaning honey) was coined by Willis in 1674\(^1\). Today diabetes is a serious and economically devastating illness that is reaching epidemic proportions\(^2\). In the year 2000 diabetes was the fifth leading cause of mortality in the United States\(^3\).

The incidence and prevalence of DM is increasing rapidly. In fact it has increased five fold from 1959 to 1993\(^4\). In 1985, approximately 30 million people were suffering from DM and it is projected that this number will increase to 300 million people, which will be 5.4% of the world population, by 2025\(^5\). Canada, though a developed country, is no exception to the incidence of DM. Approximately 3.5% of all Canadians or 0.9 million people aged between 12 or older were diagnosed as having DM by the year 1999\(^6\). Given the fact that there are large numbers of undiagnosed cases of DM\(^7\), it is believed that up to 2.6 million Canadians may presently have DM and this number is projected to reach 3 million by 2010\(^8\).

DM is thus a serious health problem throughout the world. It is the number one cause of adult blindness, end stage renal failure and nontraumatic amputations in the United States\(^9,10,11\). It also is a major risk factor for many of the cardiovascular, cerebrovascular and peripheral artery diseases and consequently it is increasing economic burden\(^12\). In 1998 it was estimated that the total annual economic burden of diabetes in Canada was around 5 billion US\$\(^13\). In the US as much as 15% of the total health care expenditure is being absorbed by DM alone\(^14\). DM therefore, is a major public health disorder and meets the essential criteria that define the term “a high disease burden”\(^15\).
Definition and classification of diabetes mellitus

DM is a chronic disorder affecting carbohydrate, fat and protein metabolism. A characteristic and prominent feature of DM is hyperglycemia, a reflection of deranged carbohydrate (glucose) utilization resulting from a defective or deficient insulin secretory response\textsuperscript{16}.

In 1997, following the recommendations of the American Diabetes Association (ADA), a revised scheme of classification and diagnostic criteria were adopted by many international health agencies including the World Health Organization (WHO) and the Canadian Diabetes Association (CDA)\textsuperscript{8,17}. Under this scheme, the classification of DM is based on the etiology rather than type of pharmacotherapy used. Accordingly the previous notions of “insulin dependent diabetes mellitus” (IDDM) and “non-insulin dependent diabetes mellitus” are being replaced by the terms “Type 1 diabetes and Type 2 diabetes”. Further changes were incorporated to this classification in 2001. Under the sponsorship of the ADA, an international expert committee further classified DM into four main etiological categories which are as follows,

- Type 1 DM caused by pancreatic $\beta$ cell destruction leading to absolute insulin deficiency (8-10\% of all cases).
- Type 2 DM resulting from insulin resistance (IR) with relative insulin deficiency or an insulin secretory defect (~ 90\% of all cases).
- Other specific types of DM consisting primarily of specific genetically related forms of diabetes or diabetes associated with other disease or drug use and
- Gestational DM related to glucose intolerance with onset during pregnancy\textsuperscript{8}.

According to the latest diagnostic criteria (ADA), diagnosis of diabetes can be made by the following 3 ways,

- Random plasma glucose values of $\geq 11.1$ mM in the presence of other diabetic symptoms such as fatigue, polyuria, polydipsia and unexplained weight loss.
- Fasting plasma glucose values of $\geq 7.0$ mM
- Two-hour postprandial (2hPG) glucose levels of $\geq 11.1$ mM during an oral glucose tolerance test (OGTT) with an oral glucose load of 75g of glucose.

In all the above cases, a confirmatory test has to be done on a subsequent day.
Pathogenesis of diabetes mellitus

Type 1 diabetes results from a severe, absolute lack of insulin caused by a reduction in the β cell mass. This type of DM usually develops in childhood but can occur at later ages as well. A great deal of research has been done to understand the pathogenesis of Type 1 DM. Most studies speculate on three common interlocking mechanisms collectively responsible for islet cell destruction. These include genetic susceptibility, autoimmunity and an associated environmental insult. It is suggested that a genetic susceptibility to altered immune regulation, linked to specific alleles in the class-II major histocompatibility complex (HLA-D), predisposes certain individual to the development of autoimmunity to islet β cells. The autoimmunity which may develop spontaneously, can be triggered by a variety of environmental factors such as viruses, chemicals or toxins, resulting in acute insulitis and damage to B cells. An autoimmune reaction directed against B cells further causes damage to the pancreatic β cells resulting in DM.

Diabetic Complications: Acute and Chronic

Acute complications of DM include polyuria, polyphagia, polydipsia, weakness, weight loss, dry skin and less often ketoacidosis. Prior to the discovery of insulin by Banting and Best in early 1900s, the outlook for this disease was very grim, as patients would succumb to fatal consequences due to severe acute complications such as ketoacidosis. However, with the introduction of insulin therapy, mortality due to acute diabetic complications decreased dramatically but the prolongation of the survival still carried a high price in terms of morbidity. Two decades after insulin had become available, the clinical impact of complications arising from chronic diabetes became much more evident when a growing number of reports documented the occurrence of chronic complications. These include macrovascular diseases (peripheral, cerebral, cardiovascular), microvascular diseases (retinopathy and nephropathy), neuropathy (peripheral and autonomic), foot problems and a host of other complications. It is of interest to note that all the above complications were observed despite efforts to attain tight metabolic control with insulin therapy. The present understanding is that, regardless of the etiology, long term diabetes often leads to a cluster of abnormalities resulting in damage, dysfunction and organ failure including heart, kidneys, nerves and eyes. Clinically these consequences are manifested in the form of end stage renal
failure, neuropathy, ocular disorders and cardiac diseases such as angina, atherosclerosis, cardiomyopathy, stroke, myocardial infarction, heart failure, hypertension etc.

**Diabetes and cardiovascular complications**

Cardiovascular disease is the leading cause of mortality among patients with DM\(^{23,24}\). The incidence of cardiovascular disease is three to four fold higher in the presence of diabetes compared to patients without diabetes. It is reported that up to 80% of deaths in diabetic patients are due to cardiovascular events. It was therefore no surprise when the American Heart Association (AHA) stated, “from the point of view of cardiovascular medicine, it may be appropriate to say, diabetes is a cardiovascular disease”\(^{25}\). For the sake of simplicity, cardiovascular complication in DM can be categorized into three major groups as follows,

- Diseases of large vessels (macroangiopathy) may lead to the development of coronary artery disease, atherosclerosis, peripheral vascular disease, stroke, hypertension and myocardial infarction\(^{26,27}\).
- Diseases of microvasculature (microangiopathy), characterized by structural and functional alterations in the vessels such as basement membrane thickening, increased vascular permeability and microaneurysms, may contribute towards the development of diabetic retinopathy, neuropathy, nephropathy, cardiomyopathy and gangrene\(^{28,29,30}\).
- Cardiac muscle disease, also known as cardiomyopathy occurs independent of any major vascular disease in diabetes\(^{31}\). It is characterized by impaired ventricular performance often manifested clinically in terms of shorter left ventricular ejection time, a longer pre-ejection period and a higher ratio of pre-ejection period/ left ventricular ejection time \(^{32,33,34}\).

**Potential mechanisms underlying diabetic complications**

Ever since the discovery of insulin, the most significant controversy in the field of diabetes has revolved around the question “does glycemic control affect the appearance and progression of chronic complications?” Evaluating this question, a number of long-term, multi-centered, randomized, prospective studies involving Type 1 diabetes patients were conducted\(^{23}\). The reports of these studies overwhelmingly suggest that effective glycemic
control is the key in preventing or reversing the metabolic derangements and complications due to long-term diabetes.

Although chronic hyperglycemia may be the most important cause of cardiovascular disease in DM, a number of equally tenable mechanisms have been proposed to initiate or mediate the cardiovascular complications of DM. While the molecular mechanisms leading to chronic complications have not been conclusively delineated, several abnormal pathways have been suggested. Most salient in this list of deleterious biochemical pathways is the production of high concentration of advanced glycosylation end (AGE) products and sorbitol.

**Advanced glycosylation end products**

Glucose forms chemically reversible glycosylation products with proteins at a rate proportional to the ambient glucose concentration. These glycosylated proteins are highly reactive, forming bonds with other glycosylated proteins, collagen and other molecules, eventually forming AGE. These products, being stable are incorporated into the basement membrane matrix of capillaries causing various cardiovascular abnormalities\(^{35,36}\). There is evidence that AGE, particularly in collagen correlate with the severity of diabetic complications\(^{37}\).

**Polyol pathway**

In insulin independent tissues such as endothelium, neurons, liver, kidney, brain etc, hyperglycemia leads to an increase in intracellular glucose concentration. This results in the formation of sorbitol by aldose reductase and eventual formation of fructose by dehydrogenase enzyme. The accumulated sorbitol and fructose in turn decrease myoinositol content resulting in decreased phosphoinositol metabolism, diacyl glycerol (DAG), protein kinase C (PKC) and Na\(^+/\)K\(^+\)-ATPase activity. This pathway has been implicated in many microvascular diseases of DM. That this pathway may contribute to the ocular and neurological complications is supported by the experimental studies using aldose reductase inhibitors\(^{38,39}\).

In addition to the above pathways, an increase in oxidative stress (discussed in detail in the later sections) in DM is being implicated in various cardiovascular complications\(^{40,41,42,43}\). As well, the activated DAG-PKC pathway and endothelin system appears to be
involved in diabetic complications\textsuperscript{44,45,46,47}. In general, abnormalities in vascular sensitivity and reactivity to various ligands, alterations in cardiac autonomic function, altered myocardial substrate utilization and abnormal ionic movements all have been implicated in diabetic cardiovascular complications\textsuperscript{48,49,50}. The list of the proposed mechanisms is long and growing and thus represents the multifactorial nature of the causes underlying diabetic complications. Perhaps each proposed pathway is a different representation of a common underlying pathogenic mechanism; or more likely, perhaps different tissues are susceptible to different mechanisms.

1.2 CARDIOVASCULAR ABNORMALITIES IN DIABETES: HEMODYNAMIC CHANGES.

Clinical studies indicate that cardiovascular dysfunction is a major cause of death in patients with Type 1 diabetes. Whether these cardiovascular dysfunctions are a consequence of direct cardiovascular deficits (such as abnormalities of the heart and vasculature apart from neural defects) and/or indirect autonomic neuropathy is still unclear. However, epidemiological studies suggest that cardiac dysfunction, either alone or in combination with vascular injury can significantly increase the morbidity and mortality in diabetic patients\textsuperscript{51,52}. Cardiac abnormalities such as cardiomyopathy and coronary artery diseases contribute significantly to the mortality rate in diabetic patients. Hemodynamic abnormalities such as changes in blood flow and vascular contractility have been extensively documented in various organs including kidneys, retina, peripheral vasculature and nerves. These abnormalities manifest themselves clinically in the form of diabetic nephropathy, retinopathy, diabetic foot and neuropathy. All the above manifestations of cardiovascular abnormalities in humans have also been studied and well documented in variety of animal models of Type 1 diabetes\textsuperscript{53}.

**Cardiac abnormalities in diabetes**

A number of \textit{in vitro} and \textit{in vivo} studies have shown depressed cardiac contractility in various models of Type 1 DM. Following injection of streptozotocin (STZ), isolated cardiac myocytes have been shown to exhibit impaired contractility both in short and long term diabetes\textsuperscript{54,55}. Studies using isolated working hearts have demonstrated impaired left


ventricular pressure development (LVP), rate of contraction (+dP/dT) and rate of relaxation (-dP/dT) in diabetic rats. In vivo studies have reported a lower resting heart rate, systolic pressure and left ventricular ±dP/dT in STZ diabetic rats.

In addition, the inotrophic effects of various adrenergic agonists have been reported to be impaired in animal models of diabetes. Sensitivity to β-adrenergic receptor (AR) agonists such as isoproterenol and noradrenaline is decreased in diabetes. Studies examining the β-AR density and activity have reported that cardiac membrane β AR receptor numbers are decreased in diabetic rats. Alterations in cardiac autonomic function and muscarinic receptors have also been implicated in reduced cardiac contractility in diabetic rats. Results of insulin treatment of STZ diabetic rats from our lab suggest that changes in heart function are due to insulin deficiency or chronic hyperglycemia. In addition, changes in other hormones and elevated levels of catecholamines have also been suggested to be involved in cardiac dysfunction in diabetes.

Diabetes associated changes in cardiac function in the STZ diabetic rats are not entirely consistent with alterations observed in diabetic patients. For example, in contrast to the depressed MABP in STZ diabetic rats, clinical studies indicate that diabetes is associated with normal blood pressure and /or high blood pressure. Hypertension is particularly seen in Type 1 diabetic patients with a major renal disease. The differences observed between the clinical and experimental scenario may be due to the variation in species and method of induction of diabetes (natural vs chemically induced). Further, treatment in patients is usually initiated almost immediately following diagnosis of diabetes, which is not the case with diabetic animals. Nevertheless, some of the cardiac abnormalities in animal models are mirrored in human diabetes. These include ventricular dysfunction with diastolic abnormalities, impaired vagal and sympathetic control of heart, blunted tachycardiac responses to exercise and to drug-induced reductions in arterial pressure and to postural changes and decrease in circadian variation in HR. All the above factors contribute significantly to higher mortality rate in diabetic patients. For example, reduced heart rate variability in diabetic patients is associated with a high mortality from sudden cardiac death.
Vascular abnormalities in diabetes

A large number of studies have looked at the effect of diabetes on vascular morphology and function using a wide variety of animal and human vascular beds\(^7\)\(^6\). There are considerable discrepancies among the results reported so far. Most of the studies have demonstrated impaired, contractile and relaxant effects to vasoactive agents in diabetic animals. Among these, altered responses to noradrenaline (NA), methoxamine and calcium have been reported in isolated diabetic arteries\(^7\)\(^7\)\(^8\). In vitro studies utilizing large conductance vessels such as aorta and smaller conductance vessels such as mesenteric arteries have shown either a reduced or enhanced contractility to vasoconstrictor agents\(^7\)\(^9\)\(^4\)\(^9\). The reasons for these inconsistencies are not apparent, but the variability is generally attributed to the differences in the type of diabetogens used, gender differences, the techniques used to measure contractility and, most important of all, is the progression, duration and severity of the disease.

In contrast, most of the in vivo studies with STZ diabetic rats have demonstrated attenuated pressor responses to vasoconstrictor agents\(^8\)\(^0\)\(^8\)\(^1\). Diminished pressor responses to NA, angiotensin-2 (AT II), phenylephrine (PE) and methoxamine have been reported in conscious STZ diabetic rats\(^5\)\(^9\)\(^6\)\(^0\)\(^6\)\(^5\). In addition, pressor responses to acetylcholine were also depressed in STZ diabetic rats\(^6\)\(^0\)\(^8\)\(^2\). These results suggest an impairment of endothelial function in diabetic arteries. Studies in humans with DM, assessing endothelial function have also reported impaired vasodilatation to the infusions of muscarinic agonists such as acetylcholine, methanachol and carbachol\(^8\)\(^3\)\(^8\)\(^4\). These results suggest that vascular relaxation and contraction are impaired both in human and animal models of Type 1 diabetes.

The impairment of cardiac and vascular function in DM thus may contribute to a state of cardiovascular depression characterized by reduced cardiac output, heart rate, depressed blood pressure and inadequate organ perfusion. Various mechanisms have been proposed to explain the pathogenesis of cardiovascular depression in DM. Among these inducible nitric oxide synthase (iNOS) mediated formation of NO and associated oxidative stress are being explored as potential mechanisms.
1.3 ANIMAL MODELS OF TYPE 1 DIABETES: THE STREPTOZOTOCIN (STZ) DIABETIC MODEL

Animal models of diabetes are enormously useful in understanding the etiologies of diabetic complications, both acute and chronic. In addition to having a shorter life span, these animals also feature many physiological and pathophysiological characteristics similar to those seen in human diabetes. Various animal models of both genetic and chemically induced diabetes have been developed. Genetic models of Type 1 diabetes include the spontaneously diabetic biobreeding (BB) Wistar rats and the non-obese diabetic (NOD) mouse. Genetic models of Type 2 diabetes include the Zucker diabetic fatty (ZDF) rat and the db/db and ob/ob mice. The genetic models allow the identification of specific genes and environmental triggers associated with the development of the disease. However the use of genetic models is limited due to high cost and inherent genetic defect in metabolic regulatory systems in their normoglycemic control littermates.

Chemically induced models such as the streptozotocin and alloxan models of diabetes allow for better control of the duration and severity of the diabetic state. These chemicals specifically cause β-cell necrosis leading to the development of hyperglycemia and a hypoinsulinemic state and mimic human Type 1 diabetes. Streptozotocin is preferred over alloxan as a diabetogenic agent due to its greater selectivity for pancreatic β-cells and for lower mortality.

Streptozotocin (2-deoxy-2- (3-methyl-3-nitrosourea) 1-D glucopyranose is a broad spectrum antibiotic obtained from Streptomyces achromogenes. A glucose molecule along with a highly reactive and cytotoxic nitrosourea in the chemical structure of STZ directs this moiety to the pancreatic islets and initiates the destruction of β-cells. Uptake across the cell membrane is mediated by GLUT2 receptors. Since it discovery as a cytotoxic agent forty years ago, various mechanisms have been proposed to explain its β-cell toxic effects. Generation of free radicals, DNA strand breaks, activation of the enzyme poly (ADP-ribose) polymerase (PARP) and depletion of intracellular nicotinamide adenine dinucleotide (NAD) are some of the factors believed to be responsible for β-cell death in the STZ induced diabetic model.
Over a dose range of 45-100 mg/kg, STZ (either intravenously or intraperitoneally) produces the characteristic symptoms of Type 1 diabetes such as polydipsia, polyphagia, decreased body weight gain, hyperglycemia, hypoinsulinemia and elevated levels of plasma lipids\textsuperscript{93,56}. In addition to the dose, the severity of diabetes also depends on the strain, gender and age of the rat\textsuperscript{94}. In our laboratory, induction of diabetes in male Wistar rats is achieved by a single tail vein injection of 60 mg/kg of STZ\textsuperscript{95}.

Chronic diabetic rats exhibit many cardiovascular abnormalities similar to those seen in human diabetic patients\textsuperscript{49}. Studies with isolated working heart have demonstrated impaired ventricular performance\textsuperscript{67,96}. In vivo studies employing rodent echocardiography have confirmed many of the cardiac functional aberrations associated with chronic STZ diabetic rats\textsuperscript{97}. Furthermore numerous \textit{in vivo} and \textit{in vitro} studies have reported the occurrence of functional abnormalities in vascular tissues of STZ diabetic rats\textsuperscript{81,77,98,99}. Thus, STZ diabetic model offers many advantages and allows for the evaluation of pharmacological interventions to gain an insight into the disease processes and therapeutic modalities that could prevent or reverse the abnormalities present in humans.

1.4 POTENTIAL MECHANISMS OF CARDIOVASCULAR DEPRESSION IN DIABETES.

A. Nitric Oxide System

Nitric oxide (NO) is a simple molecule of a 1:1 combination of the two most abundant gases in the atmosphere. Until 1987 it was thought to be a pollutant or a product of electrochemical storms but now it is the subject of vigorous and intensive research in biology. In 1980, Furchgott and Zawadski discovered the endogenous endothelium derived relaxation factor (EDRF) when they were testing the effect of muscarinic agents on an isolated blood vessel preparation\textsuperscript{100}. This EDRF was subsequently shown to be NO by Ignarro and others\textsuperscript{101}.

NO is a ubiquitous molecule generated in tissue and participates in several important physiological functions in the body including vasodilatation, neurotransmission and elimination of pathogens. It also initiates or mediates many pathophysiological processes in
several cardiovascular diseases. NO produced in the tissue is soluble in water and lipid and can easily diffuse across the membranes. Its half-life is very short, a few seconds and it is rapidly inactivated when comes in contact with hemoglobin.

Nitric oxide synthesis and actions

NO is produced from many cells. Its synthesis requires nitric oxide synthase (NOS) in addition to the substrate, L-arginine and cofactors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄), flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), oxygen and protoporphyrin IX. Three different isoforms of NOS have been cloned and characterized so far. These are neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Neuronal and endothelial NOS's are constitutively expressed and produce NO in smaller quantities and are regulated by calcium. Inducible NOS is a calcium independent NOS and can be induced by endotoxins, proinflammatory cytokines and other stimuli. iNOS induction results in the production of large quantities of NO and is being implicated in many disease processes and pathological states including both forms of diabetes. Another form of uncharacterized NOS called mitochondrial NOS (mNOS) is suggested to be localized in mitochondria and is being implicated in some cardiovascular disease processes. It is widely believed that low levels of NO produced by constitutive NOS are responsible for physiological actions while higher quantities of NO produced by iNOS produces pathophysiological effects.

NO or related reactive nitrogen species can oxidize, nitrate or nitrosylate proteins that account for the myriad of physiological responses mediated by NO including vasodilatation, oxygen consumption, neurotransmission and apoptosis. At nanomolar concentrations, NO reversibly nitrosylates soluble guanylate cyclase (sGC) and catalyzes the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). Elevated levels of intracellular cGMP can further activate various isoforms of cGMP dependent protein kinases (PKG) downstream. Relaxation of vascular smooth muscle in response to increased NO/cGMP is mediated by a specific isoform of PKG (PKG₁α). In fact the entire physiological cell signaling processes of NO occurs in the nanomolar concentration range.
At higher concentrations, NO affects a wide variety of haeme containing and redox sensitive enzymes and ion channels and has both reversible and irreversible effects. Higher steady state micromolar concentrations of NO produce irreversible toxic effects. Since NO is a free radical, its interaction with superoxide anion ($O_2^-$) leads to the generation of peroxynitrite (ONOO$^-$), a powerful peroxidant that can cause significant cell damage. This reaction is approximately 3 times faster than the dismutation of superoxide by superoxide dismutase (SOD). Peroxynitrite can be protonated further to produce peroxynitrous acid, which in turn can yield hydroxyl radical. All the above nitrogen species are highly reactive and unstable and can oxidize lipids, thiol groups of many antioxidant enzymes and damage cell membranes\textsuperscript{110}. They can also covalently modify intracellular proteins including eNOS, damaging them or rendering them less active\textsuperscript{111}. The detoxification of peroxynitrite is possible when it combines with reduced glutathione (GSH) or other thiols to form S-nitrosoglutathione or other nitrosothiols thus depleting the antioxidant concentration and their capacities. NO is thus physiological at lower concentrations and pathological at higher concentrations. The pathological actions of NO are not mediated by NO \textit{per se} but mediated by its oxidative products, most notably peroxynitrite.

\textit{Nitric oxide and cardiovascular functions in diabetes}

Several important concepts have evolved about the actions of NO in the cardiovascular system soon after the discovery of EDRF as NO. Nitric oxide plays a pivotal role in the regulation of cardiovascular and renal homeostasis. Physiologically, smaller and continuous release of NO from endothelial NOS causes vascular smooth muscle (VSM) relaxation and maintains the vasculature in a state of active vasodilatation. A basal low level of NO acts as an endogenous auto regulator of blood flow to tissues including heart and kidney in response to local changes. NO synthesis is important to maintain an increased renal blood flow, to suppress the activated renin-aldosterone-angiotensin (RAS) system and increased sympathetic nervous system (SNS) activity in diabetes\textsuperscript{112,113}. An increased dependence on a functional nitric oxide system has been reported, which prevents the development of hypertension in STZ diabetic rats\textsuperscript{114}. It has been demonstrated that non-selective blockade of NO synthesis results in increased mean arterial blood pressure (MABP) at the onset of diabetes. These findings imply the presence of increased NO to counteract the
pressor effects of endogenous mediators with the progress of diabetes. Defects in the regulation of NOS such as endothelial dysfunction (ED) and impaired NO production can thus lead to vascular diseases in diabetes. Also, NO reduces blood clotting and inhibits platelet aggregation and adhesion and lack of NO may favor and aggravate thrombosis.\(^{115}\)

NO from different sources can modulate regional cardiac contractility. NO generated from eNOS in vascular endothelium can diffuse into cardiac myocytes and may alter function and metabolism. Low levels of NO increase cardiac contractility through a cAMP dependent mechanism while high level NO decrease contractility by cGMP dependent mechanism.\(^{116}\) It is well recognized that NO exhibits potent actions on heart such as depressed cardiac contractility, inhibition of the positive inotropic effect to β-AR agonists and decreased heart rate and cardiac output associated with ventricular dysfunction. Low levels of NO can delay the progression of heart failure and shift the utilization of substrate from free fatty acids to lactate.\(^{117}\) NO overproduction however is toxic to the cardiovascular system. Increased production of NO exerts negative inotropic effects and may cause left ventricular dysfunction and heart failure. In pathological conditions such as diabetes, iNOS is activated and expressed in various tissues such as cardiac myocytes, VSM cells, macrophages, platelets and renal tissues. Large amounts of NO produced by iNOS particularly when associated with increased oxidative stress have the capacity to affect multiple enzymes and signaling systems. In fact, increased production of NO through iNOS causes severe systemic hypotension and septic shock.\(^{118}\) It is suggested that overproduction of NO in the smooth muscle cell leads to many of the key indicators of endotoxemia, including depressed vasoconstriction, a hypodynamic vascular syndrome and refractory hypotension which may contribute to multiple organ failure.

**Endothelium, vascular tone and endothelial dysfunction**

The vascular endothelium is the inner lining of all blood vessels and serves as an important paracrine and autocrine organ that regulates vascular wall function including the maintenance of vascular tone.\(^ {119}\) Because of its strategic location between the circulating blood and the vascular wall, the endothelium interacts with cellular and neurohumoral mediators, thus controlling vascular contractile state and cellular composition.\(^ {120}\) The vascular endothelium maintains vascular homeostasis by modulating blood vessel tone, by
regulating local cellular growth and extracellular matrix deposition and by controlling homeostatic as well as inflammatory response\textsuperscript{121}. The general belief that the vascular endothelium represents a mere mechanical barrier between the intravascular and interstitial compartments has been disputed and revised over the past two decades. The current understanding about the vascular endothelium, as an organ responsible for the regulation of hemodynamics, vascular remodeling, metabolic, synthetic, inflammatory, anti- and pro-thrombic processes has been largely substantiated with the discovery of nitric oxide and its role in various pathologic conditions. The vascular endothelium is now believed to be like any other organ system and is subject to dysregulation, dysfunction, insufficiency and failure. Endothelial dysfunction has been largely implicated in various pathological conditions such as stroke, myocardial infarction, hypertension, diabetes induced vascular complications and atherosclerosis\textsuperscript{122}.

The endothelium plays an important modulatory role in regulation of vascular tone and its reaction to stimuli by secreting various paracrine and autocrine substances. The underlying vascular smooth muscle undergoes contraction or relaxation to a variety of extracellular, neural and humoral signals, ionic and mechanical forces to maintain the vasomotor tone and in turn the functional capacity of the blood vessel\textsuperscript{122}. Thus vasomotor tone is the end result of a complex set of interactions that control relaxation and contraction of blood vessels or in other words vasomotor tone is maintained by an equilibrium between the cell signaling pathways that mediate the generation of force (contraction) and release of force (relaxation)\textsuperscript{122}.

Mechanisms leading to an elevation of intracellular calcium concentration in VSM can trigger VSM contraction either by direct opening of voltage dependent calcium channels or secondary to receptor mediated actions of numerous endogenous mediators such as NA, AT II, endothelin-1 (ET-1), vasopressin and thromboxane A\textsubscript{2} (TxA\textsubscript{2})\textsuperscript{123}. The endogenous vasoconstrictors bind to specific subunits (G\textsubscript{q}) of G-protein coupled receptors on VSM cells causing activation of phospholipase C (PLC) leading to the formation of inositol triphosphate (IP\textsubscript{3}) and DAG from phosphatidylinositol biphosphate (PIP\textsubscript{2})\textsuperscript{124}. IP\textsubscript{3} then causes the release of calcium from sarcoplasmic reticulum (SR) stores and DAG activates PKC, which in turn phosphorylate various downstream proteins that bring about a contraction. Subsequent to the rise in intracellular calcium induced by IP\textsubscript{3}, the calcium calmodulin pathway is activated and
initiates the phosphorylation cascade of myosin light chain kinase (MLCK) and actin-myosin crossbridging that eventually culminates in a contraction. Membrane phospholipids when acted upon by phospholipase A$_2$ (PLA$_2$) or PLC result in the formation of arachidonic acid (AA) that serves as an important precursor for various prostaglandins and thromboxane which in turn influence the vasomotor tone.

Mechanisms resulting in decreased intracellular VSM calcium concentration complement and counteract, the contractile responses of various endogenous and exogenous ligands to maintain vasomotor tone and the execution of hemodynamic adaptation in physiologic and pathologic conditions. The endothelium derived relaxing factors include nitric oxide (NO), prostacyclin and a still elusive endothelium dependent hyperpolarizing factor (EDHF)$^{125}$. NO is the one of the best-characterized and most important substances released from the endothelium. The most important stimuli for NO release represent physical factors such as shear stress$^{126}$ and pulsatile stretching$^{127}$ of the vessel wall. Also, numerous circulatory and locally released vasoactive substances such as acetylcholine, bradykinin, serotonin and thrombin influence the release of NO from endothelium$^{128}$. NO is a membrane-diffusable molecule that readily interacts with the haeme group of the cytosolic enzyme guanylate cyclase in platelets and VSM cells$^{129}$. Subsequent formation of cyclic GMP from GTP results in the stimulation of VSM cell relaxation and vasodilation. Thus, NO production or availability regulates the functions of endothelial cells and their interaction with formed elements in circulation and VSM cells. Endogenous eNOS plays an important role in synthesizing NO and therefore, a point of convergence for several stimulatory and inhibitory pathways. Activation of eNOS by stimuli such as acetylcholine, ET-1, bradykinin, vascular endothelial growth factor (VEGF), insulin, insulin like growth factor (IGF-1), endogenous or exogenous opioids and cannabinoids, L-arginine, AT II, estrogens, fluid shear stress and cyclic strain result in increased NO output$^{130,131,132,133,134}$. The inhibitory stimuli include stress-induced haeme oxygenase-1 with generation of carbon monoxide, TNF-α, hyperlipidemias, hyperhomocystenemia, endogenous NOS inhibitors (NG-monomethyl-L-arginine monoacetate, L-NMMA and dimethylarginine, ADMA), diabetes mellitus, hyperglycemia and protein modification by AGE$^{135,136,137}$. The availability of NO and its physiologic functions can be seriously compromised by impaired signal transduction along
the stimulatory pathways as well as induction of inhibitory influences together with NO scavenging by other molecules. It is therefore obvious that eNOS dysfunction has broad consequences affecting virtually every aspect of endothelial cell function including vascular tone.

**Endothelial dysfunction**

The hallmark of chronic diabetes is the impairment of endothelial function\(^\textsuperscript{138,76}\). Endothelial dysfunction (ED) plays an important role in the pathogenesis of diabetic vascular disease. There is substantial evidence indicating that impairment of endothelial nitric oxide (NO) mediated vasodilation occurs in both clinical\(^\textsuperscript{83,84}\) animal models of Type 1\(^\textsuperscript{139}\) and Type 2 diabetes\(^\textsuperscript{140,141}\). Several mechanisms of ED have been reported, including impaired signal transduction or substrate availability, impaired release of NO, increased destruction of NO, enhanced release of endothelium-derived constricting factors and decreased sensitivity of the vascular smooth muscle cells to NO. Although the exact mechanisms by which diabetes contributes to ED are currently unknown, it is likely that hyperglycemia may initiate this abnormality\(^\textsuperscript{142}\). The principal mediators of hyperglycemia-induced ED may be the activation of PKC, increased activity of the polyol pathway, non-enzymatic AGE formation and oxidative stress\(^\textsuperscript{143,144}\).

**B. OXIDATIVE STRESS IN DIABETES**

The normal physiological process of ATP generation in living cells is accompanied by the formation of minute quantities of reactive oxygen species (ROS), which are usually buffered by endogenous antioxidant defense mechanisms. These ROS are a family of molecules including superoxide anion (O\(_2^-\)), hydroxyl radical (HO'), NO and lipid radicals. However, when excess production of ROS is formed outstripping the endogenous antioxidant concentrations, a state of stress called "oxidative stress" is created that is toxic to the cell. Occurrence of oxidative stress in diabetes is extensively documented\(^\textsuperscript{145,146}\). Also, an increasing body of evidence suggests that oxidative stress is involved in the pathogenesis of many cardiovascular complications of diabetes including atherosclerosis, stroke, heart failure, myocardial infarction, hypertension and vascular diseases.
Potential sources and mechanisms of oxidative stress in diabetes

The mechanisms behind the increased oxidative stress in diabetes are not clear but many biochemical pathways associated with hyperglycemia such as glucose auto-oxidation, formation of AGE and activation of polyol pathway can all cause oxidative stress\textsuperscript{143,147}.

Glucose auto-oxidation results in the formation of non-enzymatic AGE that are known to increase the formation of superoxides, hydrogen peroxides and hydroxyl radicals\textsuperscript{148}. Cellular uptake of AGE by subendothelial macrophages results in the activation of these macrophages initiating a local inflammatory response and further contributing to the oxidative stress\textsuperscript{149,150}. Antioxidants are reported to decrease AGE cross-linking and AGE formation by reducing the oxidative stress in diabetes\textsuperscript{151}.

Prolonged hyperglycemia results in an alternative metabolism of glucose through the polyol pathway. This pathway involving the oxidation of glucose to sorbitol in the first step and sorbitol to fructose in the second step creates an altered redox state with an increased cytosolic ratio of NADH/NAD\textsuperscript{+}144,143. This in turn affects the availability of BH\textsubscript{4}, an essential cofactor for NOS. Depletion of BH\textsubscript{4} uncouples NOS resulting in an increased production of superoxides rather than NO\textsuperscript{152,153}. In diabetic animal models, BH\textsubscript{4} supplementation has been shown to improve endothelial function\textsuperscript{154}. In addition increased cytosolic NADH/NAD\textsuperscript{+} can also induce the synthesis and accumulation of DAG and activation of PKC that can cause ED as a result of increased oxidative stress\textsuperscript{155}. Increased formation of free radicals by PKC may be due to the activation of transforming growth factor β (TGF-β) and thromboxane A\textsubscript{2} (TxA\textsubscript{2})\textsuperscript{156}. TGF-β is known to induce cell proliferation, chemo-attraction and activation of monocytes to produce interleukins and cytokines. Activated monocytes can produce free radicals through NADPH oxidases\textsuperscript{157}. Also increased TxA\textsubscript{2} from arachidonic acid can produce more free radicals through cytochrome P450\textsuperscript{158}. Treatment with aldose reductase inhibitors has been shown to normalize NADPH, GSH and lipid peroxidation in STZ diabetic rats\textsuperscript{159}.
Markers of oxidative stress

The shift in the redox state as explained above can cause an increased breakdown of NO by superoxide anions resulting in the formation of RNS such as peroxynitrite. Peroxynitrite is a potent oxidizing agent and can initiate lipid peroxidation, sulfhydryl oxidation, nitration of tyrosine residues, direct damage to DNA and oxidation of antioxidants such as ascorbate and α-tocopherol. Some of the most commonly used in vivo biological markers of oxidative stress are 3-nitrotyrosine (NT) for protein modifications, F₂-isoprostanes, malonyldialdehyde (MAD) and/ or thiobarbituric acid reactive substances (TBARS) for lipid peroxidation, and 8-hydroxydeoxyguanosine for DNA damage. In addition to these, the levels of antioxidants and enzyme activities such as catalase, superoxide dismutase and glutathione peroxidase can also be measured in vivo as markers of oxidative stress.

The overproduction of peroxynitrite can be indirectly inferred by the measurement of nitrotyrosine (NT) in plasma and urine by HPLC and enzyme immunoassay (EIA) methods. Despite being a low yield process in vivo, the quantitative measurement of protein nitration in terms of NT is widely accepted as a marker of nitrosative stress. Tyrosine nitration is not only mediated by peroxynitrite but also by NO₂, which is formed as secondary product of NO metabolism in the presence of oxidants like superoxide radicals, hydrogen peroxide (H₂O₂) and transition metal ions. Several studies support a direct role of hyperglycemia in favoring the production of NT. Formation of NT is detected in the arterial tissue of monkeys during hyperglycemia, in the plasma of healthy subjects during a hyperglycemic clamp and in diabetic patients. It is also detected in the coronaries and heart tissues of STZ diabetic rats and working hearts from rats during hyperglycemia.

Isoprostanes are a group of novel biologically active prostaglandin-like compounds produced by a non-enzymatic free radical catalyzed oxidation of arachidonic acid. Several isoprostanes have been identified in biological tissues and fluids. Among these, 15-F₂ isoprostane and its metabolite 2,3-dinor-4, 5-dihydroxy-8-iso-PGF₂α are considered to be reliable in vivo markers of lipid peroxidation. Various methods including radioimmuno assay (RIA), EIA, HPLC, LCMS and GC-MS are available for measurement of isoprostanes. Several clinical studies have indicated that isoprostanes are reliable markers of oxidative stress in various cardiovascular diseases. Studies have suggested that hyperglycemia
increases the formation of $F_2$ isoprostanes and antioxidant therapies aimed at reducing oxidative stress have shown beneficial effects in diabetes$^{171,172}$.  

**Antioxidant defense mechanisms: Glutathione and N-acetylcysteine**

Chronic hyperglycemia is associated with oxidative stress created by a serious imbalance between the oxidants and antioxidant defense mechanisms. As described earlier, oxidative stress can result from glucose auto oxidation, protein glycation, polyol pathway, activation of mitochondrial NADPH oxidases and induction of iNOS. In addition to the formation of reactive oxygen and nitrogen species, depletions in antioxidant concentrations such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase etc can significantly increase oxidative stress. These antioxidants are metabolic intermediates or substrates that undergo recycling or regeneration by interacting with biological reductants and protect the cell from oxidative stress$^{173}$.  

Production and elimination of ROS by antioxidant enzymes is tightly regulated to maintain a normal redox state. However, several pathological conditions such as hyperglycemia can disturb this regulation and significantly affect the activity of these enzymes. Data from numerous studies underline the importance of antioxidant enzymes in preventing oxidative stress and diabetic complications$^{173,174}$. In experimental models of diabetes, such as STZ diabetic rats, hyperglycemia is associated with depleted GSH levels, increased superoxide anions, reduced SOD and GPX activity and increased activities of NADPH and xanthine oxidase$^{173,175,176}$. Therefore treatment with antioxidants or agents that can either mimic or increase the concentrations of endogenous antioxidants or the antioxidant enzymes can greatly reduce the oxidative stress associated with hyperglycemia$^{177,178,179}$. For example, replenishment of exogenous GSH improves the endogenous repository of GSH and significantly reduces the oxidative stress.
Glutathione (GSH)

Glutathione is a cysteine containing tripeptide thiol, a water-phase antioxidant, antitoxin and an essential cofactor for antioxidant enzymes such as GPX and glutathione transferase (GST). It exists in two forms, reduced GSH and an oxidized form, glutathione disulfide (GSSG). The ratio of GSSG and GSH is a sensitive indicator of oxidative stress\(^{180}\).

GSH has a potent electron donating capacity (high negative redox potential) that regulates a complex thiol exchange system (-SH↔-S-S-). Its high electron donating capacity, also called "reducing power" makes it a good antioxidant *per se* as well as a convenient cofactor for many enzymatic reactions that require readily available electron pairs (reducing equivalents). Reduced GSH plays a major role in the regulation of intracellular redox state by providing reducing equivalents for many biochemical pathways. The reduced GSH molecule consists of three amino acids, glutamic acid, cysteine and glycine covalently bonded to each other. The sulphydryl group (-SH) that gives the molecule its electron donating character comes from the cysteine residue. GSH status is homeostatically controlled by the enzymes that synthesize it (GSH synthetase), that recycle it (GSH reductase) and utilize it including peroxidases, transferases, transhydrogenases and transpeptidases\(^{180}\).

Since GSH is an important regulator of cellular redox state, its depletion can have a deleterious effect on various cellular processes. Its depletion, for example in hyperglycemic conditions\(^{181}\) may increase the expression of iNOS and peroxynitrite formation and cause cardiovascular complications. Studies examining the effects of GSH supplementation in hyperglycemic conditions have indicated improvement in oxidative stress and normalization of depressed blood pressure, heart rate and catecholamine levels in the plasma of diabetic patients\(^{182}\).

N-acetylcysteine (NAC)

N-acetylcysteine is the N-acetyl derivative of the amino acid cysteine, which is a part of the tripeptide molecule in GSH and is a major contributor of reducing equivalents (SH group). NAC, in addition to its antioxidant activity has also hepatoprotective and anti-apoptotic activity, particularly on pancreatic beta cells and cardiac myocytes\(^{183}\).

Several studies have indicated that NAC might be useful in treating chronic complications of diabetes. NAC has been to shown to moderately decrease blood glucose
levels and to retain glucose stimulated insulin secretion\textsuperscript{184}. Supplementation with NAC reduced glomerular damage\textsuperscript{185} and functional abnormalities in peripheral nerves of STZ diabetic rats\textsuperscript{186}. NAC has been shown to inhibit the induction of iNOS in lipopolysaccharide (LPS) models of endotoxemia both \textit{in vitro} and \textit{in vivo}\textsuperscript{187,188}. NAC inhibition of iNOS could be due to the suppression of TNF-\(\alpha\) mediated induction of nuclear factor kappa beta (NFkB). In addition, NAC treatment has been shown to reduce the expression of plasma vascular adhesion molecule-1 (VAM-1) and increase the concentration of GSH and GSH/GSSG ratio in patients with Type 2 diabetes\textsuperscript{189}. Numerous studies have suggested that NAC reduces the formation of ROS and concurrently reduces NO production, possibly by suppressing NFkB mediated activation of iNOS\textsuperscript{190}. However, no studies have reported the effect of NAC on iNOS mediated cardiovascular abnormalities, most notably the effects on blood pressure at different durations of diabetes.

C. NITROSATIVE STRESS IN DIABETES

It is very well known that NO has physiologically beneficial effects on the cardiovascular system including the regulation of vascular tone, cardiac contractility, cell growth, vascular remodeling and baroreflex functions. In the kidney, NO regulates salt and fluid absorption, hemodynamics, renin secretion and tubuloglomerular feedback and these desirable actions are compromised in diabetes\textsuperscript{191}. Most of the evidence for NO deficiency in diabetes has been derived from studies on the endothelium where NO synthesis is regulated by eNOS, which may respond differently to chronic hyperglycemia than does NO produced from other sources. NO in macrophages, monocytes, epithelial cells, VSM cells, hepatocytes and many other tissues of the body is synthesized by iNOS\textsuperscript{192}. The gene expression of iNOS is modulated by NFkB, which in turn can be activated by hyperglycemia and oxidative stress. Over expression of iNOS results in an increased generation of NO\textsuperscript{193}. Enhanced NO production in concert with overproduction of superoxides may result in the induction of nitrosative stress. Peroxynitrite, an important product of nitratative stress may cause cardiovascular depression that is characterized by depressed blood pressure, heart rate and attenuated pressor responses to various vasoactive agents in diabetes.
Inducible nitric oxide synthase (iNOS) in diabetes

A large body of evidence suggests that iNOS is activated in cardiovascular tissues in diabetes. A number of mechanisms have been proposed to explain the increased expression of iNOS in hyperglycemic conditions. As mentioned before a number of studies have suggested that oxidative stress, enhanced activation of polyol pathway and PKC isoforms and elevated AGE, as a result of chronic hyperglycemia have been shown to enhance the expression of iNOS (scheme-1). In addition, many reports have also suggested that chronic hyperglycemia is associated with changes in the expression of various cytokines such as interleukin-1β (IL-1β), TNF-α, interferon-γ, NF-κB and interleukin-6 (IL-6) all of which are known stimulators of iNOS. Following the discovery that selective iNOS inhibition attenuates hypotension and vascular hypo-reactivity to pressor agents caused by endotoxin, numerous studies have demonstrated beneficial hemodynamic effects of iNOS inhibition in animal models of septic shock and circulatory failure. These findings, together with the discovery that iNOS knock out mice were resistant to the hypotensive effects of endotoxin suggest that iNOS induction by various cytokines results in enhanced NO production. Thus it is possible that a chronic diabetic state associated with elevated levels of various cytokines might induce iNOS in various tissues that can directly influence cardiovascular homeostasis (Scheme-1).

iNOS was expressed in normal isolated rat hearts perfused with a high glucose concentrations (33.3mM) within 2 hours. Induction of iNOS was demonstrated in cardiomyocytes from rats in STZ diabetic rats and in platelets of both Type 1 and Type 2 diabetic patients. An inverse correlation with a significant down-regulation of eNOS and a concomitant upregulation of iNOS mRNA was demonstrated in hearts of four week STZ diabetic rats. Several lines of evidence also suggest that iNOS is functionally expressed in the aorta and superior mesenteric arteries of STZ diabetic rats. The studies also suggest that iNOS expressed in the arteriolar SMC play a suppressive role on the basal tone and vascular reactivity. In addition to the heart and VSM cell, iNOS is also found to be expressed in kidney, sciatic nerve and pancreatic β cells.

Increasing evidence now suggests that iNOS might play a role in the regulation of cardiovascular function in diabetes. It is suggested that iNOS contributes significantly to the attenuated vascular responses to various vasoconstrictor agents in STZ diabetic rats.
Recently, inhibition of iNOS with 1400W was demonstrated to significantly improve the arterial and venous contractility in acute STZ diabetic rats\textsuperscript{205}. Knock out mice studies have demonstrated that iNOS inhibition increases vasoconstrictor responses in diabetes\textsuperscript{206}.

**iNOS- a possible link between diabetes and cardiovascular depression?**

In hyperglycemic conditions there is an overproduction of both superoxide and NO with a 3-fold increase in superoxide generation. The simultaneous generation of NO and superoxide favors the production of the toxic reaction product, peroxynitrite anion. Peroxynitrite in turn can oxidize BH\textsubscript{4}, an iNOS cofactor to dihydrobiopterin\textsuperscript{207}. Under conditions of BH\textsubscript{4} deficiency, iNOS is in an uncoupled state, which means that electrons flowing from iNOS reductase domain are diverted to molecular oxygen rather to L-arginine, resulting in production of superoxide instead of NO. Exposure to peroxynitrite during hyperglycemia also produces an uncoupling state of eNOS, presumably via a zinc depletion of the enzyme, favoring superoxide over production\textsuperscript{208}.

The cytotoxic actions of peroxynitrite are due to its strong ability to oxidize sulphydryl groups in proteins, to cause lipid peroxidation and nitration of amino acids such as tyrosine that in turn can affect many signal transduction pathways. Nitrotyrosine, an indirect marker of peroxynitrite formation is associated with increased apoptosis in cardiac myocytes, endothelial cells and fibroblasts in heart biopsies from diabetic patients\textsuperscript{209} and in heart from STZ diabetic rats\textsuperscript{210}, and in working hearts exposed to high glucose concentrations\textsuperscript{166}. Increased formation of peroxynitrite may cause cardiovascular depression and vascular hyporeactivity in diabetes\textsuperscript{211}.

The mechanisms by which peroxynitrite causes cardiac depression are not clear but it is suggested that peroxynitrite depresses cardiac contractility by decreasing the Ca\textsuperscript{2+} sensitivity of contractile elements\textsuperscript{212}. Studies have demonstrated that infusion of peroxynitrite into working hearts impairs cardiac contractile function by decreasing cardiac efficiency\textsuperscript{213}. Other studies have reported that peroxynitrite can cause direct oxidation of catecholamines\textsuperscript{214} and binding capacity of endogenous agonists to \(\alpha\)-adrenergic receptors (\(\alpha\)-1\textsubscript{A} and \(\alpha\)-1\textsubscript{D})\textsuperscript{215} thereby decreasing the vascular reactivity to vasoactive agents. These results strongly indicate that, under conditions of increased oxidative stress, iNOS play a crucial role in abetting the formation of peroxynitrite, which in turn causes cardiovascular depression by
contributing greatly to the nitrosative stress. Interventions with strong antioxidants or inhibitors of iNOS should therefore reduce nitrosative stress and improve cardiovascular function in diabetes\textsuperscript{216}. 
Intracellular hyperglycemia induces the overproduction of superoxide at mitochondrial level. This in turn stimulates polyol pathway flux, increased advanced glycosylation end products (AGE) formation, activation of protein kinase C (PKC) and NFkB and increased hexosamine pathways flux. Superoxide overproduction stimulates NFkB mediated induction of inducible nitric oxide synthase (iNOS). In addition, under conditions of chronic hyperglycemia (CHG) an increased level of various cytokines also induces the transcription of iNOS resulting in overproduction of NO. This condition favours the formation of peroxynitrite, a strong oxidant that may cause cardiac depression, vascular hyporeactivity and endothelial dysfunction.
2. RESEARCH OUTLINE: RATIONALES, HYPOTHESES AND OBJECTIVES

Diabetes mellitus is associated with an increased incidence of cardiovascular complications, which is a major cause of morbidity and mortality in the western world\textsuperscript{21,217}. One of the important predisposing factors in the development of cardiovascular complications is the chronic hyperglycemic state, which is now recognized as an independent risk factor. Chronic hyperglycemia has been shown to directly affect the composition and structure of vascular, cardiac and renal tissues, the progressive modification of which results in a deranged cardiovascular homeostasis\textsuperscript{218}. Cardiovascular depression, characterized by depressed blood pressure and heart rate is one of the most notable manifestations of this derangement, particularly in animal models of type 1 diabetes. Although the exact mechanisms by which diabetes contributes to cardiovascular depression are currently unknown, it is likely that hyperglycemia may initiate this abnormality through the activation of PKC, increased activity of the polyol pathway, non-enzymatic AGE formation and oxidative stress\textsuperscript{143,144}.

Increased production of NO and /or RNS from iNOS together with increased formation of reactive oxygen species, particularly superoxide anions, is being documented extensively in chronic hyperglycemic conditions. Both NO and superoxide anions are highly reactive and unstable radicals that can react rapidly to form peroxynitrite, a cytotoxic compound\textsuperscript{219}. Peroxynitrite is a strong oxidant that can oxidize various biomolecules and can exert cytotoxic actions. Recent studies have suggested that peroxynitrite is a favored product under conditions such as hyperglycemia where cellular production of NO and ROS are increased. Studies exploring the biochemical mechanisms of inflammatory cytokine-induced myocardial failure and vascular hyporeactivity have strongly suggested that formation of peroxynitrite is crucial in the development of septic shock\textsuperscript{219}. It has also been suggested that deactivation of norepinephrine and $\alpha$-adrenergic receptors by peroxynitrite may contribute to systemic hypotension and cardiovascular depression\textsuperscript{215,214}. Several studies have demonstrated that under hyperglycemic conditions, iNOS expression is elevated in heart and vascular smooth muscle of STZ diabetic rats\textsuperscript{201,202}. Also, hyperglycemia is found to be associated with increased formation of AGE and activation of PKC and expression of various cytokines, all of which are known stimulators of iNOS\textsuperscript{194,195}.
The above observations strongly indicate that nitrosative stress associated with chronic hyperglycemia may contribute to cardiovascular depression in diabetes. Since iNOS is the major contributor of NO to the nitrosative stress, therapeutic strategies aimed at inhibition of iNOS or strengthening the antioxidant defense mechanisms may be a rational approach in treating cardiovascular abnormalities associated with diabetes. However, there is paucity of information about the role of iNOS per se on hemodynamics and its contribution to the overall cardiovascular depression in diabetes. In addition, the effect of the duration of diabetes on iNOS mediated cardiovascular depression is not clear. The primary goals of the present work were (1) to investigate the potential role of iNOS in modulating the hemodynamic effects of various vasoactive agents and (2) to study the effect of antioxidant therapy on iNOS mediated cardiovascular abnormalities in STZ diabetic rats. The present studies were conducted in the STZ diabetic rat model of Type 1 diabetes, which is a widely employed model that mimics most of the long-term cardiovascular complications of human diabetes.

2.1 OVERALL HYPOTHESIS

Chronic hyperglycemia results in the activation of iNOS leading to enhanced production of nitric oxide and / or RNS (nitrosative stress) that causes cardiovascular depression in STZ diabetic rats. Specific inhibition of iNOS may therefore improve the hemodynamic effects of vasoactive agents in intact conscious STZ diabetic rats. Treatment with N-acetylcysteine, a strong antioxidant and iNOS inhibitor may improve or normalize cardiovascular depression by inhibiting iNOS mediated formation of ROS / RNS and/ or strengthening the antioxidant defense mechanisms in STZ diabetes.

2.2 SPECIFIC RESEARCH OBJECTIVES AND RATIONALE

2.2.1 Study-1: Selection and standardization of the dose of 1400W (N-[-3(Aminomethyl) Benzyl] acetamidine, dihydrochloride), a specific inhibitor of iNOS.

Septic shock is a systemic inflammatory response to diverse pathological insults and is characterized by severe hypotension, profound vasodilation and multiple organ failure\(^{220}\). It is well known that large amounts on NO produced in the late phase of septic shock in lipopolysaccharide (LPS, *Escherichia coli*) treated rats are through the induction of iNOS.
Following the discovery that selective iNOS inhibition attenuates hypotension and vascular hypo-reactivity to pressor agents caused by endotoxin, numerous studies have demonstrated beneficial hemodynamic effects of iNOS inhibition in animal models of septic shock and circulatory failure\textsuperscript{196,197,198,199}. Among the various nitric oxide synthase inhibitors, 1400W is, by far, the most selective inhibitor of iNOS\textsuperscript{221}. It is a slow but tight binding and irreversible inhibitor of iNOS both \textit{in vitro} and \textit{in vivo}. It has been reported to exhibit greater than 1000-fold potency against rat iNOS relative to eNOS\textsuperscript{222}. A wide variety of doses ranging from 0.3 mg/kg to 3 mg/kg of 1400W have been reported to inhibit iNOS and prevent LPS induced endotoxic shocks. However, many of these experiments were conducted either \textit{in vitro} or in anesthetized animals or have employed different techniques for measurements of blood pressure\textsuperscript{223}. \textit{We therefore studied the effect of 1400W on LPS induced endotoxemia in intact and freely moving conscious rats, the model which we used in our subsequent experiments.} The results of the study would determine the ability of 1400W to specifically inhibit iNOS and help us select the minimal yet appropriate dose for our subsequent experiments. Single intravenous bolus doses of 10, 5 and 3 mg/kg of LPS (\textit{E coli}) against 1 and 3 mg/kg of 1400W in sterile saline were selected for the study.

\textbf{2.2.2 Study-2a: To study the effect of selective iNOS inhibition (1400W) on the hemodynamic effects of vasoactive agents (AT II and MTX) in unrestrained conscious STZ diabetic rats: Effect of the duration of diabetes.}

An increased dependence on a functional nitric oxide system has been reported, which prevents the development of hypertension in STZ diabetic rats\textsuperscript{114}. It was demonstrated that non-selective blockade of NO synthesis results in increased MABP at the onset of diabetes compared to the same in normal rats\textsuperscript{113,112}. NO synthesis is important to maintain an increased renal blood flow, to suppress the activated RAS system and increased SNS activity. These findings imply the presence of increased NO to counteract the pressor effects of endogenous mediators with the progress of diabetes\textsuperscript{224,225}. Given the fact that endothelial function is impaired in diabetes, the source of NO in such settings is still not clear but it is suggested that iNOS might contribute to the total NO pool. Another important aspect is the complex interaction between NO and superoxide, both of which are increased in diabetes and have opposite actions. A large body of evidence implicates superoxide in the
pathophysiology of hypertension, both in, humans and animals models of hypertension\textsuperscript{226,227,228, 229}. We therefore proposed that there is a balance between the hypotensive effects of NO/RNS and the hypertensive effects of superoxides\textsuperscript{227}, which may dictate the hemodynamic outcome and this, in turn may depend on the duration of diabetes\textsuperscript{228}. Although many studies have suggested an increased expression of iNOS in cardiovascular tissues of STZ diabetic rats it is still unclear how the duration of diabetes effects its expression and activation. We therefore investigated the effects of selective iNOS inhibition (1400W) on the hemodynamic effects of vasoactive agents at different stages of diabetes, starting at 1 week and continuing up to 12 weeks. This is particularly interesting in the light of attenuated pressor responses to vasoactive agents in diabetes\textsuperscript{81}. Since anesthetics\textsuperscript{230} and stress have been reported to alter the sympathetic activity, we conducted our experiments using intact, freely moving conscious rats.

\textbf{2.2.3 Study-2b: To study the effect of duration of diabetes on the differential regulation of eNOS and iNOS in STZ diabetic rats.}

Elevated glucose levels have been shown to trigger the down-regulation of eNOS accompanied by the induction of iNOS\textsuperscript{201}. It is suggested that hyperglycemia mediated activation of the PKC pathway and formation of AGE may negatively influence the activity and expression of eNOS resulting in endothelial dysfunction\textsuperscript{231}. The impairment of endothelial function as determined by attenuated vasorelaxant response to acetylcholine was improved by free radical scavengers such as superoxide dismutase\textsuperscript{178}. It has been suggested that chronic hyperglycemia produces free radicals by AGE formation, activation of NADPH oxidase and alteration of the polyol pathway\textsuperscript{232}. The same pathways are also involved in the activation of iNOS\textsuperscript{233}. In addition, elevated expression of iNOS is reported to generate free radicals. Thus it is possible that nitrosative stress resulting from increased production of NO/RNS from iNOS may downregulate the expression of eNOS resulting in cardiovascular depression. Since the level of nitrosative stress depends on the duration of diabetes, we studied the differential expression of eNOS and iNOS and formation of peroxynitrite at various time points in heart, aorta and superior mesenteric artery.
2.2.4 Study-3: To study the effect of N-acetylcysteine, an antioxidant on iNOS mediated cardiovascular changes in acute and chronic STZ diabetic rats.

Increased superoxide generation concurrent with increased NO production is known to produce peroxynitrite. This reaction, which is three times faster than the dismutation of superoxide by SOD, is a favored reaction under conditions of cellular stress such as hyperglycemia. In experimental diabetes and also endotoxemic models of septic shock, increased peroxynitrite content has been found in cardiovascular tissues. Treatments aiming at inhibition of iNOS or reduction of oxidative stress have been beneficial in experimental models of septic shock and diabetes respectively. It is not clear how antioxidant therapy aimed at reducing oxidative stress and iNOS inhibition affect hemodynamics in acute and chronic diabetes. We therefore studied the effects of N-acetylcysteine, a strong antioxidant and inhibitor of iNOS on cardiovascular depression in STZ diabetic rats.
3. MATERIALS AND METHODS

3.1 MATERIALS

A. CHEMICALS AND DRUGS

N-[3-((Aminomethyl) benzyl] acetamidine, dihydrochloride (1400W) was obtained from Calbiochem (San Diego, CA). Angiotensin-2 acetate, calcium chloride, dexamethasone (water soluble), dextrose, disodium ethylene diamine tetra-acetic acid (EDTA), ethyl alcohol (absolute), lipopolysaccharide (Escherichia coli), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), magnesium sulphate, methoxamine hydrochloride, N-acetylcysteine (NAC), neutral buffered formalin (10% NBF), potassium chloride, potassium hydrogen phosphate, sodium bicarbonate, streptozotocin (STZ), sodium chloride and t-butyl hydroperoxide (t-BHP) were purchased from Sigma Aldrich Company (St Louis, MO). Halothane was obtained from Benson Laboratories, Sodium pentobarbital (Somnotol®) was from MTC pharmaceuticals (Cambridge, ON). Heparin sodium (Hepalean®) was obtained from Organon Teknika Inc (Toronto, ON). Sterile saline was obtained from a local pharmacy. Unless otherwise stated, all other chemicals were reagent grade and obtained from Sigma, St Louis, MO or BDH Inc., ON or Fischer Scientific, Nepean, ON.

B. ASSAY KITS

Enzymatic colorimetry assay kits for glucose, triglyceride and cholesterol were purchased from Roche Diagnostics, Laval, Quebec. Radioimmunoassay kits for insulin were from Linco Research Inc., St Charles, MO. Plasma nitrite and nitrate (NOx) assay kit and 8-Isoprostane EIA kits were obtained from Cayman Chemical Company, Ann Arbor, MI. Total antioxidant status kits were obtained from Calbiochem, San Diego, CA. Rabbit anti-iNOS polyclonal antibody and mouse anti-eNOS monoclonal antibody kits were obtained from BD Biosciences Canada, Mississauga, ON. Rabbit anti-NT polyclonal antibodies were from Upstate. Secondary antibodies (Labelled polymer, Envision System, K1491) were obtained from DakoCytomation Inc., Mississauga, ON. Chromagens were obtained from Vector Laboratories (Canada), Inc, Burlington, ON. Nonspecific mouse and rabbit immunoglobulins (IgG) were obtained from Jacksons Immuno- Research Laboratories, West Grove, PA.
3.2 RESEARCH METHODOLOGY AND EXPERIMENTAL PROTOCOLS

3.2.1 INDUCTION OF DIABETES

Male Wistar rats weighing between 200-250 g were obtained from the Animal Care Center, University of British Columbia, Vancouver, BC or from Charles River Laboratories Inc., Laval, Quebec and allowed to adapt to the local *vivarium*. For acclimatization the rats were housed on 12-hour light-dark cycle and received rat chow and water *ad libitum*. Care was given in accordance with the principles and guidance of the Canadian Council on Animal Care. Rats, initially were assigned into two groups. One group received a single tail vein injection (under halothane anesthesia) of streptozotocin (STZ, 60 mg/kg, i.v) and served as the diabetic group. The other group was injected with saline and served as age-matched control. The rats were considered diabetic and used for the study only if they had hyperglycemia (set at ≥ 15mM) at 72 hr after STZ injection. Blood glucose levels were determined using Accusoft glucose test strips read on a Glucometer (Roche Diagnostics, Laval, Quebec).

3.2.2 METHODOLOGY

3.2.2.1 Surgical Procedures

In the presence of light pentobarbital anesthesia (20mg/kg), each animal was cleanly shaven using an electric hair clipper (Oster, Sunbeam Corporation, Mississauga, ON) and their ventral and dorsal neck regions were sterilized with 70% ethanol. All surgical procedures were performed on a sterilized surgical table equipped with an anesthetic machine for a constant supply of oxygen and anesthesia (Fluotec-3, Yorkshire, England). In the presence of light halothane anesthesia and a local anesthetic (Lidocaine), a fluid filled (heparinised saline, 20U/ml) catheter (PE 50, Intramedic® Clay Adams, Becton Dickinson and Company, Sparks, MD) was placed in the left carotid artery to record mean arterial blood pressure (MABP) and heart rate (HR). A second catheter (PE 50) was placed in the left jugular vein for drug administration. All the catheters were exteriorized at the nape of the neck, passed through a harness and tether and connected to a swivel (Instec Lab Inc., PA) mounted above the cage for free movement of the animal. The surgical wounds were closed using wound clips (Michel wound clips, Propper Manufacturing Co, NY) and the animals were allowed to recover from surgery and anesthesia in cages.
The venous catheter was connected, by a swivel, to a syringe for drug administration. The arterial catheter was connected, also by a swivel, to a disposable pressure transducer (DTX®, Viggo-Spectramed, Oxnard, CA) mounted on the cage exterior at the level of the rat. The systolic, diastolic, mean arterial blood pressure and the heart rate were simultaneously recorded on Gould TA 2000 Thermal Array Recorder (Gould Instrument System Inc., Ohio, USA) and a computer, using custom made data acquisition software. In order to achieve normalization of cardiac baroreflexes, the animals were allowed to recover from anesthesia and surgery for at least 4 hours.

3.2.2.2 Biochemical Measurements

Blood samples were collected from the tail vein, except at termination when samples were collected by carotid artery bleeding into centrifuge tubes containing 10% v/v EDTA solution (20mg/ml). Blood samples were centrifuged either in a desktop centrifuge (Beckman Allegra 21R centrifuge, Beckman Instruments Inc., Spinco Division, CA) at 14000 rpm for 4500 rpm at 4°C for 25 minutes. Plasma samples were aliquoted and stored at −70°C until assayed.

MEASUREMENT OF GLUCOSE, INSULIN, TRIGLYCERIDES and CHOLESTEROL

Plasma glucose was measured either with an enzymatic colorimetric assay kit (Roche Diagnostics, Laval, Quebec) using a Beckman Glucose Analyzer 2 or Accusoft glucose test strips read on a Glucometer (Roche Diagnostics, Laval, Quebec). Plasma insulin levels were measured using double antibody based radioimmunoassay kits from Linco Research Inc., St Charles, MO. Triglyceride and cholesterol levels were determined by using colorimetric assay kits from Boehringer Mannheim and Sigma, St Louis, MO.

MEASUREMENT OF PLASMA NITRITE AND NITRATE (NOx) LEVELS

Plasma NOx levels were determined using a commercially available colorimetric assay kit (Cayman chemical Company, Ann Arbor, USA) based on the Griess reaction. The assay was performed according to the instructions of the manufacturer. Prior to the assay plasma samples were ultrafiltered though a 30kDa molecular weight cut-off filter (Ultrafree®-MC centrifugal filter units, Millipore Corporation, Bedford, MA). Ultrafiltration
is necessary to reduce the background absorbance due the presence of hemoglobin and to improve the color formation. Briefly, 200 µl of plasma was placed in each ultra filtration unit (pre-rinsed with ultrapure water) and centrifuged for 40 minutes at 14000 rpm using a desktop centrifuge (Beckman Allegra 21R centrifuge, Beckman Instruments Inc., Spinco Division, CA) at 5°C. The filtrate collected in the filtration tube was used for NOx analysis. On a 96 well plate, in the first step plasma nitrate was converted to nitrite using nitrate reductase and in the second step Griess reagent was added to convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this azo chromophore, the intensity of which is proportional to quantity of NOx, was done in a plate reader.

**MEASUREMENT OF PLASMA FREE 8-ISOPROSTANE LEVELS**

8-Isoprostane (8-iso PGF\textsubscript{2\alpha}), which is considered as a marker of antioxidant deficiency and oxidative stress was measured by using a competitive enzyme immunoassay kit (Cayman chemical Company, Ann Arbor, USA). The assay relies on the competition between 8-Isoprostane in the plasma (free) and an 8-isoprostane–acetylcholinesterase (AChE) conjugate (8-Isoprostane tracer) for a limited number of 8-Isoprostane specific rabbit antiserum binding sites. This rabbit antiserum - 8 - isoprostane (either free or tracer) complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate was then washed to remove any unbound reagent and then Ellman’s reagent (which contains substrate for AChE) was added to the well. The final product of this enzymatic reaction, which is yellow in color, was read at 412 nm using a spectrophotometer (Ultrospec 4050, LKB Biochrom, Cambridge, England). The intensity of this color, which is proportional to the amount of the tracer bound to the well, is inversely proportional to free 8-Isoprostane (plasma) in the well during incubation.

**MEASUREMENT OF PLASMA TOTAL ANTIOXIDANT STATUS**

Plasma total antioxidant concentration was measured by using a commercially available kit (Calbiochem, San Diego, CA, USA). The assay is based on the ability of antioxidants in the plasma to inhibit the oxidation of ABTS\textsuperscript{\textregistered} (2,2’-azino-di- [3-ethylbenzodiazoline sulfonate]) to ABTS\textsuperscript{\textcircled{+}} by metmyoglobin (a peroxidase). The amount of
ABTS®+ formed (that depends on the total antioxidant concentration in plasma) was read at 600 nm using a temperature controlled spectrophotometer (water bath maintained at 37°C).

3.2.2.3 Determination of Myocardial Antioxidant Capacity

Antioxidant capacity of myocardial tissue was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) using in vitro forced peroxidation method as described elsewhere. Tissue samples that have lower antioxidant capacity result in the formation of lipid byproducts when exposed to oxidizing agents such as t-butylhydroperoxide (t-BHP). These byproducts when incubated with thiobarbituric acid form chromogens (TBARS), the concentration of which can be determined spectrophotometrically at 532 nm\textsuperscript{234}.

Briefly, each frozen heart tissue sample (300mg) was thawed and homogenized in Tris-EDTA buffer (0.05-0.1M) on ice using a polytron homogenizer (PT 3100, Brinkmann Instruments Canada Ltd, ON) for 30s at 25% power. Tissue homogenates measuring 400 µl were combined with 400 µl of t-BHP (in saline with 2mM sodium azide to produce final concentrations of 0.5 to 5mM t-BHP). The mixture was then incubated at 37\textdegree C for 30 minutes, after which 400 µl of cold trichloroacetate (28% w/v) with 0.1M sodium arsenite was added to this and centrifuged at 12000g for 5 min at 4\textdegree C. Supernatant (800 µL) was removed and added to 400 µL of thiobarbituric acid (0.5% in 25 mM NaOH). The samples were boiled for 15 min, and the absorbance at 532 nm was measured spectrophotometrically.

3.2.2.4 Immunohistochemical Localization of eNOS, iNOS and NT

Immunohistochemical analysis of eNOS, iNOS and NT was performed in the heart (ventricle), aorta (thoracic) and mesenteric (superior) artery tissues of all study groups.

Ventricles, aortas and superior mesenteric arteries were cleaned and fixed in 10% neutral buffered formalin overnight and transferred to 70% ethanol. This was followed by paraffin processing through increasing grades of ethanol, xylene and paraplast (Fischer Scientific, Nepean, ON). Paraffin embedded tissue blocks were sectioned at 3 µm and sections were mounted on positively charged slides. For immunostaining, sections were deparaffinized, rehydrated, treated with target retrieval buffer (DAKO, S1699), blocked with 3% hydrogen peroxide (H$_2$O$_2$, to block endogenous peroxidase activity), washed with
phosphate buffered saline (PBS) and blocked with 5% normal goat serum (NGS) in PBS for 30 minutes. The slides were subsequently incubated with primary mouse monoclonal eNOS antibody (1:600) or rabbit polyclonal iNOS antibody (1:100) or rabbit polyclonal NT (1:200) in PBS containing 1% NGS overnight at 4°C. The primary antibody was rinsed off with PBS and the sections were incubated with the secondary antibody (Dako Envision®, Dakocytomation Inc., ON) for 30 minutes. This contains goat anti-mouse (eNOS) and goat anti-rabbit (iNOS) and anti-NT antibodies conjugated to an enzyme (HRP, horseradish peroxidase) labeled special water-soluble polymer (dextran). After three washing steps in PBS, the sections were stained using NovaRED® (Vector Labs, SK-4800) for 10 minutes, washed with distilled water (5 min) and counter stained using hematoxylin (Mayers). The sections were dipped in lithium carbonate (to blue for 90 seconds), washed in running water, dehydrated in increasing grades of alcohol and cleared in xylene before being mounted in resinous mounting medium with Permount (Fischer) coverslips. Some sections incubated with nonspecific mouse and rabbit immunoglobulins (IgG) (Jacksons Immuno Research Laboratories, PA) served as negative controls.

A digital imaging system was used during the observation of sections. Slides were viewed through a Leitz Orthoplan high power microscope. A video camera (Leica Microsystems, Cambridge) attached to this microscope and connected to a computer with an image analysis software (Leica IM 1000 Image Manager, Leica Microsystems Imaging Solutions Ltd., Cambridge, UK) was used for image analysis and photography. Each section was observed individually at 10X and later at 25X magnification under a fixed and constant light exposure before a final photograph was taken at 25X.
3.2.3 EXPERIMENTAL PROTOCOLS


This study was designed to select an appropriate dose of 1400W, a specific inhibitor of iNOS to be used in study-2.

Male Wistar rats weighing between 300-350 g were randomly divided into four groups. Each group received an i.v administration of either 1 or 3 mg/kg of 1400W (in sterile saline) in the presence of 10, 5 or 3 mg/kg of lipopolysaccharide (E coli). In each rat (conscious) following surgical implantation of catheters, basal MABP and HR were measured. This was followed by i.v administration of 10, 5 or 3 mg/kg of LPS (in sterile saline) over a period of 2 minutes. MABP and HR were constantly monitored and recorded every 5 minutes for the first 15 minutes and every 15 minutes later till the end of the experiment. Two hours post LPS administration, each rat was administered bolus doses of 1 or 3 mg/kg of 1400W slowly over a period of 2 minutes. MABP and HR were recorded till the end of experiment (4 hours) or death, whichever was the earliest.


This study was designed to elucidate the role of iNOS in cardiovascular hemodynamics and to study the effect of the duration of diabetes on iNOS mediated cardiovascular changes.

One hundred and twenty eight male Wistar rats weighing 250 ±10 g were obtained from Charles River Laboratories Inc., Montreal, Quebec and allowed to acclimatize to the local vivarium. Studies were conducted in 4 stages, wherein the animals were divided into 4 study groups of 32 animals each (1, 3, 9 and 12 week study groups). In each group, 16 animals were made diabetic by a single bolus i.v injection of STZ (60 mg/kg) via the tail vein under light halothane anesthesia. Body weights and serum glucose were measured in the beginning and the animals were allowed free access to food and water till the surgery.

At 1,3,9 or 12 weeks following STZ or saline injection, surgical implantation of catheters was done as explained in the sections of methodology. For normalization of cardiac baroreflexes, the animals were allowed to recover from anesthesia and surgery for at least 4
Following recovery, baseline hemodynamic measurements were taken. The pressor responses (MABP and HR) to isovolumeic bolus doses of methoxamine (100-300 nmol/kg) and AT II (20-320-pmol/kg) were measured separately in 8 control and 8 diabetic rats of each study group. The following protocol was adopted in each experiment,

<table>
<thead>
<tr>
<th>STZ/ Vehicle 1, 3, 9 or 12 week</th>
<th>Surgery</th>
<th>Recovery</th>
<th>1st DRC AT II/ MTX</th>
<th>1400W</th>
<th>2nd DRC AT II/ MTX</th>
<th>L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collect blood samples</td>
<td>15-20min</td>
<td>4 hrs</td>
<td>50 min</td>
<td>50 min</td>
<td>50 min</td>
<td>15-20min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MABP and HR recorded</td>
<td>MABP and HR recorded</td>
<td>MABP and HR recorded</td>
<td></td>
</tr>
</tbody>
</table>

For each bolus dose of methoxamine (MTX) and AT II, MABP and HR were recorded. Sufficient time (10 min) was allowed for the MABP and HR to return to normal before administration of the next dose. Subsequent to the final dose of methoxamine or AT II, a single bolus dose of 1400W (3 mg/kg) was administered through the jugular vein. Fifty minutes later, a second dose response curve (DRC) for methoxamine or AT II similar to the first one was constructed over a period of 50 minutes. This was followed by a single bolus dose of L-NAME (10 mg/kg). Once again, MABP and HR were recorded over a period of 15-20 minutes.

At the end of each experiment, animals were anesthetized (pentobarbital) and blood was collected from the carotid artery into tubes containing 10% v/v EDTA. The collected blood samples were immediately centrifuged (4500 rpm), plasma separated, collected, aliquoted into small microcentrifuge tubes and stored at -70°C till assayed. Subsequent to blood collection, thoracotomy was performed in each rat. The heart, aorta and superior mesenteric artery (SMA) were immediately removed and placed in ice-cold Krebs solution.
(120 mM NaCl, 5.9 mM KCl, 25 mM NaHCO₃, 11.5 mM glucose, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂). To prevent the induction of iNOS in vitro during the course of cleaning, water-soluble dexamethasone (0.1 μM) was added to the Krebs solution. The heart, aorta and SMA were cleared of all adherent tissues and divided into two halves. A portion of the heart’s left ventricle, aorta and mesenteric artery from each rat was fixed in 10% neutral buffered formalin (NBF) and processed for immuno-histochemical localization of NT and NOS isoforms. The remaining tissues were snap frozen in liquid nitrogen and stored at -70 °C for various other biochemical measurements.

**STUDY-3: Effects of N-acetylcysteine on iNOS mediated cardiovascular abnormalities in acute and chronic STZ diabetic rats.**

This study was designed to evaluate the acute and long-term effects of NAC treatment on oxidative stress and iNOS mediated cardiovascular changes.

Sixty-four male Wistar rats weighing 250 ± 25 g were obtained from Charles River Laboratories Inc., Laval, Quebec and allowed to acclimatize to the local vivarium. They were randomly divided into two major groups, 3 week and 9-week study groups. In each study group, 16 rats were made diabetic (STZ, 60 mg/kg, i.v) and further subdivided into four small groups namely Control (C), Control treated (CT), Diabetic (D) and Diabetic treated (DT). One week after induction of diabetes, NAC, a strong antioxidant and iNOS inhibitor was administered to the CT and DT groups in the drinking water at a concentration of 4.8 and 2.4 g/L respectively for 3 or 9 weeks. Five hour fasted blood samples were collected from the tail vein at basal and every week of NAC treatment for biochemical measurements. At termination, each animal was surgically prepared for measurement of MABP and HR.

After the measurement of MABP and HR, each rat was anesthetized (pentobarbital) and blood was collected from the carotid artery. The collected blood samples were immediately centrifuged (4500 rpm), plasma separated, collected, aliquoted into small microcentrifuge tubes and stored at -70°C till assayed. Subsequent to blood collection, thoracotomy was performed in each rat. The heart, aorta and superior mesenteric artery (SMA) were immediately removed and placed in ice-cold Krebs solution (120 mM NaCl, 5.9 mM KCl, 25 mM NaHCO₃, 11.5 mM glucose, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂). To prevent the induction of iNOS in vitro during the course of cleaning, water-
soluble dexamethasone (0.1µM) was added to the Krebs solution. A portion of the heart’s left ventricle, aorta and mesenteric artery from each rat was fixed in 10% neutral buffered formalin (NBF) and processed for immuno-histochemical localization of NT and NOS isoforms. The remaining tissues were snap frozen in liquid nitrogen and stored at -70 °C for various other biochemical measurements.

3.2.4 STATISTICAL ANALYSIS

All values are expressed as mean ± SEM. “n” denotes the sample size in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA) or repeated measures ANOVA (general linear model ANOVA), followed by a post-hoc test, the Newman-Keuls test. One-way ANOVA with “Newman-Keuls” post test was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA and repeated measure ANOVA was done using NCSS 2000, Kaysville, Utah, USA. For all results the level of significance was set at P<0.05.
4. RESULTS

4.1 STUDY-1: 1400W and LPS induced endotoxemia

This study was conducted to select an appropriate dose of 1400W, using the LPS-induced endotoxemic model of septic shock. Intravenous administration of 10, 5 or 3 mg/kg of LPS (E. coli) was tested against 1 or 3 mg/kg of 1400W. Administration of LPS resulted in characteristic symptoms of endotoxemia including systemic hypotension and circulatory collapse and eventual death in some cases. The results of the study are summarized in Table 4.1. Treatment with a low dose (1 mg/kg, i.v) of 1400W, 2 hours after LPS administration (10 or 5 mg/kg) did not prevent the circulatory collapse triggered by iNOS induction. However when the dose of LPS was reduced to 3 mg/kg and 1400W increased to 3mg/kg, the mortality was reduced in these animals and mean arterial blood pressure remained normal (Fig 4.1). The results of the study suggest that a dose of at least 3 mg/kg of 1400W is required to inhibit the activation of iNOS in LPS treated rats.

Table 4.1: Effect of 1400W administration on LPS induced endotoxemic shock.

<table>
<thead>
<tr>
<th>Number of Animals</th>
<th>Dose of LPS mg/kg (i.v)</th>
<th>Dose of 1400W mg/kg (i.v)</th>
<th>Percent Survival = Number of animals survived / Total number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>10</td>
<td>1</td>
<td>0%</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>1</td>
<td>0%</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>1</td>
<td>66%</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3</td>
<td>100%</td>
</tr>
</tbody>
</table>
Fig. 4.1: Effect of (N-[-3(aminomethyl) benzyl] acetamidine, dihydrochloride), 1400W (1 or 3 mg/kg, i.v) administered at 2 hours after LPS (10, 5 or 3 mg/kg, i.v) injection on the mean arterial blood pressure (MABP, mmHg). All values are expressed as mean ± SEM. This figure represents the pattern of blood pressure change following the administration of LPS in the surviving animals.
4.2 STUDY-2a: Effect of iNOS inhibition on the hemodynamic effects of methoxamine and angiotensin-2 at different durations of diabetes.

General Characteristics

The body weights and blood glucose levels of all rats were measured (Fig. 4.2.1a and 4.2.1b) at termination. All STZ injected rats were hyperglycemic (>20mM) compared to their age matched controls. The body weights of diabetic rats were significantly lower than the age matched control rats by 3 weeks of diabetes.

Basal Mean Arterial Blood Pressure (MABP) and Heart Rate (HR)

The MABP and HR were measured at 1, 3, 9 and 12-week post STZ injection (Fig. 4.2.2a and 4.2.2 b). By 3 weeks, all STZ diabetic rats had depressed MABP and HR. In control rats there was a progressive increase in the MABP upto 9 weeks of age. The MABP of 1-week control rats was significantly lower than MABP’s of 3W, 9W and 12-week control rats. The results of this observation suggest that MABP increases with age in normal control rats. On the other hand, diabetic rats had similar blood pressures but significantly lower than their corresponding age matched control groups at all time points starting from 3 weeks. The heart rate in diabetic groups decreased significantly compared to their age matched control animals, starting from 3rd week of diabetes. The effect of 1400W administration on the basal MABP and HR at different stages of diabetes were recorded and summarized in table 4.2. The results suggest that administration of 1400W did not alter the basal MABP and HR at any time point either in control or diabetic groups.
Fig.4.2.1a and b: Body weights (A) and blood glucose levels (B) measured at 1, 3, 9 and 12 weeks following induction of diabetes. Diabetes was induced by an i.v injection of 60 mg/kg STZ. All values are expressed as mean ± SEM. * different from their respective control groups (P < 0.01). n = 16-17.
Fig. 4.2.2a and b: Basal mean arterial blood pressure (MABP, mm Hg) and heart rate (HR, beats/min) measured at 1, 3, 9 and 12 weeks following induction of diabetes. All values are expressed as mean ± SEM. * different from their respective control groups (P<0.001), @ different from 3W, 9W and 12W control groups (P<0.001), # different from 12 W diabetic group (P<0.05), $ different from 1W, 9W and 12 W control groups (P<0.01). n = 11 to 14.
Table 4.2: Effect of 1400W (3mg/kg, i.v) administration on basal mean arterial blood pressure (MABP, mm Hg) and heart rate (HR, beats/min) in rats of different durations of diabetes.

<table>
<thead>
<tr>
<th>DURATION OF DIABETES</th>
<th>BASAL MEAN ARTERIAL BLOOD PRESSURE (mm Hg)</th>
<th>HEART RATE (beats / min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>DIABETIC</td>
</tr>
<tr>
<td></td>
<td>Before 1400W</td>
<td>After 1400W</td>
</tr>
<tr>
<td>1 WEEK</td>
<td>101 ± 2.0</td>
<td>99 ± 2.0</td>
</tr>
<tr>
<td>3 WEEK</td>
<td>114 ± 1.5</td>
<td>112 ± 1.8</td>
</tr>
<tr>
<td>9 WEEK</td>
<td>126 ± 1.0</td>
<td>122 ± 1.2</td>
</tr>
<tr>
<td>12 WEEK</td>
<td>122 ± 1.5</td>
<td>121 ± 1.8</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. The above parameters were measured at basal (prior to DRC for vasoactive agents). A single bolus dose of 3mg/kg (i.v) of 1400W was used to inhibit iNOS. * different from their respective control group (P<0.05). n = 11-14.
Effect of iNOS inhibition with 1400W on the pressor responses (MABP) of methoxamine and angiotensin-2: Effect of the duration of diabetes.

Pressor responses (changes in MABP) to bolus doses of methoxamine (100-300 nmol/kg) and AT II (20-320 pmol/kg) were tested in rats of different durations of diabetes. The pressor responses to methoxamine were significantly attenuated in 12-week diabetic rats compared to 1-week diabetic rats (Fig.4.2.3a). Similarly, the pressor responses to AT II were also significantly reduced in 9W and 12 W diabetic rats compared to 1-week diabetic rats (Fig.4.2.3b). The results suggest that the duration of diabetes significantly affect the pressor responses of both methoxamine and AT II.

Administration of bolus doses of methoxamine increased the pressor responses in a dose dependent manner both in control and diabetic rats at different time points (Fig.4.2.4 a-d). Compared to the age matched control rats, the pressor responses to methoxamine were attenuated in diabetic rats by 3 weeks. Pretreatment with 1400W did not significantly affect the MABP and HR responses of methoxamine in control rats. However, the pressor responses to methoxamine in 1400 treated rats were significantly augmented in 3, 9 and 12-week diabetic rats.

Similar changes in MABP were also observed for bolus doses of AT II (Fig.4.2.5 a-d). The pressor responses were increased in a dose dependent manner both in control and diabetic rats to AT II at different time points. Compared to age matched control rats, the pressor responses to AT II in diabetic rats were significantly reduced in 3, 9 and 12 week diabetic rats. Pretreatment with 1400W did not show any effect in control rats however there was a significant improvement in the pressor responses to bolus doses of AT II in 3, 9 and 12 week diabetic rats.

Further analysis of the results indicates that, the pressor responses to methoxamine were depressed to a greater extent when compared to responses of AT II in untreated diabetic rats (Fig.4.2.3 c and d). Selective inhibition of iNOS by 1400W completely restored the pressor responses to both AT II and methoxamine in all diabetic groups except the 12-week diabetic group, where the methoxamine response increased but did not reach control levels.
Fig. 4.2.3 a and b: Effect of the duration of diabetes on the pressor responses of methoxamine (A) and AT II (B). All values are expressed as mean ± SEM. * different from 1 week diabetic rats (P<0.05), # different from 9 and 12 week diabetic rats. n=6-8.
Fig. 4.2.3 c and d: Effect of the duration of diabetes on the AUC formed by the pressor responses of methoxamine (MTX) and angiotensin-2 (AT II). All values are expressed as mean ± SEM. * different from their corresponding age matched AT II group (P<0.001). @ different from 1, 3 and 12 week AT-II groups. # different from 1 week methoxamine group.
Fig. 4.2.4 a and b: Pressor responses to bolus doses of methoxamine in 1 week (A) and 3 week (B) diabetic rats treated with or without 1400W (3mg/kg). CB and CA denote control before and after 1400W administration and DB and DA denote diabetic before and after 1400W administration respectively. All values are expressed as mean ± SEM. * different from DA, CB and CA groups (P<0.05). n=6-8.
Fig. 4.2.4 c and d: Pressor responses to bolus doses of methoxamine in 9 week (C) and 12 week (D) diabetic rats treated with or without 1400W (3mg/kg). CB and CA denote control before and after 1400W administration and DB and DA denote diabetic before and after 1400W administration respectively. All values are expressed as mean ± SEM. * different from DA, CB and CA groups (P<0.05). n=6-8.
Fig. 4.2.5 a and b: Pressor responses to bolus doses of AT II in 1 week (A) and 3 week (B) diabetic rats treated with or without 1400W (3mg/kg). CB and CA denote control before and after 1400W administration and DB and DA denote diabetic before and after 1400W administration respectively. All values are expressed as mean ± SEM. * different from DA group (P<0.05). n=6-8.
Fig. 4.2.5 c and d: Pressor responses to bolus doses of AT II in 9 week (C) and 12 week (D) diabetic rats treated with or without 1400W (3mg/kg). CB and CA denote control before and after 1400W administration and DB and DA denote diabetic before and after 1400W administration respectively. All values are expressed as mean ± SEM. * different from DA, CB and CA groups (P<0.05). n=6-8.
**Endothelial function: Effect of L-NAME administration on MABP**

The effect of duration of diabetes on endothelial function was tested by a single bolus dose of L-NAME (10 mg/kg, i.v), a non-selective inhibitor of NOS. The effect of L-NAME on changes in MABP is shown in fig.4.2.6. By three weeks, the diabetic animals showed attenuated pressure response (Δ MABP) compared to their age matched control rats to a non-selective blockade of NOS. Similar results were also observed in 9 and 12 week diabetic rats indicating endothelial dysfunction.

![Graph](image)

**Fig. 4.2.6:** Effect of L-NAME administration (10mg/kg) on mean arterial blood pressure (MABP, mm Hg) in control and diabetic rats of different durations of diabetes. All values are expressed as mean ± SEM. * different from their respective control groups (P<0.05), @ different from all other diabetic groups (P<0.05). n=14-17.
Plasma nitrite and nitrate (NOx) levels.

The basal plasma NOx values were measured in all of the groups (Fig. 4.2.7). No significant difference in plasma NOx values was observed in 1 and 3 week diabetic rats compared to their age matched control rats. However, by 9 weeks the plasma NOx values decreased significantly in diabetic rats compared to their control rats. Interestingly, there was a significant elevation of plasma NOx values in 12-week diabetic rats compared to their age matched control rats.

Fig. 4.2.7: Effect the duration of diabetes on plasma nitrite and nitrate (NOx) levels in control and diabetic rats of different durations of diabetes. All values are expressed as mean ± SEM. * different from 9C group (P<0.05), # different from 12C group (P<0.05). n=14-17.
4.2 STUDY-2b: To study the effect of duration of diabetes on the differential expression of eNOS and iNOS in STZ diabetic rats.

Immunohistochemistry, to detect and localize eNOS and iNOS proteins was performed on the paraffin sections of heart (left ventricle), thoracic aorta and superior mesenteric artery. In control rats, strong positive immunostain for eNOS was observed in the vascular endothelium (monolayer) of all the tissue sections tested. Similar to control rats, there was no change in eNOS immunostain in the endothelium of 1 or 3 week diabetic rats (representative fig.4.2.8 and 4.2.9). However, the intensity of positive immunostain for eNOS was relatively lower in aortic and mesenteric arteries of 9 and 12 week diabetic rats compared to their age matched control rats. By 12 weeks, positive staining for eNOS was markedly lower in diabetic rats and almost completely disappeared in the vascular endothelium of all the tissues tested (representative fig.4.2.10 and 4.2.11). Sections incubated with nonspecific mouse immunoglobulins (IgG) did not demonstrate immunostaining indicating the specificity of the eNOS antiserum.

In contrast there was a striking difference between control and diabetic tissues for iNOS immunostain. iNOS protein expression was not evident in any of the tissue sections tested in 1 week control and diabetic rats (representative fig.4.2.12). The first trace of iNOS protein, although not very strong, was detected in the myocardial tissue of 3-week diabetic rats (representative fig.4.2.13). However, a strong positive signal for iNOS protein was observed in the myocardial and vascular (aortic and superior mesenteric) tissues of 9 and 12-week diabetic rats (representative fig.4.2.14 and 4.2.15). In addition, localization of iNOS protein could not be established in the tissues tested with the available photomicrographs. No positive immunostaining for iNOS was observed in any of the tissues tested in control rats at any time point. Sections incubated with nonspecific rabbit immunoglobulins (IgG) did not demonstrate immunostaining for iNOS indicating the specificity of the iNOS antiserum.

The results of this study demonstrate that the expression of eNOS and iNOS are differentially regulated in experimental diabetes where the prolonged diabetic state led to the down regulation of eNOS with a concurrent upregulation of iNOS.
Fig.4.2.8: Immunohistochemical detection of eNOS in 1 week control and diabetic rat tissues
Fig. 4.2.9: Immunohistochemical detection of eNOS in 3 week control and diabetic rat tissues
Fig. 4.2.10: Immunohistochemical detection of eNOS in 9 week control and diabetic rat tissues
Fig. 4.2.11: Immunohistochemical detection of eNOS in 12 week control and diabetic rat tissues
Fig. 4.2.12: Immunohistochemical detection of iNOS in 1 week control and diabetic rat tissues
Fig. 4.2.13: Immunohistochemical detection of iNOS in 3 week control and diabetic rat tissues
Fig.4.2.14: Immunohistochemical detection of iNOS in 9 week control and diabetic rat tissues
Fig. 4.2.15: Immunohistochemical detection of iNOS in 12 week control and diabetic rat tissues
**Immunohistochemical identification of nitrotyrosine in the heart and arteries.**

In addition to the detection of NOS protein isoforms, immunohistochemistry to determine the NT expression was performed in all the groups. Analysis of the results indicate a marked increase in the intensity of NT stain (reddish brown) in all the diabetic tissues by 3 weeks of diabetes. The pink stain observed in some of the photographs is due to the H & E counterstain and not due to NT immunostain. Further, this intensity increased as the diabetic state progressed from week 3 to week 12 with the strongest intensity observed in 12 week diabetic rats. Also, NT was more prominently stained in the diabetic myocardium than aorta and mesenteric arteries. Although the control groups were stained appreciably, they were less intense when compared to the diabetic tissues (representative fig.4.2.16). Sections incubated with nonspecific rabbit immunoglobulins (IgG) did not demonstrate immunostaining for iNOS indicating the specificity of the iNOS antiserum.
Fig. 4.2.16: Immunohistochemical detection of NT in 12 week control and diabetic rat tissues
4.3 STUDY-3: To study the effect of N-acetylcysteine (NAC) on iNOS mediated cardiovascular abnormalities in acute and chronic STZ diabetic rats.

General physical and biochemical characteristics

The effect of NAC on the general physical and biochemical characteristics at termination of 3 and 9 weeks STZ diabetic rats are summarized in tables 4.3 and 4.4. Injection of STZ to rats resulted in characteristic symptoms of diabetes including hyperglycemia, hypoinsulinemia, decreased body weight gain and increased food and fluid intake when compared to age matched control rats (Table.4.3 and 4.4). Plasma triglyceride or cholesterol levels were not different in 3 week control and diabetic rats. NAC treatment did not affect the fluid intake, plasma glucose, insulin and cholesterol levels in either control or diabetic rats. However, treatment with NAC significantly reduced the plasma triglyceride levels in 3 week diabetic rats.

In 9 week diabetic rats there was a significant elevation in plasma triglyceride and cholesterol levels compared to their age matched control rats. Treatment with NAC normalized these values in diabetic rats. Also, food and fluid intake was markedly reduced in NAC treated diabetic rats.

Effect of NAC on MABP and HR

The aim of this study was to investigate if the uncoupling of oxidative stress from nitrative stress would provide protection against diabetes induced cardiovascular depression. At termination the MABP and HR were measured in all rats of both study groups. As observed in previous studies, the MABP and HR were significantly reduced in untreated diabetic rats by 3 weeks of diabetes (Fig.4.3.1 a and b). Similar results were observed in 9 week untreated diabetic rats. Treatment with NAC prevented these changes in both 3 week and 9 week diabetic rats. The results suggest that STZ diabetic rats develop cardiovascular depression by 3 weeks and antioxidant treatment prevented these changes.
Table 4.3: Effect of NAC treatment on general physical and biochemical characteristics in 3 week STZ diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control C, (n=8)</th>
<th>Diabetic D, (n=8)</th>
<th>Control Treated CT, (n=8)</th>
<th>Diabetic Treated DT, (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>389±7</td>
<td>319±6</td>
<td>382±10</td>
<td>321±11</td>
</tr>
<tr>
<td>Plasma Glucose (mM)</td>
<td>8.3±0.5</td>
<td>27±2.3*</td>
<td>7.9±0.3</td>
<td>22.2±0.6*</td>
</tr>
<tr>
<td>Fluid Intake (mL/day)</td>
<td>54±3</td>
<td>209±24*</td>
<td>42±2</td>
<td>189±10*</td>
</tr>
<tr>
<td>Plasma Insulin (ng/mL)</td>
<td>1.5±0.1</td>
<td>0.5±0.04*</td>
<td>2.1±0.2</td>
<td>0.6±0.06*</td>
</tr>
<tr>
<td>Plasma Cholesterol (mM)</td>
<td>0.7±0.06</td>
<td>0.5±0.03</td>
<td>0.8±0.07</td>
<td>0.5±0.02</td>
</tr>
<tr>
<td>Plasma Triglycerides (mM)</td>
<td>0.9±0.15</td>
<td>0.9±0.15</td>
<td>0.7±0.14</td>
<td>0.4±0.1*</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM. The above parameters were measured at termination of 3 weeks of NAC treatment (2.4 or 4.8 g/L in drinking water). Diabetes was induced by a single i.v injection of 60 mg/kg of streptozotocin. * different from control group (P<0.05), # different from D group (P<0.05).
Table 4.4: Effect of NAC treatment on general physical and biochemical characteristics in 9 week STZ diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control C, (n=8)</th>
<th>Diabetic D, (n=8)</th>
<th>Control Treated CT, (n=8)</th>
<th>Diabetic Treated DT, (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>507±8</td>
<td>372±21*</td>
<td>495±14</td>
<td>343±11*</td>
</tr>
<tr>
<td>Plasma Glucose (mM)</td>
<td>8.3±0.1</td>
<td>33.2±1.9*</td>
<td>8.3±0.3</td>
<td>28.2±1.1*</td>
</tr>
<tr>
<td>Fluid Intake (mL/day)</td>
<td>58±4</td>
<td>281±14*</td>
<td>41±4</td>
<td>207±4#</td>
</tr>
<tr>
<td>Food Intake (g/day)</td>
<td>31±0.3</td>
<td>59±2*</td>
<td>31±0.3</td>
<td>46±1.2#</td>
</tr>
<tr>
<td>Plasma Insulin (ng/mL)</td>
<td>1.3±0.15</td>
<td>0.2±0.05*</td>
<td>1.0±0.09</td>
<td>0.3±0.06*</td>
</tr>
<tr>
<td>Plasma Cholesterol (mM)</td>
<td>1.1±0.1</td>
<td>2.1±0.3*</td>
<td>1.0±0.05</td>
<td>1.1±0.07#</td>
</tr>
<tr>
<td>Plasma Triglycerides (mM)</td>
<td>0.4±0.1</td>
<td>3.1±0.8*</td>
<td>0.6±0.1</td>
<td>0.8±0.1#</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM. The above parameters were measured at termination of 9 weeks of NAC treatment (2.4 or 4.8 g/L in drinking water). Diabetes was induced by a single i.v injection of 60 mg/kg of streptozotocin. * different from control group (P<0.05), # different from D group (P<0.05).
**Fig. 4.3.1 a and b:** Effect of NAC treatment on MABP (A) and HR (B) in 3 and 9 week diabetic rats. All values are expressed as mean ± SEM. *different from their respective control group, # different from their respective untreated diabetic group (P<0.05). n=6-8.
Effect of NAC treatment on plasma isoprostane (15-F_2t) and NOx concentrations.

In this experiment the effect of NAC treatment on plasma isoprostane, a marker of lipid peroxidation and oxidative stress and concomitant nitrative stress was observed.

Measurement of plasma free isoprostanes in 3-week study groups did not show any significant difference between untreated control and diabetic rats (Fig. 4.3.2). Also NAC treatment did not alter these values in control and diabetic rats. However, nine weeks of STZ-diabetes resulted in a significant elevation in isoprostane values suggesting an increased lipid peroxidation and oxidative stress. Treatment with NAC significantly decreased the plasma isoprostane values in diabetic rats without altering values in control rats.

![Fig.4.3.2: Effect of NAC treatment on plasma free isoprostane levels in 3 and 9 week diabetic rats. All values are expressed as mean ± SEM. *different from all other groups and also 9C group (P<0.001), # different from 9 week untreated diabetic group (P<0.01). n=6-8.](image-url)
Plasma nitrite and nitrate (NOx) levels, an indicator of NO metabolism were measured at termination in all rats of both 3 and 9 week study groups (Fig.4.3.3). The NOx values were not different from each other in untreated control and diabetic rats in the 3 week study. Also, NAC treatment did not affect the plasma NOx values either in control or diabetic rats. However, as observed in our previous studies, 9 weeks of STZ- diabetes resulted in a significant reduction in plasma NOx values in untreated diabetic rats. The results suggest that there is either a decreased formation of NO or increased scavenging activity of oxidative free radicals in untreated diabetic rats. Treatment with NAC normalized these values in diabetic rats but did not alter the same in control rats.

The above results suggest that by 9 weeks of diabetes, there may be an increased level of oxidative stress concomitant with decreased plasma NOx values and NAC treatment normalized both of these values in diabetic rats.

**Fig.4.3.3:** Effect of NAC treatment on plasma nitrite and nitrate (NOx) levels in 3 and 9 week diabetic rats. All values are expressed as mean ± SEM. *different from 9 week control group (P<0.001), # different from 9 week untreated diabetic group (P<0.01). n=6-8.
Effect of NAC treatment on plasma total antioxidant concentrations and myocardial antioxidant capacity.

To study the effect of NAC on the total antioxidant capacity we measured the plasma total antioxidant concentrations in both 3 and 9 week study groups. Three weeks of hyperglycemia did not alter the plasma total antioxidant concentrations and NAC treatment did not change these values in any of the treated group (Fig.4.3.4). Nine weeks of STZ diabetes however, resulted in a marked reduction in the total antioxidant concentrations. Treatment with NAC significantly improved the total antioxidant capacity both in control and diabetic rats.

![Graph showing the effect of NAC treatment on plasma total antioxidant concentrations in 3 and 9 week diabetic rats. All values are expressed as mean ± SEM. * different from their respective 3D, 9C and 9CT groups (P<0.01), # different from their respective 9 week untreated diabetic group (P<0.01). n=6-8.](image)

Fig.4.3.4: Effect of NAC treatment on plasma total antioxidant concentrations in 3 and 9 week diabetic rats. All values are expressed as mean ± SEM. * different from their respective 3D, 9C and 9CT groups (P<0.01), # different from their respective 9 week untreated diabetic group (P<0.01). n=6-8.
Myocardial antioxidant capacity as determined by the formation of TBARS in response to increasing concentrations (Fig-22) of t-butylhydroperoxide (t-BHP) is represented in the Fig. 4.3.5. This study was performed in 3 week diabetic rats. Analysis of the results suggests that the myocardial antioxidant capacity was lower (increased TBARS formation) in untreated diabetic rats compared to control rats. NAC treatment significantly improved the myocardial antioxidant capacity in diabetic rats. The treatment was effective against the oxidant effects of t-BHP concentrations as high as 5 mM.

The results suggest that NAC treatment significantly improved the antioxidant defense mechanisms by increasing the plasma total antioxidants concentration and myocardial antioxidant capacity in diabetic rats.

**Fig.4.3.5:** Effect of NAC treatment on myocardial antioxidant capacity in 3 week diabetic rats. All values are expressed as mean ± SEM. *different from C and CT groups (P<0.05), # different from D (P<0.05). n=6-8.
Effect of NAC treatment on eNOS, iNOS and NT protein expression in heart, aorta and mesenteric arterial tissues.

This experiment was conducted to study the effect of NAC treatment on the eNOS, iNOS and NT protein expression in different cardiovascular tissues.

The expression of eNOS protein, as assessed by immunohistochemistry was abundant in heart (ventricle), aortic (thoracic) and superior mesenteric artery tissues of all groups in the 3-week study. Neither NAC treatment nor diabetes altered the eNOS protein expression in 3 week rats (images not shown). However, by 9 weeks of STZ diabetes there was a marked reduction in the intensity of eNOS protein stain in heart (representative fig.4.3.6), aortic (representative fig.4.3.7) and superior mesenteric artery tissues (representative fig.4.3.8) of untreated diabetic rats. This is in comparison with the corresponding tissues of untreated and treated control rats. Treatment with NAC significantly improved the eNOS protein expression in heart, aortic and mesenteric artery tissues of diabetic rats. Sections incubated with nonspecific mouse immunoglobulins (IgG) did not demonstrate immunostaining indicating the specificity of the eNOS antiserum.

A marked increase in the protein expression of iNOS by 9 weeks (representative fig.4.3.9 to 4.3.11) and NT by 3 weeks (representative fig.4.3.12), was observed in all the tissues of untreated diabetic rats compared to treated and untreated control rats. Treatment with NAC reduced both the iNOS and NT protein expression in all the tissue sections tested. Some sections stained with pink colour (CT group) are due to H&E counterstain and not due to NT immunostaining. Sections incubated with nonspecific rabbit immunoglobulins (IgG) did not demonstrate immunostaining indicating the specificity of the iNOS and NT antiserum.

Data from this study showed that the expression of eNOS and iNOS are inversely regulated at 9 week of diabetes where increased expression of iNOS is associated with a corresponding decrease in eNOS. The results of this study demonstrate that NAC treatment reverses this imbalance by normalizing the eNOS and NT protein expression and at the same time prevented the induction of iNOS in diabetic tissues.
Fig. 4.3.6: Effect of NAC treatment on eNOS expression.
Representative photomicrographs of eNOS immunostaining in myocardial tissues of 9 week control (C), diabetic (D), control treated (CT), diabetic treated (DT) and negative control (IgG).
Magnification (X25)
Fig. 4.3.7: Effect of NAC treatment on eNOS expression.
Representative photomicrographs of eNOS immunostaining in aortic tissues of 9 week control (C), diabetic (D), control treated (CT), diabetic treated (DT) and negative control (IgG). Magnification (X25)
Fig. 4.3.8: Effect of NAC treatment on eNOS expression.
Representative photomicrographs of eNOS immunostaining in superior mesenteric arterial tissues of 9 week control (C), diabetic (D), control treated (CT), diabetic treated (DT) and negative control (IgG). Magnification (X25)
Fig. 4.3.9: Effect of NAC treatment on iNOS expression. Representative photomicrographs of iNOS immunostaining in myocardial tissues of 9 week control (C), diabetic (D), control treated (CT), diabetic treated (DT) and negative control (IgG). Magnification (X25)
Fig. 4.3.10: Effect of NAC treatment on iNOS expression.
Representative photomicrographs of iNOS immunostaining in aortic tissues of 9 week control (C), diabetic (D), control treated (CT), diabetic treated (DT) and negative control (IgG). Magnification (X25)
Fig. 4.3.11: Effect of NAC treatment on iNOS expression.
Representative photomicrographs of iNOS immunostaining in superior mesenteric arterial tissues of 9 week control (C), diabetic (D), control treated (CT), diabetic treated (DT) and negative control (IgG). Magnification (X25)
Fig.4.3.12: Effect of NAC treatment on myocardial NT expression.
Representative photomicrographs of NT immunostaining (reddish brown) in heart tissues of 3 week control (C), diabetic (D), control treated (CT), diabetic treated (DT) and negative control (IgG). Magnification (X25)
5. DISCUSSION

5.1 Overview

Diabetes mellitus is associated with an increased incidence of cardiovascular complications, which are the major causes of morbidity and mortality in the western world\textsuperscript{23,217}. Despite the increasing number of pharmacological and surgical interventions, mortality among diabetic patients has remained high\textsuperscript{215,236}. It is now recognized that diabetes mellitus is "a cardiovascular disease" and that diabetes is an independent risk factor for a cluster of cardiovascular diseases. An important and independent predisposing factor in the development of diabetic cardiovascular complications is chronic hyperglycemia. Hyperglycemia has been shown to directly affect the composition and structure of cardiac, vascular and renal tissue, the progressive modification of which results in deranged cardiovascular homeostasis\textsuperscript{218}.

The etiologies of cardiovascular complications, as mentioned in the introduction, are complex and multifactorial and involve a number of factors including specific vascular, renal, neuropathic and myopathic alterations. Altered vascular responses to vasoactive agents, defective cardiac autonomic function, altered substrate utilization, structural alterations in cardiovascular tissues, abnormal ionic movements, changes in transmembrane and sarcolemmal proteins and many more mechanisms have been implicated in cardiovascular complications of diabetes\textsuperscript{237}. Excess oxidative stress, one of the sequel of hyperglycemia has received enormous attention in the etiology of diabetic cardiovascular diseases. Recent studies have suggested that superoxide production at the mitochondrial level during hyperglycemia is the initiating event that can activate all other major pathways involved\textsuperscript{232,238}. These include the most widely believed mechanisms such as an activated polyol pathway, increased formation of AGE and activation of the PKC and hexosamine pathways. Increased formation of superoxide anions in concert with increased formation of NO from iNOS lead to peroxynitrite formation and lipid peroxidation, which synergistically compromise ATP synthesis, damage mitochondria, decrease cellular viability and promote apoptosis\textsuperscript{239,240}. These studies have emphasized that glucose-induced formation of free radicals is an early step in the cell perturbation and that suppression of intracellular oxidants using either low molecular weight inhibitors or by expression of antioxidant enzymes such as SOD can prevent the cascade of events that lead to diabetic complications\textsuperscript{232}. 
Since formation of peroxynitrite is an important mediator and unifying mechanism and since iNOS derived NO may be a major contributor of NO in hyperglycemic conditions, it is possible that iNOS induction and activation might play an important role in the pathophysiology of cardiovascular complications. The main focus of the work presented in this thesis was to study the effect of the duration of diabetes on the expression of iNOS in different tissues of cardiovascular system. Using the STZ model of diabetes we examined the effects of diabetes on the iNOS, eNOS and NT protein expression and the role of iNOS in modulating the hemodynamic effects of vasoactive agents. In addition we studied the effects of acute and chronic antioxidant treatment on iNOS-mediated cardiovascular abnormalities.

5.2 Selective iNOS inhibition with 1400W in endotoxemic model of septic shock

Septic shock is a systemic inflammatory response to diverse pathological insults and is characterized by severe hypotension, profound vasodilation, cardiac depression and multiple organ failure. Pathological insults include pro-inflammatory cytokines such as interleukin-1β (IL-1β), interferon-γ (IF-γ) and tumor necrosis factor-α (TNF-α) produced by macrophages, endothelial and myocardial cells. It is well known that large amounts of NO produced in response to these bacterial endotoxins (lipopolysaccharide from Escherichia coli) and cytokines in the late phase of septic shock occurs through the induction of iNOS. Concomitant with the enhanced NO production is an increase in cellular superoxide anion production from various sources such as xanthine oxidase, NAD(P)H oxidase, mitochondria, arachidonic acid metabolism, eNOS and iNOS. NO reacts rapidly with $O_2^-$ to form peroxynitrite at a rate that is approximately 3 times faster than the rate of SOD catalyzed dismutation of $O_2^-$. An increasing body of evidence suggests that peroxynitrite irreversibly oxidizes or nitrates proteins, lipids and DNA resulting in cardiovascular dysfunction in septic shock.

Following the discovery that iNOS knockout mice are resistant to the hypotensive effects of endotoxin and that selective iNOS inhibition attenuates hypotension and vascular hypo-reactivity to pressor agents, numerous studies have demonstrated beneficial hemodynamic effects of iNOS inhibition in animal models of septic shock and circulatory failure. Among the various nitric oxide synthase inhibitors, 1400W is by far the most selective inhibitor of iNOS. It is a slow but tight binding and irreversible inhibitor of
iNOS both *in vitro* and *in vivo*. It has been reported to exhibit greater than 1000-fold selectivity against rat iNOS relative to eNOS\(^{222}\). Other iNOS inhibitors include S-ethylisothiourea (EIT), 7-chloro-3-imino-5-methyl-2-azabicyclo heptatane hydrochloride (ONO-1714), aminoguanidine (AG) and L-NMMA, which are 40, 10, 4.8 and 0.3 fold more selective respectively for iNOS than eNOS\(^{243}\).

Induction of peripheral iNOS by LPS has been used as a rapid procedure to evaluate 1400W-inhibition activity\(^{244}\). A wide variety of doses ranging from 0.3 mg/kg to 3 mg/kg of 1400W have been reported to inhibit iNOS and prevent LPS induced endotoxic shock in peripheral tissue, and a dose of 20 mg/kg is reported to inhibit iNOS in the central nervous system\(^{245}\). However, many of these experiments were conducted either *in vitro* or in anesthetized animals and have employed different techniques for measurements of blood pressure. In our investigation we studied the iNOS inhibitory effects of 1400W on MABP in intact and freely moving conscious rats.

To examine the ability of 1400W to selectively inhibit iNOS, we administered 1400W two hours after LPS injection and monitored MABP constantly for 4 hours or until death, whichever was the earliest. Administration of LPS caused a severe but a brief decline in MABP and HR (not reported) in the first 10 minutes. This was immediately followed by a recovery period and another phase of severe hypotension (early phase, 60-90 min). In next 3 to 4 hours, a significant drop in MABP was observed (late phase) in all LPS injected rats and all the deaths reported in the study were observed in this phase of hypotension. This phenomenon has also been noted in other studies and has been suggested that the early phase of depressed MABP (60-90 min) is due to eNOS activation whereas the late phase of septic shock that is characterized by severe hypotension and or death is due the induction of iNOS \(^{246,247,248}\). The severe and transient hypotension observed in the beginning of LPS administration may be explained by the direct cardiac depressant actions of LPS\(^{249,219}\) and/or due to a direct administration of LPS into the heart via the jugular vein. Restoration to normal MABP and HR in these rats probably involves a compensatory mechanism (baroreceptor sensing) resulting in increased sympathetic discharge and increase catecholamine levels. This is possible because septic shock is reported to be associated with increased plasma concentrations of vasoconstrictor agents such as NE, which can help overcome hypotension \(^{250,251,252,253,254}\).
Administration of low doses of 1400W (1 mg/kg) did not prevent the decline in the late phase MABP against the large doses of LPS (10 or 5 mg/kg). All the animals that died in our study were in the late phase of septic shock (>2 hrs). We therefore reduced the dose of LPS to 3 mg/kg and still noticed a similar pattern of MABP changes in the late phase of septic shock. Hence we used this dose to test the effect of 1400W to selectively inhibit iNOS. When the dose of 1400W was increased to 3 mg/kg, it completely abolished the late phase fall in MABP and thus indicated inhibition of iNOS. This is consistent with other studies, which have demonstrated selective inhibition of iNOS in similar range of 1400W doses in the LPS model of septic shock\textsuperscript{205,223,255}. Hence, it is reasonable to conclude that 1400W at a dose of 3 mg/kg inhibits iNOS activation. We therefore used this dose in our subsequent experiments.

5.3 Effect of the duration of diabetes on cardiovascular homeostasis

Administration of STZ to rats resulted in characteristic features of uncontrolled Type 1 diabetes similar to that seen in humans, including hyperglycemia, hypoinsulinemia, hyperlipidemia, polyphagia, polyuria and deceased body weight gain. Cardiovascular homeostasis was also demonstrated to be compromised in those rats with depressed blood pressure, heart rate and abnormal vascular reactivity, most notably impaired endothelial function.

Although we did not determine if STZ directly affected the cardiovascular homeostasis including MABP and HR, previous studies have reported no significant effects on cardiovascular tissues\textsuperscript{256,225}. Moreover, histological studies have failed to discover any sign of direct toxic effects of STZ on organs other than pancreatic β cells\textsuperscript{257}. The short half-life (15 min) of STZ further rules out any prolonged effects on cardiovascular tissue. In addition, numerous studies have demonstrated that correction of STZ mediated biochemical changes with insulin or other glucose lowering agents was able to prevent the cardiovascular abnormalities\textsuperscript{258,259,260}. Thus it is reasonable to conclude that any changes observed in STZ diabetic rats were not likely due to a direct effect of STZ \textit{per se} but rather were due to the consequences of the long-term hyperglycemia and hypoinsulinemia.

Administration of 60 mg/kg STZ in our rats resulted in depressed mean arterial blood pressure and heart rate within 3 weeks. No significant difference in the basal MABP or HR was observed in 1-week diabetic rats compared to age-matched control rats. However, in all
other study groups, namely 3, 9 and 12 week diabetic rats, the basal MABP and HR was significantly lower than in the corresponding age-matched controls. Cross analysis of the results suggests that diabetic rats actually did not show any decrease in MABP (from 1 week to 12 week) but rather failed to increase MABP with age. In control rats, the MABP increased while HR decreased progressively with age. It has been suggested that the increase in MABP is normal and is required to maintain an adequate tissue perfusion with age and is often described as a compensatory mechanism to the loss of arterial compliance \(^{261}\). Similarly, the decrease in heart rate is usually attributed to a diminution in the efficacy of \(\beta\) adrenergic stimulation of the heart, which again is normal and an age-dependent process \(^{262, 263}\).

Diabetes-associated changes in cardiovascular function in the STZ-induced rat model are not entirely consistent with alterations observed in diabetic patients. In contrast to the absence of changes in MABP, some clinical studies indicate that diabetes is associated with hypertension \(^{264, 265}\) while others show no significant changes in blood pressure \(^{266, 69}\). The etiology of hypertension in diabetes is unknown but is typically associated with long-term disease. The patients often have atherosclerotic changes, impaired vascular compliance and/or diabetic nephropathy, all potential mediators of elevated arterial pressure \(^{69}\). In addition, unlike STZ diabetic rats, most diabetic patients have better glycemic control with insulin treatment. These may contribute to disparities between humans and the rat model.

The depressed MABP observed in our diabetic rats is in good agreement with many other studies in STZ diabetic rats \(^{267, 268, 59, 258, 259, 260, 203, 269}\). Previous studies from our own laboratory have shown lower heart rates and reduced systolic blood pressure in conscious STZ diabetic rats. However, a few studies from other laboratories have reported unchanged or increased blood pressure in diabetic rats \(^{270, 205, 271}\). For example, in rats with STZ diabetes under thiobarbitone anesthesia, the MABP was not different from control rats after 3-4 weeks \(^{273}\). Similarly the mean arterial pressure in conscious rats of 3 weeks diabetes was not different from control rats \(^{205}\). However, in another study an increase in arterial blood pressure was observed as early as one week and reached maximum by 4 weeks of diabetes \(^{274}\). Although the reasons for these discrepancies are not very clear, they may be explained by the variations in the strain, the duration of diabetes, anesthesia or different techniques for measuring blood pressure \(^{275}\). For example, many studies have reported that relatively short-term diabetic rats are normotensive \(^{276, 277}\). Our data also reveal a similar observation in 1-
week diabetic rats in which the MABP was not different from control rats. Furthermore, use of anesthetics that have depressant effects on sympathetic activity can affect accurate blood pressure measurement. It has been recently shown that α-chloralose reduces sympathetic activity in rats and leads to unstable systemic hemodynamics in the presence of urethane anesthesia. In addition, the method or techniques employed for blood pressure measurement can also significantly affect the results. It was demonstrated that in normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), injection of STZ resulted in increased blood pressure in diabetic WKY rats when measured by an indirect method (tail cuff) and decreased BP when measured by a direct cannulation method. A similar study in STZ diabetic rats showed no change in blood pressure when measured by an indirect method while it decreased when measured by a direct method. A study employing a biotelemetry technique that involved implantation of a pressure sensor in the artery of an intact animal did not show any differences in the mean arterial pressure between control and STZ diabetic rats. However, the pulse pressure and heart rate was significantly reduced in the diabetic rats. It is suggested that even the mild stress that is associated with the indirect (tail cuff) blood pressure determination can significantly affect the accuracy of the results. We therefore used a direct measurement of blood pressure thus eliminating the concerns about handling induced stress and the well know cardiovascular and autonomic effects elicited by commonly used anesthetic agents.

The basal HR in our STZ diabetic rats was depressed by 3 weeks of diabetes. The mechanisms underlying the diabetes-associated decrease in HR (bradycardia) are not known and beyond the scope of our studies. It may be mediated by changes in the heart and/or neuroendocrine control. Studies in isolated working heart preparations suggest that STZ-induced diabetes is associated with a depression in basal spontaneous pacemaker rate. In addition it could also be mediated in part by alterations in autonomic nervous system control such as an increase in vagal or a decline in sympathetic tone that would diminish heart rate.
The mechanisms underlying the development of cardiovascular depression (depressed MABP and HR) in STZ diabetic rats are unknown. It is possible that, following diabetes changes in sensitivity and bioavailability of endogenous NO in the cardiovascular and renovascular systems may contribute to the cardiovascular depression. It is well known that NO plays an important role in the maintenance of cardiovascular and renal homeostasis in diabetes. It is involved in the regulation of vascular tone, cardiac contractility and baroreflex function. In addition, in the kidney, it regulates salt and fluid absorption, hemodynamics, renin secretion and tuberoglomerular feedback and other processes, all of which are important factors are involved in the regulation of arterial blood pressure and cardiovascular homeostasis.

However, the role of NO in cardiovascular homeostasis in diabetes is controversial. An increased dependence on a functional nitric oxide system has been reported, which prevents the development of hypertension in STZ diabetic rats. It has been demonstrated that non-selective blockade of NO synthesis results in increased MABP at the onset of diabetes. It was suggested that an increased NO synthesis is important to maintain an increased renal blood flow and to suppress the activated renin-aldosterone-angiotensin (RAS) system and increased sympathetic nervous system (SNS) activity. However, in contrast to this, our studies demonstrated a decreased elevation in MABP in diabetic rats compared to their age-matched controls (in 3, 9 and 12 week diabetic rats) when NOS was blocked non-selectively by an acute administration of L-NAME. This discrepancy may be due to the difference in the duration of diabetes and because of differences in the method of L-NAME administration (chronic vs acute). Our results, which indicate an impairment of endothelial function by 3 weeks is in agreement with many other studies that have demonstrated an impaired NO mediated endothelium dependent relaxation in experimental diabetes.

Despite the overwhelming evidence of impaired endothelium dependent relaxation in diabetes, there are sporadic reports of enhanced or unaltered relaxation. These disparities may be due to the different types of arteries, different methodologies used in tissue preparation and most importantly, the duration of diabetes. A temporal study examining the duration of diabetes on endothelial function reported an increased (24 hours), unaltered (1 week) and an impaired (8 weeks) endothelial function. The mechanisms involved in endothelial dysfunction are not clear but may involve inhibition of eNOS and...
quenching of bioavailable NO by superoxides. The later mechanism fits with the evidence that eNOS derived NO production may be increased in diabetes at least in the early stages, but its vasodilator action can be suppressed below normal as a result of oxidative stress in the later stages of diabetes. This was supported by studies from Brands et al who demonstrated an increased dependence on NO for the maintenance of normal blood pressure at the onset of diabetes. However, with the progression of the disease state, elevated oxidative stress involving increased formation of free radicals may quench NO and can cause endothelial dysfunction.

This notion is supported by many studies, which have demonstrated that the exposure of arteries to elevated glucose levels impairs acetylcholine-induced relaxation and treatment with free radical scavengers improves or normalizes the endothelial function. These observations suggest that hyperglycemia increases the degradation of NO secondary to its enhancement of oxygen derived free radical production. The increased levels of free radicals in turn may quench NO and impair endothelial function. However, there is also evidence for increased NO production both in clinical studies and experimental models of diabetes and an important consideration probably is the duration of diabetes at the time of testing. Consistent with this, a recent clinical study on Type 1 diabetic patients demonstrated significantly higher plasma NOx levels in diabetic patients without late complications compared to patients with late complications. In addition, a higher level of lipid peroxidation end products (TBARS) was also observed in diabetic patients compared to control. These results suggest that increased oxidative stress might quench the bioavailable NO, reduce plasma NOx and cause impairment of endothelial function. In addition, free radicals themselves, such as hydroxyl radical can also cause endothelial dysfunction.

Observation of plasma NOx values in our study indicates no significant change between control and diabetic rats in the first 3 weeks of diabetes. However, there was a significant reduction by 9 weeks, which was followed by a reversal in 12 weeks diabetic rats where the NOx values were elevated. The reduction in plasma NOx levels in 9-week diabetic rats may be due to increased formation of reactive oxygen species that can quench NO. This was supported by our findings of increased plasma isoprostanes concomitant with a decreased total plasma antioxidant concentration in another study of diabetic rats of the same duration (9 weeks). Thus, it is likely that increased oxidative stress may cause a reduction in
plasma NOx levels. However, the increase in plasma NOx in 12-week diabetic rats was somewhat surprising. This intriguing observation in the later stage of diabetes is difficult to explain with the limited data available from these studies. It is possible that increased production of large quantities of NO from other sources such as iNOS may contribute to the increased NOx levels. However, since we do not have quantitative data for iNOS protein abundance and/or mRNA expression and also since the MABP’s were not different between 9 and 12 week diabetic rats, it is difficult to explain the increased plasma NOx levels in 12-week diabetic rats.

Endothelial dysfunction as measured by a failure to increase MABP in the presence of L-NAME suggests that diabetic rats had impaired endothelial function by 3 weeks. Increasing evidence now suggest that diabetes induced endothelial dysfunction results, in part, from a paradoxical increase of NO production from iNOS. This was supported by a study that showed long term treatment with a NO scavenger prevented the impairment of endothelial function in diabetes\textsuperscript{299}. In addition, a few other studies have demonstrated that long term treatment with aminoguanidine (iNOS inhibitor) prevents diabetes impaired endothelial dysfunction both in aorta and mesenteric artery\textsuperscript{82}. The earlier notion that endothelial dysfunction is associated with decreased NO production therefore needs re-evaluation particularly with relevance to the duration of diabetes. Our data suggest the possibility that the duration of diabetes and associated oxidative stress as important regulators of endothelial function and cardiovascular homeostasis. In addition, increased expression of iNOS observed in the heart and arteries of 9 and 12 week diabetic rats in our present and previous studies\textsuperscript{202} support the hypothesis that increased nitrosative stress (reactive nitrogen species) might be involved in cardiovascular depression.

Induction of iNOS results in 50-100 fold more NO than is produced from the constitutive forms of NOS. It has been demonstrated that under hyperglycemic conditions, iNOS expression is elevated in heart and vascular smooth muscle (VSM) of STZ diabetic rats. Some investigators have noted that induction of iNOS is associated with down-regulation or decreased expression of eNOS gene\textsuperscript{300}. A recent study using gene transfer techniques, demonstrated that iNOS gene was transferred to normal arteries, the NO dependant relaxation was impaired, suggesting endothelial dysfunction\textsuperscript{301}. These results
suggest that induction of iNOS may result in endothelial dysfunction. In other studies, in the presence of hyperglycemia, expression of iNOS in cardiac, arterial, and sciatic nerve was associated with a pronounced reduction of eNOS both in human and animal models of diabetes\textsuperscript{201,302,303,203}. The results of our immunohistochemistry study also demonstrate a similar pattern of differential expression of iNOS and eNOS wherein a prolonged diabetic state led to down-regulation of eNOS with a concomitant upregulation of iNOS. This pattern of differential or inverse regulation of NOS enzymes was more prominent in the later stages of diabetes (9 and 12 week diabetic rats). It therefore is possible that hyperglycemia may act to reduce constitutive NOS expression and, at the same time, increase iNOS expression with enhanced NO release\textsuperscript{304}. Furthermore, prior long-term exposure to NO donor agents also has been shown to decrease eNOS production of NO. These results suggest the likelihood of enhanced production of NO possibly from iNOS and not high glucose \textit{per se} being the main reason for down regulation of eNOS. However, quantitative estimation of eNOS and iNOS protein and/or mRNA expression may be required to validate the above conclusion.

The molecular mechanisms of NOS regulation in hyperglycemia are not fully understood, but recent studies point to a decisive role of the PKC pathway\textsuperscript{305}. Hyperglycemia can markedly activate the $\beta_2$ isoform of PKC in endothelial cells by promoting \textit{de novo} synthesis of DAG and increasing mitochondrial superoxide production via NADPH oxidase\textsuperscript{232}. LY 333531, specific inhibitor of PKC $\beta$ has been demonstrated to reduce oxidative stress generation in the retina: this is consistent with the evidence that PKC activation may increase superoxide generation through NADPH oxidase\textsuperscript{306}. In addition, iNOS itself is known to produce superoxides in settings with limited availability of substrate or cofactors\textsuperscript{307} such as L-arginine depleted macrophages\textsuperscript{308}. Thus in long term diabetes, iNOS may be induced in cardiac and vascular tissues and the increased production of NO may be involved in the down regulation of eNOS causing endothelial dysfunction and cardiovascular depression.

The mechanisms underlying the iNOS mediated cardiovascular depression are not clear but may involve the formation of peroxynitrite. This is because under cellular stress such as hyperglycemia there is an increased production of both NO (iNOS) and reactive oxygen species. Both NO and superoxide are highly reactive and unstable and can interact to form peroxynitrite at a rate 3 times faster than dismutation of superoxide by SOD\textsuperscript{304}. This would be analogous to the observations that after endotoxin treatment there are increases in
iNOS activity, peroxynitrite formation and an eventual cardiovascular depression. A large number of studies have implicated iNOS mediated formation of peroxynitrite as the major contributor to the cardiac depression, severe hypotension, profound vasodilatation and vascular hyporeactivity associated with endotoxin induced septic shock\textsuperscript{212,214,215,219,309}. 

The downstream cellular targets of peroxynitrite are attributed to its oxidizing capacity\textsuperscript{211}. Its highly reactive decomposition products at physiological or acidic pH can attack protein (oxidation of SH, nitration of tyrosine), lipids (formation of lipid peroxides) and DNA (strand breakage). This results in depletion of low molecular weight antioxidants such as GSH, and inhibition of several enzyme activities including the enzymes of the mitochondrial respiratory chain\textsuperscript{310}. In fact peroxynitrite has been shown to cause irreversible inhibition of mitochondrial respiratory chain and to trigger apoptosis in cardiomyocytes\textsuperscript{239,240}. 

The formation of peroxynitrite can be determined indirectly by measuring the levels of NT, a major marker of protein nitration (tyrosine nitration) in plasma and tissue. We used immunohistochemistry to determine the nitrotyrosine formation in heart and arteries. The result of our study demonstrates an increased expression of NT in both hearts and arteries of 3, 9 and 12-week diabetic rats. The mechanisms by which peroxynitrite causes cardiovascular depression are not clear but it is suggested that peroxynitrite depresses cardiac contractility by decreasing the Ca\textsuperscript{2+} sensitivity of contractile elements\textsuperscript{212}. Studies have demonstrated that infusion of peroxynitrite into working hearts impairs cardiac contractile function by decreasing cardiac efficiency\textsuperscript{213}. Incubation of isolated cardiac myocytes and rat papillary muscles with peroxynitrite showed impaired contractility\textsuperscript{311,312}. Administration of the peroxynitrite generator SIN-1 exerted a cardiac depressant action in rat hearts\textsuperscript{313}. Many of these studies showed a strong correlation between endogenous peroxynitrite formation and deterioration of cardiac function\textsuperscript{211}. 

A number of studies have also reported peroxynitrite-mediated abnormalities in vascular and neural (central and peripheral) tissues in diabetes. Coppey et al\textsuperscript{314} reported that NT staining of the endothelium was associated with endothelial dysfunction. Peroxynitrite is toxic to the central\textsuperscript{315} and peripheral nervous system as well\textsuperscript{316}. A recent clinical study has demonstrated that nitrosative stress (peroxynitrite) is associated with deterioration of peripheral nerve function in diabetes\textsuperscript{317}. 
Other studies have reported that peroxynitrite can cause direct oxidation of catecholamines (NA)\textsuperscript{214} and also reduce the binding capacity of endogenous agonists to α-adrenergic receptors (α-1\textsubscript{A} and α-1\textsubscript{D}), thereby decreasing the vascular reactivity to vasoactive agents\textsuperscript{215}. Many of the cardiovascular abnormalities can be prevented by inhibiting the formation of peroxynitrite in cardiac, neuronal and endothelial cells\textsuperscript{178,177,179,318}. FP15 a potent peroxynitrite decomposition catalyst has been shown to inhibit tyrosine nitration and reduce the toxicity to β cells and vascular endothelium in diabetes\textsuperscript{319}. These results strongly indicate that, under conditions of increased oxidative stress, iNOS play a crucial role in abetting the formation of peroxynitrite, which in turn causes cardiovascular depression by contributing greatly to the nitrosative stress.

5.4 Effect of iNOS inhibition on the hemodynamic effects of vasoactive agents

Diabetes mellitus is associated with vascular complications including an impairment of endothelium dependent vasodilation and alterations in the reactivity of blood vessels to vasoconstrictors in micro and macro vasculature. A number of \textit{in vitro} studies have examined the endothelium dependent relaxation of vascular smooth muscle in experimental diabetes. However, these studies are conflicting and report decreased, unchanged or increased responses to acetylcholine, which is a endothelium dependent vasodilator. Similarly \textit{in vivo} studies in STZ diabetic rats have also presented conflicting results such as decreased or unchanged vasodilatory responses to acetylcholine in STZ diabetic rats\textsuperscript{81,80}. The discrepancies noted above may be due to variations in experimental conditions such as the type of artery preparation used, techniques employed in the measurement of muscle contraction and, most importantly, the duration of diabetes. For example, previous studies examining the endothelium dependant relaxation to acetylcholine or bradykinin in STZ diabetic rats have shown impairment of endothelial function within 1 week in intestinal arteries, 2 weeks in hind quarter arteries, 3 weeks in cremaster muscle arterioles, 4-6 weeks in mesenteric arteries or 4 weeks in aorta\textsuperscript{225}. The results of our studies have demonstrated a similar effect when L-NAME was used to test endothelial function. We observed endothelial dysfunction only after 3 weeks of STZ diabetes. Interestingly, these animals are also hypotensive (depressed MABP and HR) despite endothelial dysfunction.
Similarly a number of *in vitro* studies have demonstrated altered contractile responses to various vasoactive agents in diabetic arteries. Contractile responses to noradrenaline or phenylephrine are increased, decreased or unchanged in STZ diabetic rats\textsuperscript{81,80}. The precise mechanism for the discrepancy is not clear at the moment but it is possible that the duration and severity of diabetes may influence the vasocontractility to noradrenaline. Previous studies from our laboratory and elsewhere have demonstrated that the sensitivity of vasoactive agents in mesenteric arteries depends on the duration of diabetes\textsuperscript{320,321}. Increasing and overwhelming evidence now suggests that contractile responses to vasoconstrictor agents are increased, particularly in small conduit arteries such as the mesenteric artery. Studies in the past have demonstrated increased contractility to norepinephrine in both endothelium intact and denuded mesenteric arteries of 10-12 week STZ diabetic rats\textsuperscript{202}. Non-selective inhibition of NOS in isolated diabetic arteries (endothelium intact and denuded) resulted in an increased sensitivity to norepinephrine. It was suggested that the enhanced contractile response to α-adrenergic agents in diabetic arteries may be due to decreased NO release\textsuperscript{322}. However this is unlikely because, even in the presence of a nonselective NOS inhibitor, the diabetic arteries with intact endothelium exhibited an increased contractile response to these agents\textsuperscript{246,203}. Some of the most widely accepted mechanisms of increased vascular contractility in isolated diabetic arteries include enhanced calcium influx, increased activity of G protein and phospholipase C\textsuperscript{323}, and/or increased Ca\textsuperscript{2+} sensitivity to the contractile protein\textsuperscript{324}.

In contrast to this, most of the *in vivo* studies with STZ diabetic rats have shown only decreased or attenuated pressor response to noradrenaline, methoxamine and various other vasoactive agents\textsuperscript{59,81,80,325}. In our present studies we noticed a similar pattern of pressor responses to methoxamine and angiotensin-2 in diabetic rats. Administration of bolus doses of methoxamine or AT II resulted in increased MABP in a dose dependent manner both in control and diabetic rats. However, the pressor responses to these agents were significantly attenuated in diabetic rats compared to the control rats by 3 weeks of diabetes. An interesting observation was that this attenuation was more prominent to methoxamine than to angiotensin-2, particularly in the rats with a longer duration (12 week) of diabetes. Improved pressor responses to angiotensin-2 in diabetic rats may be attributed to the higher sensitivity of vascular tissues, particularly the renal arteries, to the actions of AT II\textsuperscript{326}. It is reported that
infusion of low doses of AT II that do not affect systemic BP can induce a substantial constriction of renal arteries\textsuperscript{327}. The reasons for the attenuated response to methoxamine are not clear but it is possible that hyperglycemia-mediated impairment of sympathetic function can reduce the pressor responses to $\alpha_1$-adrenergic agonists \textsuperscript{328}. Also at the post receptor level, stimulation of the $\alpha_1$ receptor by methoxamine may involve increased production of prostaglandin I\textsubscript{2} (PGI\textsubscript{2}) and/or decreased formation of TxA\textsubscript{2} in diabetes\textsuperscript{325}, both of which can contribute to the depressed-pressor responses to $\alpha$ adrenergic stimulation.

Clinical studies have reported that Type 1 diabetes is associated with alterations in the autonomic nervous system (ANS). The depressed resting blood pressure and heart rate together with a blunted bradycardiac response to methoxamine observed in our studies may indicate impairment of both sympathetic and parasympathetic systems. In fact, clinical evidence suggests that both branches of the ANS are impaired in Type 1 diabetic patients. In addition, studies have demonstrated a disturbed sympathetic innervation of blood vessels, which might underlie hyporesponsiveness of blood pressure and of macro-circulatory and micro-circulatory neuronal blood flow to sympatho-adrenergic neuronal blood flow\textsuperscript{369}.

The mechanisms underlying depressed SNS activity are not clear and beyond the scope of our studies but it is suggested that NO may suppress the sympathetic activity in diabetes\textsuperscript{112}. On the other hand many reports have indicated that glucose (or hyperglycemia) actually stimulates the release of NO\textsuperscript{329,330}. Thus it is unclear how diabetes influences the release of NO, which in turn suppresses the SNS activity. Recent studies by Brands et al\textsuperscript{113,331} have tried to answer this baffling interrelationship between diabetes, NO and SNS. They hypothesized that at the onset, diabetes stimulates increased synthesis of NO (possibly from eNOS), which in turn may suppress the otherwise activated SNS and RAS activity and prevent the development of hypertension. They demonstrated that an increased dependence on a functional NO system is required to suppress activated RAS and SNS activity and to prevent the development of hypertension at the onset of diabetes. However, the results provide no explanation for the depressed MABP and HR and endothelial dysfunction observed in the later stages of diabetes.
A large number of studies examining the effect of diabetes on vascular morphology and functions have reported increased expression and activity of iNOS in diabetes. Studies from our lab are among the first to demonstrate the functional expression of iNOS in vascular smooth muscle of mesenteric arteries\textsuperscript{202}. Our results raise the possibility that iNOS plays a significant role in the attenuated pressor responses to vasoactive agents and etiology of endothelial and cardiac dysfunction thus contributing greatly to the cardiovascular depression. To test the hypothesis that iNOS contributes to the attenuated pressor response to methoxamine and AT II, we inhibited iNOS acutely \textit{in vivo} with 1400W and measured the pressor responses to these agents. The results of our study demonstrate a significant improvement in the pressor response to both AT II and methoxamine when iNOS was inhibited. Our results are consistent with recent reports that have suggested the involvement of iNOS in modulating the pressor responses to vasoactive agents \textit{in vivo}\textsuperscript{205} and \textit{in vitro}\textsuperscript{203,202}. The \textit{in vitro} studies suggest that iNOS, expressed in VSM of mesenteric artery suppresses the basal tone and reactivity in diabetes\textsuperscript{203}. In addition, studies involving carotid artery iNOS gene knock-out mice have confirmed that iNOS not only suppresses the increased vasoconstrictor responses in diabetes but also causes endothelial dysfunction\textsuperscript{206}.

Our studies, in addition to confirming endothelial dysfunction, also suggest that iNOS may be involved in suppressing the pressor responses to vasoactive agents. However, the results of our study cannot explain the reasons for the increased contractility seen with vasoactive agents \textit{in vitro} and the lower blood pressure seen \textit{in vivo} in diabetic rats with endothelial dysfunction. These discrepancies are probably, due to the differences between the \textit{in vitro} and \textit{in vivo} experimental settings and also due to the fact that diabetic rats also have cardiac dysfunction (perhaps due to peroxynitrite) that may contribute to depressed MABP and HR.

Our findings suggest that hyperglycemia may be associated with induction of iNOS in cardiovascular tissue (immunohistochemical evidence) that contributes significantly to the depressed blood pressure and heart rate and also to the depressed pressor responses to vasoactive agents. In addition, induction of iNOS and occurrence of cardiovascular depression depends on the duration of diabetes (3 weeks). The depressed blood pressure may be due to the cardiac dysfunction or due to a decreased contractility of resistance vessels such as those of mesenteric arterial bed or both. It is also possible that induction of iNOS may
cause endothelial dysfunction (due to increased nitrosative stress) and yet prevents the development of high blood pressure in diabetes. To test the latter hypothesis, we treated STZ diabetic rats acutely (3 weeks) and chronically (9 weeks) with an antioxidant and iNOS inhibitor (NAC), to reduce oxidative stress and to uncouple the interaction between oxidative free radicals and NO. Preventing the formation of peroxynitrite or nitrotyrosine may improve cardiovascular depression in diabetes.

5.5 Effect of NAC treatment on iNOS mediated cardiovascular abnormalities

Increased superoxide generation concurrent with increased NO production is known to produce peroxynitrite. This reaction, which is three times faster than the dismutation of superoxide by SOD, is a favored reaction under conditions of cellular stress such as hyperglycemia. In experimental diabetes, and also endotoxemic models of septic shock, increased peroxynitrite content has been found in cardiovascular tissues. Many studies, particularly endotoxemic models of septic shock, have suggested a strong and negative correlation between myocardial peroxynitrite content (NT) and cardiac depression. Treatments aiming at inhibition of iNOS or reduction of oxidative stress have been beneficial in experimental models of septic shock and diabetes respectively. It is not clear how antioxidant therapy aimed at reducing oxidative stress and iNOS inhibition affect hemodynamics in acute and chronic diabetes. We therefore, tested this by treating our STZ diabetic rats with NAC in drinking water for 3 and 9 weeks. N-acetylcysteine (NAC) which is a precursor in the synthesis of glutathione (GSH), a free radical scavenger, is also an inhibitor of TNF-α and NFkB mediated induction of iNOS.

In order to assess the effect of NAC on the formation of ROS in diabetic rats we measured total plasma free isoprostanes and antioxidant concentrations. As shown in our experiments, oxidative stress is markedly increased in diabetic rats. This conclusion was based on our finding that (a) plasma concentration of isoprostanes were significantly elevated in diabetic rats, and (b) plasma concentrations of total antioxidants were markedly decreased in diabetes. Treatment with NAC improved the antioxidant defense mechanisms by increasing the total antioxidant concentrations and reducing the formation of isoprostanes. These results are in line with the well known glutathione-replenishing properties of NAC,
that can be due both to its role as a stable precursor of cysteine, which then enters into the GSH synthesis cycle, and to its GSH sparing effect due to its antioxidant properties.

Measurement of nitrite/nitrate (NOx) levels in plasma as NO based by-products has been established as an indirect marker of increased NO production in diabetes. There is a possibility that STZ, being a NO donor can contribute to the total NO pool in the plasma. However, owing to its short half-life, it doesn’t seem to contribute to the measured plasma NOx concentration after 3 and 9 weeks of diabetes. The results of our NOx studies indicate a significant reduction of NOx concentrations in 9-week diabetic rats. Although, it is unclear why the plasma NOx values were significantly lower in diabetic rats it is possible that different NOS isoforms showing different capacities of NO production (pico-to nano molar over seconds and minutes for eNOS and nano- to micromolar over hours for iNOS) contribute to the differences in NOx values in diabetes. In addition, increased levels of oxidative stress may scavenge the bioavailable NO and therefore reduce the plasma NOx levels. Treatment with NAC normalized the plasma NOx concentration to the control levels. The results suggest that NAC, by reducing the levels of oxidative stress may help spare the bioavailable NO.

In another set of experiments we tested the myocardial antioxidant capacity by challenging the myocardial tissue with an increasing concentrations of oxidant (t-BHP). The results suggest that NAC treatment substantially improved the myocardial antioxidant capacity of diabetic hearts. The mechanisms by which NAC improved myocardial antioxidant capacity were beyond the scope of our studies. However, it is possible that NAC supplementation may replenish intracellular GSH stores and improve the ratio of GSH/GSSG in diabetic cardiomyocytes contributing to an increased antioxidant capacity.

At termination the MABP and HR were measured. Similar to the results of our previous studies, the MABP and HR were significantly reduced in untreated diabetic rats by 3 weeks of diabetes. Treatment with NAC in drinking water prevented these changes. The results suggest that STZ diabetic rats develop cardiovascular depression by 3 weeks and treatment with NAC prevented these changes. The mechanisms underlying the development of cardiovascular depression (depressed MABP and HR) are not clear but increasing evidence suggests that reactive oxygen species resulting from mitochondria, glucose autooxidation and other sources (see introduction part) may contribute significantly to the
cellular stress. In addition increased production of NO from iNOS when associated with oxidative stress results in the formation of peroxynitrite and hydroxyl radicals. Numerous studies suggest that increased production of NO concomitant with increased levels of oxidative stress in diabetes results in the formation of peroxynitrite and other products. These toxic products are deleterious to the cardiovascular tissue and may involve in the etiology of cardiovascular dysfunction\textsuperscript{211}. Our immunohistochemistry data suggest an increased expression of iNOS and NT with a concurrent reduction of eNOS in the heart and arteries of 9-week diabetic rats. Treatment with NAC significantly reduced the iNOS and NT protein expression together with an increased expression of eNOS in vascular endothelium of the tissues tested. The mechanisms by which NAC prevented the induction of iNOS though not very clear could be due to the inhibition of TNF-\(\alpha\), NF-\(\kappa\)B mediated induction of iNOS\textsuperscript{188} and or by reducing the oxidative stress. Similarly as discussed before, reduced expression of eNOS observed in diabetic tissues may be due to increased nitrosative stress.

The results of our studies suggest that activation of iNOS, which depends on the duration of diabetes, may assume a critical role under oxidative conditions. The interactions between iNOS and reactive oxygen species that favour the formation of reactive nitrogen species (nitrosative stress) could thus influence the subsequent hemodynamic outcomes in diabetes. Treatment with NAC strengthens the antioxidant defense mechanisms and effectively prevents the development of cardiovascular depression in diabetes (Scheme-2).
Prolonged diabetic state increases oxidative and nitrative stress resulting in overproduction of both superoxide anions \([O_2]\) and reactive nitrogen species such as peroxynitrite \((\text{ONOO}^-)\). Supplementation with N-acetylcysteine \((\text{NAC})\) inhibits the formation of peroxynitrite and in addition detoxifies already formed peroxynitrite by converting it into S-nitrosoglutathione or other nitrosothiols that can act as NO donor molecules. Nitrosothiols are suggested to be protective in cardiovascular system and thus may prevent cardiovascular depression in diabetes.
5.6 Limitations and future research directions

In the experiments described in this thesis using rats with different durations of diabetes, we did not use methods other than immunohistochemistry to detect and localize isoforms of NOS and nitrotyrosine. Since, analysis of data in immunohistochemical technique is subjective and since the data is not quantitated, the inferences based on the results may not provide definitive evidence. Although we did use a large sample size (6 slides per group) in our experiments, further investigations may be necessary to confirm the expression and activity of these proteins. Quantification of individual protein abundance by western blot technique and determination of mRNA expression by RT-PCR, in addition to the assay of individual enzyme activities of proteins, may validate the results and arguments in the discussion.

Furthermore, determination of the prophylactic effects of iNOS inhibition (acute vs chronic treatment similar to NAC treatment) on the cardiovascular function would be important in elucidating the role of iNOS in exacerbating nitrosative stress in diabetes. Also, studies involving the investigation of hemodynamic effects of vasoactive agents (methoxamine and AT II) in rats, treated chronically with an iNOS inhibitor would be interesting. In addition, in diabetic rats, the effect of iNOS inhibition on cardiac performance and vascular function needs to be examined. Other aspects of our future research include the investigations of the mechanisms underlying the induction of iNOS and iNOS mediated cardiovascular abnormalities in diabetes, particularly the mechanism by which peroxynitrite causes cardiovascular depression.
6. SUMMARY AND CONCLUSIONS

1. Studies have previously shown that chronic hyperglycemia in STZ diabetic rats is associated with functional expression of iNOS in superior mesenteric arteries, suggesting an important role for iNOS in the regulation of cardiovascular homeostasis. We implicated the increased production of NO from iNOS and concurrent oxidative stress collectively referred to as "nitrosative stress" in the etiology of cardiovascular depression. The results of our study indicate depressed mean arterial blood pressure and heart rate in rats of 3-week STZ diabetes. In addition, the pressor responses to methoxamine (α₁-adrenergic agonist) and angiotensin-2 (AT II) were both attenuated in rats from 3 to 12 week duration of diabetes. The attenuation was more prominent to methoxamine than AT II, particularly in the later stages of diabetes. Endothelial function as evaluated by a single bolus dose of L-NAME on changes in MABP suggested an apparent occurrence of endothelial dysfunction by 3 weeks of diabetes. These results suggest that diabetic rats develop cardiovascular depression and endothelial dysfunction with reduced pressor responses to vasoactive agents as early as 3 weeks.

2. The specificity and selectivity of 1400W, an iNOS inhibitor was evaluated by using lipopolysaccharide-induced septic shock. A minimum dose of 3mg/kg was found to inhibit the late phase fall in MABP suggesting its selective inhibitory effect on iNOS in vivo. This dose was used to inhibit iNOS in the subsequent studies. These results indicate that 1400W is a selective inhibitor of iNOS and a minimum dose of 3 mg /kg is required to prevent its activation in vivo.

3. Administration of 1400W did not affect the basal MABP and HR in any of the groups tested. However, pressor responses to both methoxamine and AT II were significantly augmented in diabetic rats treated with 1400W in 3, 9 and 12-week duration diabetes. No significant change in the pressor responses to these agents was observed in 1-week diabetic rats and in all other age matched control rats. These results suggest that iNOS is functionally active in the cardiovascular system as early as 3 weeks of
diabetes and modulate the pressor responses to vasoactive agents. The results also indicate that iNOS may not be involved in the maintenance of basal tone in diabetes.

4. Immunohistochemistry data revealed a differential regulation of iNOS and eNOS protein expression wherein prolonged hyperglycemia (9 weeks) led to the down-regulation of eNOS with a concomitant upregulation of iNOS. iNOS protein was detected in the myocardial tissue as early as 3 weeks after induction of diabetes, the intensity of which increased with the duration of diabetes. This upregulation of iNOS showed an inverse relationship to eNOS in prolonged diabetes leading to decreased eNOS expression in diabetic rat heart, aorta & superior mesenteric arteries. In addition, increased expression of nitrotyrosine (NT), an indirect marker of peroxynitrite formation was observed in heart and blood vessels of diabetic rats.

5. A triphasic response of unchanged, decreased and elevated levels of plasma nitrite/nitrate (NOx) concentrations was observed in 3, 9 and 12-week diabetic rats respectively. Although the results are difficult to explain with the limited data, it may be possible that a simultaneous increase of reactive oxygen species (ROS) and NO in the beginning (unchanged), followed by increased scavenging of NO by ROS (decreased) by 9 weeks and finally leading to an uncontrollable production of NO from iNOS (increased) in 12 weeks may contribute to this change. However, further studies are required to validate the above conclusion.

6. Acute and chronic treatment with N-acetylcysteine (NAC), an antioxidant and iNOS inhibitor corrected lipid abnormalities in addition to the depressed MABP and HR in diabetic rats. NAC treatment reduced the nitrosative stress in diabetic rats as demonstrated by normalization of plasma 15-F_{2t} isoprostanes, NOx and total antioxidant concentrations with a significant improvement in myocardial antioxidant capacity. In addition, NAC treatment markedly reduced the expression of iNOS and NT along with a significant improvement in eNOS protein expression. These results demonstrate the beneficial effects of a combined antioxidant and iNOS inhibitor in reducing the nitrosative stress associated with diabetes.
Results presented in this thesis therefore suggest that depressed MABP and HR in STZ diabetic rats may be due to increased levels of nitrosative stress. The activation of iNOS, which depends on the duration of diabetes, may assume a critical role under oxidative conditions in modulating pressor responses to vasoactive agents. The interactions between iNOS and oxidative stress in diabetes could thus influence the subsequent hemodynamic outcomes.
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