THE EFFECTS OF PHENTOLAMINE ON FRUCTOSE-FED RATS

by

Kangbin Zhou

B.Sc, The University of British Columbia, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

September 2011

© Kangbin Zhou, 2011

Abstract

The present project was designed to establish a pharmacological profile of phentolamine, a non-selective α adrenergic receptor antagonist, to provide information as to whether phentolamine might be effective on preventing the blood pressure elevation in fructose fed rats, a well-established rodent model of metabolic syndrome. Male Wistar rats fed with a 60% fructose diet for 14 weeks were found to have elevated blood pressure and insulin resistance. Phentolamine treatment prevented this increase in blood pressure without affecting insulin resistance. To further investigate the mechanism of action of phentolamine in preventing the blood pressure elevation, the levels of plasma noradrenaline and angiotensin II, two proposed contributors to the development of elevated blood pressure, were examined. Neither noradrenaline nor angiotensin II was affected by phentolamine.

Phentolamine is known to cause tachycardia mediated by indirect β adrenergic receptor activation. Cardiac tissue apoptosis was used as a marker to evaluate the extent of possible cardiac toxicity. The results suggest that there was no significant increase of apoptosis caused by in the phentolamine-treated rats. To examine the effects of phentolamine on cardiac β receptors, the activities of the dominant β receptors (β_1) were examined by measuring the phosphorylation of two downstream effectors, protein kinase A (PKA) and phospholamban since both proteins are activated through phosphorylation upon β_1 receptor activation. The phosphorylation levels of PKA were similar among all four animal groups while those of phospholamban were decreased significantly by phentolamine, suggesting chronic administration of phentolamine may have desensitized these receptors leading to a decrease in their activities.

Plasma 8-isoprostane and total nitrate/nitrite were chosen as markers to evaluate the state of oxidative stress, a proposed mechanism of fructose-induced elevated blood pressure and insulin resistance. Plasma levels of 8-isoprostane were similar among all four animal groups while those of total nitrate/nitrite (an index of total nitric oxide production) index of were about 7 folds higher in the fructose fed animals regardless of the treatment of phentolamine. This suggests an increased level of oxidative stress since overproduction of nitric oxide has been shown to lead to an elevation in peroxynitrite which can cause oxidative damage.

Preface

All animal work conducted in this thesis was approved by the University of British Columbia Animal Care Ethics Committee, protocol # A07-0730.

Table of Content

Abstract.....	ii
Preface.....	iv
Table of Contents.....	v
List of Tables.....	viii
List of Figures.....	ix
List of Abbreviations.....	x
Acknowledgements.....	xi
1: Introduction and Objectives.....	1
1.1 Introduction.....	1
1.1.1 Metabolic Syndrome.....	1
1.1.2 Fructose & Fructose-Fed Rats: An Animal Model of Metabolic Syndrome...	3
1.1.3 Two Major Proposed Mechanism(s) of Linking Insulin Resistance and Elevated Blood Pressure in Fructose-Fed Rats: Sympathetic Nervous System and Renin-Angiotensin System.....	5
1.1.4 Phentolamine.....	9
1.1.5 Apoptosis of Cardiac Myocytes.....	10
1.2 Objectives.....	11
1.2.1 Objective 1: To investigate whether chronic use of phentolamine can prevent the elevation of blood pressure induced by fructose feeding in Wistar rats.....	11
1.2.2 Objective 2: Determine whether phentolamine will increase the release of endogenous noradrenaline into the circulation and indirectly measure whether there is an abnormality in presynaptic α_2 receptor activity in fructose fed rats.....	12

1.2.3	Objective 3: Investigate whether phentolamine alters the expression of β_1 adrenergic receptors in the kidneys, the level of plasma angiotensin II, and insulin resistance. Determine whether an increase in insulin resistance is linked to an increased level of plasma angiotensin II which is mediated by activation of renal β_1 adrenergic receptors.....	13
1.2.4	Objective 4: Determine the possible changes chronic phentolamine treatment may produce in β adrenergic receptor expressions and whether β_1 adrenergic receptor-mediated apoptosis of cardiac myocytes is enhanced by chronic phentolamine treatment.....	15
1.2.5	Objective 5: Investigate the state of oxidative stress in fructose-fed rats.....	17
2:	Materials and Methods.....	19
2.1	Development of Fructose-Fed Rats.....	19
2.2	Measurement of Systolic Blood Pressure.....	20
2.3	Plasma Sample Collection.....	20
2.4	Biochemical Measurements.....	21
2.5	Oral Glucose Tolerance Test, Assessment of Insulin Resistance/Sensitivity.....	22
2.6	Western Blot Analysis.....	22
2.7	Measurement of Apoptosis of Cardiac Tissues.....	24
2.8	Statistical Analyses.....	25
3:	Results.....	26
3.1	The Effects of Fructose and Phentolamine on Blood Pressure and Metabolic Parameters.....	26

3.2	The Effects of Fructose and Phentolamine on Plasma Noradrenaline and Angiotensin II.....	27
3.3	The Effects of Fructose and Phentolamine on Cardiac Tissue Apoptosis.....	27
3.4	The Effects of Fructose and Phentolamine on the Expression and Activities of β receptors in the Heart and the Expression of these Receptors in the kidneys.....	28
3.5	The Effects of Fructose and Phentolamine on Oxidative Stress.....	29
4:	Discussion.....	53
4.1	General Overview.....	53
4.2	Fructose-Fed Rats: A Rodent Model of Metabolic Syndrome.....	55
4.3	Effects of Phentolamine in Fructose-Induced Metabolic Disturbances and Elevated Blood Pressure.....	59
4.4	Effect of Fructose Feeding and Phentolamine on Plasma Noradrenaline.....	63
4.5	Effect of Fructose Feeding and Phentolamine on Plasma Angiotensin II	65
4.6	Effects of Phentolamine on β_1 Receptor-Mediated Apoptosis in the Heart and β Adrenergic Receptor Expressions.....	68
4.7	Effects of Phentolamine on Oxidative Stress.....	74
5:	Conclusion and Future Experiments.....	76
5.1	Conclusion.....	76
5.2	Future Experiments in Fructose-Fed Rats in the Upcoming Investigation.....	78
	References.....	79

List of Tables

Table 1: Plasma Metabolic Parameters in Control and Fructose-Fed Rats following 4 Weeks and 13 Weeks of Study.....	32
Table 2: Body Weight of Control and Fructose-Fed Rats.....	32

List of Figures

Figure 1	Effect of Phentolamine on Systolic Blood Pressure.....	31
Figure 2	Effect of Phentolamine on Insulin Glucose Response during OGTT.....	33
Figure 3	Effect of Phentolamine on Insulin Sensitivity Index.....	34
Figure 4	Effect of Phentolamine on Plasma Noradrenaline Level.....	35
Figure 5	Effect of Phentolamine on Plasma Angiotensin II Level.....	36
Figure 6	Effect of Phentolamine on the Expression of Cardiac Angiotensin II Receptor Type I.....	37
Figure 7	Effect of Phentolamine on Apoptosis of Cardiac Tissues.....	38
Figure 8	Effect of Phentolamine on Cardiac PKA Phosphorylation.....	39
Figure 9a	Effect of Phentolamine on Phospholamban Phosphorylation (Serine 16).....	40
Figure 9b	Effect of Phentolamine on Phospholamban Phosphorylation (Threonine17).....	41
Figure 10	Effect of Phentolamine on Cardiac β_2 Adrenergic Receptors.....	42
Figure 11	Effect of Phentolamine on Cardiac β_3 Adrenergic Receptors.....	43
Figure 12	Effect of Phentolamine on Renal β_2 Adrenergic Receptors.....	44
Figure 13	Effect of Phentolamine on Renal β_3 Adrenergic Receptors.....	45
Figure 14	Effect of Phentolamine on Renal PKA Phosphorylation.....	46
Figure 15	Effect of Phentolamine on Plasma 8-Isoprostane.....	47
Figure 16	Effect of Phentolamine on Plasma Total Nitrate/Nitrite.....	48
Figure 17a	Effect of Phentolamine on the Expression of Cardiac eNOS.....	49
Figure 17b	Effect of Phentolamine on the Expression of Cardiac iNOS.....	50
Figure 18a	Effect of Phentolamine on the Expression of Renal eNOS.....	51
Figure 18b	Effect of Phentolamine on the Expression of Renal iNOS.....	52

List of Abbreviations

ACE	:	Angiotensin II Converting Enzyme
ARB	:	Angiotensin II Receptor Blocker
AT ₁	:	Angiotensin II Receptor Type 1
AUC	:	Area Under the Curve
C	:	Normal Chow Fed
CaMKII	:	Calcium Camodulin Kinase II
CCB	:	Calcium Channel Blocker
CP	:	Normal Chow Fed and Phentolamine Treated
F	:	Fructose Diet Fed
FP	:	Fructose Diet Fed and Phentolamine Treated
NO	:	Nitric Oxide
OGTT	:	Oral Glucose Tolerance Test
eNOS	:	Endothelial Nitric Oxide Synthase
iNOS	:	Inducible Nitric Oxide Synthase
PKA	:	Protein Kinase A
PBS	:	Phosphate Buffer Saline
RAS	:	Renin-Angiotensin System
SNS	:	Synpathetic Nervous System
VSM	:	Vascular Smooth Muscle
ROS	:	Reactive Oxygen Species

Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr. John McNeill for his patience, guidance, encouragement and support which have given me the strength and faith to get me through my graduate research training. His knowledge and wisdom have greatly enlarged my vision of science. This thesis would not be possible without his endless support. I would also like to thank my co-supervisor Dr. Ujendra Kumar for his guidance and advice to assist me in developing my experimental skills and a proper method of scientific reasoning. I am grateful to my supervisory committee members, Dr. Mac Levine (Chair), Dr. Kathleen MacLeod, Dr. Ismail Laher, and Dr. Michael Walker for their critiques and suggestions throughout the course of this thesis project.

I would also like to thank my lab manager Violet Yuen in providing me technical support and guiding in the learning process of statistical analysis. I would also like to thank the members of Kumar and MacLeod Labs for their constant assistance and valuable advice in solving technical problems.

Special thanks are owed to my parents, whose have supported me throughout my years of education, both mentally and financially.

1. Introduction & Objectives

1.1 Introduction

1.1.1 Metabolic Syndrome

A universally accepted definition of metabolic syndrome has not yet been developed as debate is still continuing on the most appropriate set of criteria to be used in clinical diagnosis (Balkau & Charles, 1999; Einhorn *et al.*, 2003; Alberti *et al.*, 2005; Grundy *et al.*, 2005). Nevertheless, metabolic syndrome is characterized as a cluster of risk factors that increase the risk of the development of cardiovascular diseases. These factors include central obesity, insulin resistance, atherogenic dyslipidemia, and hypertension (Reaven, 1988; Wajchenberg *et al.*, 1994; Grundy *et al.*, 2004; Grundy *et al.*, 2005). Recent statistics reveal at least one of these risk factors is found in 90% of the Canadians (Heart and Stroke Foundation of Canada, 2009). Metabolic syndrome was estimated to affect 27% of the population in the US in 2004 (Ford *et al.*, 2004) and increase mortality in patients suffering cardiovascular diseases (Lakka *et al.*, 2002). A worldwide estimation of the prevalence of the syndrome has been suggested ranging between 10 and 25% (Wild & Byrne, 2005). It has also been suggested that metabolic syndrome increases with age and body mass index (BMI) and varies by race, ethnicity and sex (Ervin, 2009).

The mechanism of how metabolic syndrome is triggered remains to be discovered. Therefore, health scientists and pharmacologists have been engaged in developing therapeutic agents to treat the individual risk factors to improve the overall conditions of patients suffering this syndrome. Efforts have been made in searching for effective

treatments for obesity and insulin resistance, the former of which is an important risk factor of atherosclerotic cardiovascular diseases (Grundy *et al.*, 2004), while the latter is a dominant characteristic of Type 2 diabetes. Continuing epidemics of obesity and Type 2 diabetes have raised serious health concerns (Mokdad *et al.*, 2001). Attention has been drawn to investigating the possible contribution of diet to the development of these metabolic disturbances. Diet has been extensively studied as a major contributor to the development of obesity in developed countries across the world. A study of changes in the American diet revealed a dramatic increase in fructose consumption in the past three decades (Putnam & Allshouse, 1999). It has been noted that there has been a significant increase in fructose consumption primarily in the form of high-fructose corn syrup (55% fructose content) in beverages (Bray *et al.*, 2004). Health scientists suggest that fructose might play an important role in the increasing prevalence of obesity in children (Dennison *et al.*, 1997) and adolescents (Ludwig *et al.*, 2004), and type 2 diabetes in young and middle-age women (Schulze *et al.*, 2004) consuming large amount of soft drinks on a daily basis. Investigations of dietary consumption in humans have found that individuals consuming large amounts of fructose in their diet are more prone to develop obesity (Elliott *et al.*, 2002), dyslipidemia (Parks & Hellerstein, 2000), and cardiovascular diseases (Vasdev *et al.*, 2004). Dietary management via lowering fructose in and increasing the consumption of vitamins such as vitamin B6, C and E has been suggested to be able to prevent insulin resistance and hypertension (Vasdev *et al.*, 2004).

1.1.2 Fructose & Fructose-Fed Rats: An Animal Model of Metabolic Syndrome

Fructose is one of the three dietary monosaccharides, while the other two are glucose and galactose. Although it has the same molecular formula as glucose ($C_6H_{12}O_6$), its chemical cyclic structure (the major structural conformation) possesses a five-member ring, differing from glucose which possesses a six-member ring in its cyclic structure. It is commonly found in a variety of fruits and vegetables; it can also be found in the form of sucrose in which one molecule of fructose is linked one molecule of glucose through a glycoside bond. In the United States, total fructose consumption in the form of sucrose and high fructose corn syrup (HFCS, containing 55% fructose and 45% glucose) has significantly increased in the past two decades and has been suggested to contribute to the growing epidemic of obesity (Bray *et al.*, 2004). The 2000 Dietary Guidelines for Americans advises the public to choose appropriate beverages and foods to moderate sugar intake, as high fructose sweeteners in soft drinks account for almost half of the total added sugars in the US diet and consumption of soft drinks has increased dramatically in recent years (Johnson & Frary, 2001). A series of epidemiologic investigations have suggested increased consumption of soft drinks and fruit juice is associated with an increasing prevalence of obesity in children and adolescents (Dennison *et al.*, 1997; Ludwig *et al.*, 2001) and Type 2 diabetes in young and middle-age women (Schulze *et al.*, 2004). It has been shown that fructose-enriched diets cause dyslipidemia, impaired glucose tolerance, and insulin resistance in hepatic and adipose tissues (Faeh *et al.*, 2005). Diets high in fructose or sucrose have also been suggested to increase the risk of developing cardiovascular diseases in humans (Vasdev *et al.*, 2004).

Numerous studies have been conducted to investigate the adverse effects of high fructose consumption in rodents. Fructose fed rats have been developed as a characteristic model of metabolic syndrome, since these animals develop typical features of the syndrome such as insulin resistance, elevated blood pressure (Hwang *et al.*, 1987 & 1989), and hypertriglyceridemia (Thorburn *et al.*, 1989; Park *et al.*, 1992). The development of insulin resistance and hypertension in the fructose-fed rats is influenced by the concentration of fructose in the diet, the form of diet (food or drinking water), and the duration of dietary exposure to fructose (Dai & McNeill, 1995). An early study in our laboratory showed that drinking water containing 10% (weight/volume, or w/v) fructose produced the greatest increase in insulin resistance and hypertension (Dai & McNeill, 1995), which has been confirmed by others (Shahraki *et al.*, 2011). Equivalent to such a concentration in drinking water, a 60% fructose diet has been found sufficient to induce insulin resistance and hypertension (Verma *et al.*, 1994; Dai & McNeill, 1995; Juan *et al.*, 1998; Galipeau *et al.*, 2001; Hsieh *et al.*, 2005). The onset of fructose-induced hypertension also appears to depend on the strain of the animal. Previous studies conducted in our laboratory have found that male Sprague Dawley rats can acquire insulin resistance and hypertension following 3 weeks of being fed with a 66% fructose diet (Verma *et al.*, 1994) while male Wistar rats do not normally acquire hypertension until 6 to 8 weeks after the initiation of a 60% fructose diet (Galipeau *et al.*, 2001). Similarly, other studies demonstrated that male Sprague Dawley rats develop hypertension following 4 weeks of receiving 60% fructose diet (Iyer & Katovich, 1994 & 1996; Juan *et al.*, 1998; Hsieh *et al.*, 2005) while their Wistar counterparts do not until 6 weeks (Anuradha & Balakrishnan, 1999) when given the same diet. In general, Sprague

Dawley rats develop hypertension much earlier than Wistar rats if given the same fructose diet. Studies using female rats do not demonstrate insulin resistance and hypertension following fructose feeding (Galipeau *et al.*, 2002a), suggesting sex hormones may play an important role in the development of insulin resistance and hypertension (Galipeau *et al.*, 2002b). The presence of androgens, especially testosterone, is essential for the development of fructose-induced hypertension (Song *et al.*, 2004; Vasudevan *et al.*, 2005), while estrogens may play a protective role (Song *et al.*, 2005). Therefore, male fructose-fed rats have been used to investigate the mechanism(s) of the development of insulin resistance and hypertension in metabolic syndrome, and the relationship between insulin resistance and hypertension without the confounding interference of obesity, since fructose feeding does not result in excessive weight gain in the male rats (Verma *et al.*, 1994; Dai & McNeill, 1995; Galipeau *et al.*, 2001; Iyer & Katovich, 1994 & 1996b).

1.1.3 Two Major Proposed Mechanism(s) of Linking Insulin Resistance and Elevated Blood Pressure in Fructose-Fed Rats: Sympathetic Nervous System and Renin-Angiotensin System

Endothelial dysfunction has been suggested to be a link between insulin resistance and elevated blood pressure in this model (Miller *et al.*, 1998). Tran *et al.* (2009a) suggest that it is likely insulin resistance precedes the elevation of blood pressure and contributes to the development of the latter via triggering endothelial dysfunction, since evidence has suggested that the occurrence of hyperinsulinemia/insulin resistance

precedes the detection of endothelial dysfunction which is followed by the appearance of elevated blood pressure (Katakam *et al.*, 1998).

Various mechanisms have been suggested to be involved in linking insulin resistance to the development of elevated blood pressure in fructose fed rats. These include chronic activation of sympathetic nervous system (Verma *et al.*, 1999; Mayer *et al.*, 2006 & 2008), increased expression and/or activity of vasoconstrictors such as endothelin-I (Verma *et al.*, 1995 & 1997; Cosenzi A *et al.*, 1999), angiotensin II (Kobayashi R *et al.*, 1993; Iimura O *et al.*, 1995; Navarro-Cid *et al.*, 1995; Mayer *et al.*, 2008), and thromboxane A₂ (Galipeau *et al.*, 2001), and a defective NO pathway that leads to impaired endothelium-dependent relaxation (Tay *et al.*, 2002; Behr-Roussel *et al.*, 2008).

Chemical sympathectomy has been found to prevent the development of elevated blood pressure in the fructose fed rats (Verma *et al.*, 1999). Although in another study sympathectomy induced by guanethidine only significantly reduced but did not prevent the elevation of blood pressure in these animals (Hsieh & Huang, 2001), there is no doubt that the sympathetic nervous system (SNS) plays an important role in the development of fructose-induced blood pressure elevation. The exact mechanism of how the SNS contributes to the development of elevated blood pressure remains to be uncovered. Researchers have speculated that abnormal α_2 adrenergic receptor activities might be an important contributory factor. Takagawa *et al.* (2002) have found an impairment of α_2 adrenoceptor-mediated endothelium-dependent vasorelaxation in the animals in their early stage of fructose feeding. Mayer *et al.* (2006) have observed a decrease in presynaptic α_2 adrenergic receptor activity in the hypothalamus of the fructose fed rats.

These scientists suggest that this physiological change might be responsible for maintaining the hypertensive state in these animals, since the normal physiological function of α_2 adrenergic receptor activation is to decrease SNS activity and blood pressure. However, the same group of scientists has found an increase in the responsiveness of α_2 adrenergic receptors in the hypothalamus in a subsequent experiment (Mayer *et al.*, 2007). Other major adrenergic receptors such as α_1 , β_1 , and β_2 have not yet been fully investigated in the fructose-fed rat model, and there has been no report on any abnormality or physiological change of these receptors in these animals. The only finding with α_1 adrenergic receptors is that selective antagonists such as bunazosin (Kamide *et al.*, 2002) and prazosin (Tran, 2009) have been found to prevent the increase in blood pressure in this model, suggesting α_1 selective blockers might be a potential treatment for hypertension in patients with metabolic syndrome.

The renin-angiotensin system (RAS, in which angiotensin II is a key player) has been suggested to play an important role in the development of elevated blood pressure (Iyer *et al.*, 1996) and insulin resistance (Iyer & Katovich, 1996(1)) in fructose fed rats. A recent study conducted in our laboratory has demonstrated an increase in plasma angiotensin II levels in male Wistar rats following 6 weeks of fructose feeding (Tran *et al.*, 2009b). Inhibitors of the angiotensin II receptor (ATR) and angiotensin converting enzyme (ACE) have been found to prevent the elevation of blood pressure (Iyer & Katovich, 1994; Navarro-Cid *et al.*, 1995; Tran *et al.*, 2009b) and reduce insulin resistance (Iimura *et al.*, 1995; Iyer & Katovich, 1996a; Higashiura *et al.*, 2000; Uchida *et al.*, 2002). However, Tran *et al.* (2009b) found that L-158,809, an angiotensin type 1 receptor (AT₁) antagonist, did not increase insulin sensitivity which is attenuated by

fructose feeding in rats. A human study conducted in hypertensive patients with metabolic syndrome has revealed similar findings: neither telmisartan nor losartan, both of which are AT₁ antagonists, are able to improve insulin resistance in these patients (Bahadir *et al.*, 2007).

Researchers have obtained evidence of interactions between the SNS and RAS in the development of fructose-induced blood pressure elevation. The hypothalamus of fructose fed rats has been shown to exhibit an increase in the activity of both angiotensin II receptor 1 (AT₁) and β_1 adrenergic receptors, which is suggested to be related to the increase in blood pressure in this model (Mayer *et al.*, 2008). Mayer *et al.* have demonstrated that intrahypothalamic perfusion of irbesartan, an AT₁ antagonist, and metoprolol, a β_1 receptor blocker, lowered mean arterial pressure (MAP) in only fructose fed rats but not the control rats. They have also shown that the elevation of MAP induced by intrahypothalamic microinjection of angiotensin II was significantly higher in the fructose fed rats than in the controls. Pre-administration of metoprolol via the same route dramatically decreased this pressor response in the fructose fed animals. This suggests that the fructose fed rats show a heightened sensitivity to the stimulation of angiotensin II in elevating their blood pressure, and such a physiological change appears to require the participation of β_1 adrenergic receptors.

The current study aimed to investigate any alteration in the activities of SNS and RAS, and the potential interaction between the two in the fructose fed rats in the presence of phentolamine, an anti-hypertensive that can potentially activate both systems indirectly.

1.1.4 Phentolamine

Phentolamine, an imidazoline derivative, is a potent α_1 and α_2 adrenergic receptor antagonist. It reduces peripheral resistance via inhibiting α_1 receptors and possibly α_2 receptors on vascular smooth muscle (VSM) to cause VSM relaxation, which has been summarized by Katzung (2007). α blockade can markedly reduce the interaction between α receptors and endogenous released noradrenaline. As a result, interaction between β adrenergic receptors and noradrenaline and the subsequent activation of these receptors are greatly enhanced. In the central nervous system, potent blockade of α_2 adrenergic receptors at the presynaptic terminal may potentially increase the release of noradrenaline from sympathetic nerves, since they are now relieved from the inhibition previously exerted by α_2 adrenergic receptors. Consequently, activation of β adrenergic receptors is further enhanced. Therefore, phentolamine can potentially cause activation of β_1 adrenergic receptors in the heart and kidneys, which will lead to an increase in cardiac contractility and heart rate in the former, and production of renin in the latter, respectively. As renin is an important mediator of RAS responsible for the production of angiotensin I, a precursor of angiotensin II, increased production of renin caused by phentolamine might increase the circulating angiotensin II in the plasma.

Phentolamine would be expected to decrease fructose-induced blood pressure elevation as it can lower blood pressure via its vasorelaxation mechanism through the inhibition of α_1 receptors in VSM. However, caution must be taken since there may be potential side effects. Cardiac toxicity, such as tachycardia or arrhythmia, induced by chronic activation of β_1 receptors in the heart might occur if the drug is used chronically. As angiotensin II is known to be a potent vasoconstrictor, the potential increase of its

release indirectly caused by phentolamine might result in an increase in vasoconstriction, counteracting the hypotensive effects produced by inhibition of α_1 receptors. In addition, as angiotensin II has been suggested to be a mediator of the development of insulin resistance in metabolic syndrome, chronic use of phentolamine might worsen the insulin sensitivity in patients with this syndrome. The potential side effects of phentolamine can be examined in the fructose fed rats treated with this drug, as this animal model is a representative of metabolic syndrome.

1.1.5 Apoptosis of Cardiac Myocytes

Abnormal or excessive activation of cardiac β adrenergic receptors has been suspected to be a contributor to the development of cardiac diseases. Blockade of β adrenergic receptor activity in the heart has been proposed as a treatment for heart failure (Metra *et al.*, 2000; Bristow, 2000) and cardiac arrhythmias (Anh & Marine, 2004). Recent literature suggests that excessive β adrenergic stimulation appears to be linked to apoptosis and survival of cardiac myocytes. Evidence reveals that chronic activation of β_1 adrenergic receptors leads to an increase of apoptosis of cardiac myocytes (Communal *et al.*, 1999; Zaugg *et al.*, 2000; Bisognano, 2000; Zhu *et al.*, 2003). On the other hand, stimulation of β_2 adrenergic receptors appears to provide cardiac myocytes with protection from apoptosis (Communal *et al.*, 1999; Zaugg *et al.*, 2000; Zhu *et al.*, 2001).

1.2 Research Outline: Objectives, Rationales, Hypotheses

1.2.1 Objective 1: To investigate whether chronic use of phentolamine can prevent the elevation of blood pressure induced by fructose feeding in Wistar rats.

Rationale

The sympathetic nervous system has been proposed as a factor involved in the development of elevated blood pressure in fructose fed rats, although the exact mechanism has yet to be discovered. Nevertheless, inhibition of α_1 and stimulation of β_2 adrenergic receptors can decrease vasoconstriction and increase vasorelaxation, respectively, under normal physiological conditions. Furthermore, selective α_1 receptor antagonists such as bunazosin (Kamide *et al.*, 2002) and prazosin (Tran, 2009) have been shown to prevent the increase in blood pressure induced by fructose feeding in rats. As phentolamine is a non-selective α receptor antagonist that inhibits the activities of α_1 receptors and indirectly enhances the activation of β_2 receptors, it should be able to prevent the elevation of blood pressure in fructose fed rats.

However, indirect activation of β_1 adrenergic receptors in the kidneys enhanced by phentolamine might produce a pressor effect that counteracts the hypotensive effect of this drug. Activation of renal β_1 receptors would stimulate the production of renin, an enzyme that facilitates the production of angiotensin II which is a potent vasoconstrictor. Such an effect might not be significant in the use of bunazosin and prazosin because these antagonists do not cause an increased release of noradrenaline, since they exert no inhibition on presynaptic α_2 receptors. In addition, angiotensin II has been found to be involved in the development of insulin resistance (Iyer & Katovich, 1996a) in fructose

fed rats. Insulin resistance has been suspected to contribute to the development of elevated blood pressure by triggering endothelial dysfunction (Miller *et al.*, 1998), since it has been shown that the appearance of insulin resistance precedes the detection of endothelial dysfunction which is followed by the occurrence of blood pressure elevation (Katakam *et al.*, 1998). As a result, the overall effect of phentolamine on altering blood pressures is difficult to predict theoretically.

Hypothesis 1: Chronic treatment of phentolamine will prevent the increase in systolic blood pressure induced by fructose feeding.

1.2.2 Objective 2: Determine whether phentolamine will increase the release of endogenous noradrenaline into the circulation and indirectly measure whether there is an abnormality in presynaptic α_2 receptor activity in fructose fed rats.

Rationale

In the central nervous system, inhibition of α_2 adrenergic receptors by phentolamine at the presynaptic terminal can reduce inhibitory effects of these receptors on the postsynaptic neurons and potentially increase the release of noradrenaline from postsynaptic terminals of the sympathetic nerves into the circulation. Different levels of plasma noradrenaline in the animals from control and treatment groups should reflect the inhibitory effect of phentolamine on α_2 adrenergic receptors.

However, recent findings have suggested the existence of abnormal α_2 receptor activity in the central nervous system in fructose fed rats, although the evidence is

somewhat contradictory about whether the activity is increased or decreased (Mayer *et al.*, 2006 & 2007). By measuring the level of plasma noradrenaline, one can obtain indirect evidence whether the inhibitory function of α_2 adrenergic receptors has been altered by fructose feeding.

Hypothesis 2: Chronic treatment of phentolamine can indirectly increase the plasma level of noradrenaline by blocking the inhibitory effects of presynaptic α_2 adrenergic receptors on the release of this endogenous agonist.

1.2.3 Objective 3: Investigate whether phentolamine alters the expression of β_1 adrenergic receptors in the kidneys, the level of plasma angiotensin II, and insulin resistance. Determine whether an increase in insulin resistance is linked to an increased level of plasma angiotensin II which is mediated by activation of renal β_1 adrenergic receptors.

Rationale

Antagonism of α adrenergic receptors produced by phentolamine may potentially increase the interaction between the circulating catecholamines and β adrenergic receptors. In addition, inhibition of the inhibitory effects of presynaptic α_2 receptors removes the inhibition of postsynaptic neurons, leading to an increased release of noradrenaline from sympathetic nerve fibers and the subsequent enhancement of interaction between noradrenaline and β adrenergic receptors (Katzung, 2007). As a result of these two mechanisms, the overall stimulation of β adrenergic receptors will be increased. Chronic stimulation of β_1 adrenergic receptors might induce a homeostatic

regulatory mechanism, resulting in a decrease in receptor expression in the animals treated with phentolamine. Depending on the biological and physiological characteristics of each treated individual, the extent of changes in receptor expression might be different.

Increased stimulation of renal β_1 adrenergic receptors can lead to an increase in the production of renin, an important enzyme that converts angiotensinogen into angiotensin I which can be subsequently converted to angiotensin II by angiotensin converting enzyme or ACE, (Dinh *et al.*, 2001). Therefore, the level of angiotensin II released into the blood stream may be elevated following an increased activation of β_1 adrenergic receptors in the kidneys at the presence of phentolamine.

Angiotensin II has been proposed a contributor of the development of insulin resistance in fructose fed rats (Iimura *et al.*, 1995; Iyer & Katovich, 1996a; Higashiura *et al.*, 2000; Uchida *et al.*, 2002; Tran *et al.*, 2009a). If the use of phentolamine triggers an increased production of angiotensin II via indirect activation of renal β_1 adrenergic receptors, insulin sensitivity should be further decreased in the fructose-fed rats treated with phentolamine compared to those without the treatment. Whether phentolamine will decrease insulin sensitivity in the control animals remains unknown, since angiotensin II might not necessarily cause insulin resistance but serves as a contributor to its development. Since insulin regulates glucose uptake in muscles, liver and fat tissue by activating the insulin receptors in these tissues, fasting elevated plasma level of insulin and glucose are the two common indicators of insulin resistance.

Hypothesis 3: Chronic treatment of phentolamine will increase plasma angiotensin II level via an increase in renal β_1 adrenergic receptor-mediated production of renin, which in turn will lead to an increase in insulin resistance.

1.2.4 Objective 4: Determine the possible changes chronic phentolamine treatment may produce in β adrenergic receptor expressions and whether β_1 adrenergic receptor-mediated apoptosis of cardiac myocytes is enhanced by chronic phentolamine treatment.

Rationale

Blockade of α receptors by phentolamine can indirectly enhance the interaction between the endogenous agonists, such as adrenaline and noradrenaline, and β adrenergic receptors. Depending on the extent and the duration of the enhanced interactions between the agonists and the receptors, the characteristics and physiological functions of receptor subtypes, and the influences they receive from their regulators, the changes in receptor expression and activity may be subtype-specific. Since the distribution of β adrenergic receptors is dependent on the type of tissue, the effects phentolamine may produce on β adrenergic receptor expression and activity can be expected to be tissue-specific.

Cardiac β adrenergic receptors have been suggested to play an influential role in the death and survival of cardiac myocytes. Evidence has suggested that stimulation of β_1 receptors appears linked to an increase in apoptotic cardiac myocytes while activation of β_2 receptors seem to enhance the survival of cardiac myocytes (Communal *et al.*, 1999;

Zaugg *et al.*, 2000; Li *et al.*, 2007). Although phentolamine can indirectly activate both β receptor subtypes, it is more likely to produce β_1 receptor-mediated effects since β_1 receptors are known to be dominant in cardiac tissues. Although the exact mechanism of β_1 receptor-mediated apoptosis of cardiac myocytes is not yet elucidated, important cardiac proteins such as calcineurin (Saito *et al.*, 2000, Kakita *et al.*, 2001), Ca^{2+} /calmodulin kinase II or CaMKII (Zhu *et al.*, 2003), L-type calcium channel (Wang *et al.*, 2010). Recent evidence has suggested that CaMKII appears to link β_1 receptor activation to apoptosis of cardiac myocytes (Zhu *et al.*, 2003). CaMKII, which undergoes autophosphorylation to become activated, is known to phosphorylate phospholamban on amino acid Threonine 17 (Thr^{17}) thereby activating it (Hagemann *et al.*, 2000). Therefore, the level of Thr^{17} residue phosphorylated phospholamban might be increased if CaMKII-mediated apoptotic pathway is activated by β_1 receptor activation. On the other hand, β_1 receptor activation also triggers the activation of protein kinase A (PKA) which subsequently phosphorylates phospholamban on Serine 16 (Ser^{16}) residue (Vittone *et al.*, 1990 & 1996). In addition to direct phosphorylation, PKA also regulates phospholamban through its regulation of protein phosphatase inhibitor-1 (I-1) (Neumann 2002) which regulates protein phosphatase-1 (PP-1), a protein which dephosphorylates phospholamban (MacDougall *et al.*, 1991; Steenaart *et al.*, 1992). Therefore, it is important to measure the level of PKA, CaMKII, and phospholamban phosphorylated both on Ser^{16} and Thr^{17} , if one is to investigate whether phentolamine triggers β_1 receptor-mediated cardiac myocyte apoptosis through PKA-independent activation of CaMKII. The total level of phospholamban and CaMKII should be measured to eliminate the possibility that the increased level of phosphorylated phospholamban and

autophosphorylated CaMKII is due to an increase in the total production of these proteins. Phospholamban has also been reported to be indirectly regulated by protein kinase C- α (PKC- α) which directly phosphorylates I-1 and subsequently leads to change in the activity of PP-1 (Bray *et al.*, 2004). Change in expression and activity of PKC- α has been associated with heart failure (Bowling *et al.*, 1999; Wang J *et al.*, 2003) and ventricular hypertrophy (Bayer *et al.*, 2003). Therefore, altered expression and activity of PKC- α might lead to changes in the phosphorylation of phospholamban.

Hypothesis 4: Chronic phentolamine treatment will produce receptor subtype- and tissue-specific changes in the expression of all β receptors and decrease the activity of β_1 receptors in both the heart and the kidney. Phentolamine may increase cardiac tissue apoptosis via chronic indirect stimulation of β_1 receptors in the heart.

1.2.5 Objective 5: Investigate the state of oxidative stress in fructose-fed rats.

Rationale

Increased oxidative stress has been suggested to be a contributor to the pathological development in fructose fed rats (Tran *et al.*, 2009a). It has been proposed to play a role in the development of insulin resistance (Eriksson, 2007) and elevated blood pressure (de Champlain *et al.*, 2004). Plasma 8-isoprostane will be used as an indicator of oxidative stress in this study, since it has been suggested to be a marker of free radical-induced injury in certain oxidative stress related diseases (Greco *et al.*, 2000; Praticò *et al.*, 2001).

Oxidative stress and abnormal nitric oxide (NO) activity have been proposed to be a link to the development of elevated blood pressure (Wilcox, 2005). NO has been suggested to trigger oxidative stress and the subsequent development of blood pressure elevation or hypertension in various animal models. Tran *et al* (2009a) propose that fructose feeding causes a decrease in endothelial nitric oxide synthase (eNOS) activity, which leads to a reduced production of NO and impaired endothelium dependent relaxation, and the eventual development of hypertension. On the contrary in the spontaneous hypertensive rats (SHRs), an animal model of hypertension, an increased expression of eNOS in the kidneys has been found (Welch *et al.*, 1999). Another type of NOS, Type II or inducible NOS (iNOS), has been shown to be over-expressed and to exhibit increased activity in the kidneys of SHRs (Kumar *et al.*, 2005). Kumar *et al.* (2005) speculate that over-expression of iNOS leads to excessive release of NO which reacts with reactive oxygen species (ROS) and causes oxidative stress. Whether such mechanism applies to the increase in oxidative stress and development of elevated blood pressure in fructose fed rats remains to be investigated; it is worthwhile to measure the production of NO and the expression of eNOS and iNOS in the kidneys in these animals to obtain evidence to support or reject such a hypothesis.

Hypothesis 5: If chronic phentolamine treatment increases oxidative stress, it will result in an increased plasma level of 8-isoprostane and changes in plasma total NO production and the expressions of eNOS and iNOS in the heart and kidney.

2. Materials and Methods

2.1 Development of Fructose-Fed Rats

Twenty four male Wistar rats weighing 180 to 200g at the age of 6 weeks were purchased from Charles River (Montreal, Canada) and randomly assigned into four groups of six individuals (n=6), each of which was designated as control (C), control phentolamine treated (CP), fructose (F), and fructose phentolamine treated (FP), respectively. Upon arrival, the rats were allowed to acclimatize in the animal care facility located in the basement of Cunningham Building, Faculty of Pharmaceutical Sciences, UBC, and were taken care of according to the guidelines given by the Canadian Council for Animal Care (CCAC). One week following acclimatization (at the age of 7 weeks), the animals were trained for the tail cuff procedure of blood pressure (described below). The basal systolic blood pressures of all the animals were recorded at the end of the week which was marked at Week 0. On the first day of the following week, blood samples from all the animals were collected from the tail vein. Fructose feeding (Harlan TekLad, Madison) and phentolamine (Sigma-Aldrich, Saint Louis) treatment were then started simultaneously. All the animals were maintained with *ad libitum* access to food (regular diet for C and CP group, 60% fructose diet for F and FP group) and water (tap water for C and F group, phentolamine water solution for CP and FP group) in a room with constant humidity and temperature and with a light/dark cycle of 12 hours. The concentration of phentolamine in water was adjusted on a daily basis to maintain a final dose of 5 mg/kg in the treated animals assuming the drug was 100% absorbed into the circulation. The animals were terminated when the animals in the F groups showed a significant increase in the systolic blood pressure in Week 14.

2.2 Measurement of Systolic Blood Pressure

All the animals were preconditioned to the tail cuff procedure prior to the actual measurements. The animals were placed in the plexiglas rodent restrainers of suitable sizes, in which they were allowed to rest within the restrainers on the bench at a relatively constant temperature of 24 °C. Then they were transferred (within the restrainers) to a chamber whose temperature was maintained at 24 °C and again allowed to rest. The tail of each animal was inserted into an inflatable cuff containing an electric sensor which was connected to a multi-sensor manual scanner (Model 64-120) and blood pressure amplifier attached to an analog/digital recorder (Model 179) from IITC Life Sciences Inc. (Woodland Hills, California). The animals were allowed to rest till their tails were no longer in motion. The cuffs were then inflated to a maximum pressure of 150 mmHg followed by a gradual deflation. A reappearance of pulse was detected by the sensor which was subsequently recorded as the systolic blood pressure. A minimum of 6 readings were recorded for each rat at any given time point in the study. For each animal, 5 consistent values were collected; the smallest and the largest values were eliminated and the mean systolic blood pressure was calculated based on the three intermediate values.

2.3 Plasma Sample Collection

On the first day of Week 1 and Week 5, blood samples were withdrawn from all the animals via tail vein following a fasting period of 16 hours. The samples were centrifuged in a Beckman centrifuge (Beckman Allegra 21R) at 4 °C at the speed of

14000 rpm for 25 minutes. Following the centrifugation, the plasma was immediately collected and stored at the -80 °C freezer for further analysis of plasma levels of glucose, insulin, triglyceride, and cholesterol. At termination, all the animals were euthanized with an overdose of pentobarbital (60 mg/kg, intraperitoneal injection) and blood samples were collected via cardiac puncture. For the determination of plasma levels of angiotensin II, blood samples were collected in plastic tubes containing a cocktail of 0.44 mM o-phenanthroline, 25 mM ethylenediaminetetraacetic acid (EDTA), 1 mM p-hydroxymercuribenzoic acid and 0.12 mM pepstatin A. For the determination of plasma levels of noradrenaline, blood samples were collected in glass tubes containing heparin. For the measurement of plasma 8-isoprostane and total nitrate/nitrite, the blood samples were aliquoted in plastic Eppendorf tubes. The samples were centrifuged in the Beckman centrifuge at 4 °C at the speed of 14000 rpm for 25 minutes and the plasma was collected and stored at the -80 °C freezer until assayed.

2.4 Biochemical Measurements

Plasma glucose levels were determined using a Beckman Glucose Analyzer II (Beckman, Fullerton, California). Plasma insulin levels were measured using a sensitive rat insulin RIA kit (Millipore, Billerica, Massachusetts). Plasma triglyceride and cholesterol levels were determined using an enzymatic colorimetric assay (Teco Diagnostics, Anaheim, California). Plasma angiotensin II and noradrenaline levels were measured using an ELISA kit from Cedarline (Hornby, Ontario) and IBL Hamburg (Toronto, Ontario), respectively.

2.5 Oral Glucose Tolerance Test and Assessment of Insulin Resistance/Sensitivity

Three days prior to the termination, all the animals were fasted overnight (16 hours) and subjected to an oral glucose tolerance test (OGTT). A 60% glucose solution was administered to all the animals via oral gavage. Blood samples were collected from the animals via the tail vein at 0, 10, 20, 30, 60, and 90 minutes after the oral administration. The samples are then centrifuged in the Beckman centrifuge at 4 °C at the speed of 14000 rpm for 25 minutes and the plasma was collected and stored in the -80 °C freezer until assayed. The samples collected at time point 0 minute were also used for analysis of plasma glucose and insulin in Week 14. The plasma levels of glucose and insulin were determined as described above. Insulin sensitivity index (ISI) was calculated using the formula given by Matsuda and DeFronzo (1999) using 100 as constant:

$$ISI = \frac{100}{\sqrt{(FPG \times FPI) \times (MPG \times MPI)}}$$

The fasting plasma glucose (FPG) and insulin (FPI) values were obtained the measurement of the plasma glucose and insulin levels of the samples at time point 0 minute, while the mean plasma glucose (MPG) and insulin (MPI) values were calculated as the mean of the plasma glucose and insulin levels of the samples at all the time points (0 to 90 minutes).

2.6 Western Blot Analysis

Following the cardiac puncture procedure at termination, the hearts and kidneys from all the animals were isolated. The hearts from three animals from each group and the left

kidneys from all the animals were washed with cold saline and immediately frozen in liquid nitrogen and then stored in the -80 °C freezer until assayed.

The frozen tissues were homogenized with a homogenization buffer containing 62.5 mM Tris-HCl, 50 mM dithiothreitol [DTT], 2% SDS, 10% glycerol, protease inhibitor (1:100 dilution), and phosphatase inhibitor (1:100 dilution). The total tissue protein concentration of each sample was then estimated by Bradford protein assay. 40 µg of total protein of each sample was solubilized in Laemmli sample buffer with 5% 2-mercaptoethanol and subsequently fractionated by electrophoresis on a 7% (for eNOS and iNOS), 10% (for β adrenergic receptors, CaMKII, PKA, AT₁) or 12% (for phospholamban) polyacrylamide gel based on the size of the protein of interest. The sample proteins were then transferred onto 0.2 µm nitrocellulose membranes in transfer buffer (20 mM Tris, 192 mM glycine and 20% methanol). The membranes were blocked with 5% bovine serum albumin (BSA, diluted in Tris-buffer saline with (2% tween) or TBST) at room temperature for one hour and then subsequently incubated with specific primary antibodies diluted in 5% BSA at 4 °C overnight (17 to 20 hours). The dilution for the primary antibodies of β_1 adrenergic receptor (Abcam, Cambridge, MA & Santa Cruz, California), β_2 adrenergic receptor (Abcam, Cambridge, MA), β_3 adrenergic receptor (Santa Cruz, California), phosphor- and total PKA (Santa Cruz, California), CaMKII (Cell Signaling, Ontario), phospho (Ser 16)-phospholamban (Abcam, Cambridge, MA), phospho (Thr 17)- and total phospholamban (Santa Cruz, California), AT₁ (Abcam, Cambridge, MA), eNOS (Abcam, Cambridge, MA), and iNOS (Santa Cruz, California) was 1:500 while that for GAPDH primary antibody (Cell Signaling, Ontario) was 1:3000. Following the overnight incubation, the membranes were washed TBST with for three

times and incubated with corresponding secondary antibodies (goat anti-rabbit, anti-mouse or anti-chicken) prepared in 5% BSA with a dilution of 1:2000 at room temperature for one hour. Following another series of three washes using TBST, the membranes were incubated with a chemiluminescence reagent for one minute and the protein bands on the membranes were detected using an Alpha Innotech FluorChem 8800 (Alpha Innotech Co., San Leandro, California) gel box imager. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein.

The expression of any protein of interest was determined by measuring the intensity of the protein band against the intensity of its corresponding GAPDH using the software FluorChem (Alpha Innotech Co., San Leandro, California). The results were expressed as a percentage of the mean value of the control group (% control) assuming the control mean to be 100%.

2.7 Measurement of Apoptosis of Cardiac Tissues

At termination, the hearts from other three animals from each group were collected, washed with cold saline and immediately fixed in 4% paraformaldehyde and stored at 4 °C for further processing. The fixed tissues were sectioned in paraffin into 5 µm slides vertically to preserve the integrity of both atria and ventricles. Each paraffin embedded slides contained complete sections of both left and right atria and ventricles.

The sections were deparaffinized with xylene, and rehydrated with a series of ethanol with diluting concentration (100%, 90%, 80%, and 70%, diluted in double distilled water). The sections were then washed with phosphate buffer saline (PBS) and permeabilized

with 0.1% Triton X. The sections were incubated with Hoescht dye for 15 minutes at room temperature in the dark followed by a washing period of 10 minutes. The sections were mounted and examined under Leica DMLB microscope attached with the Retiga 2000R camera.

For each section of atria or ventricles, 12 fields were randomly selected. The numbers of apoptotic and total cells were counted in each field and a ratio of apoptotic/total cells was calculated. The mean percentage apoptosis for each section was then calculated based on the 12 values obtained.

2.8 Statistical Analyses

All the data are presented as mean $\pm$ standard error of the mean (SEM) in the figures generated using Sigma Plot. The analyses were initially accomplished with two-way ANOVA. One-way ANOVA followed by Newman-Keuls multiple comparison post hoc test was used to compare individual group means to identify the source of the difference detected by two-way ANOVA. The mean values were considered statistically significant at $p < 0.05$. All statistical analyses were performed using the Number Cruncher Statistical System (NCSS) software package.

3. Results

3.1 The Effects of Fructose and Phentolamine on Blood Pressure and Metabolic Parameters

The animals fed with the 60% fructose diet for 13 weeks were found to have an elevated level of systolic blood pressure (**Figure 1**, control: 98 ± 1 mmHg, fructose-fed: 113 ± 4 mmHg) and plasma metabolic parameters (**Table 1**). In contrast to systolic blood pressure, the four plasma metabolic parameters including triglyceride, glucose and insulin were elevated as early as by 4 weeks following the start of fructose feeding. Phentolamine treatment was found to have prevented the increase in systolic blood pressure without affecting these metabolic parameters (**Figure 1**, **Table 1**). 2-way ANOVA analysis suggests there was a significant interaction between the diet and the drug. However, phentolamine treatment significantly increased plasma glucose level in both the control and fructose-fed rats at an early stage of the study, though such an increase was relatively small (**Table 1**). Neither fructose nor phentolamine had any effect on body weight (**Table 2**).

Following the oral glucose challenge, both plasma insulin and glucose rose to their maximum level as early as 10 minutes and gradually returned to the normal level (**Figure 2**). Consistent with previous studies conducted in our laboratory, both plasma insulin and glucose was elevated by fructose feeding throughout the course of the OGTT; however, only the elevation in plasma insulin was significant (**Figure 2**). Interestingly, phentolamine treatment appeared to slightly increase plasma insulin and glucose in the fructose-fed animals but exert an opposite effect on both parameters in the control

animals, although the effects were not significant. Calculations of the area under the curve (AUC) suggested that fructose feeding increased the AUC of both insulin and glucose. Phentolamine had no effect on the AUC of either insulin or glucose in either the control or fructose-fed animals (**Figure 2 insets**). Evaluation of insulin sensitivity index revealed that insulin sensitivity was attenuated by fructose feeding, which was not affected by chronic treatment of phentolamine (**Figure 3**).

3.2 The Effects of Fructose and Phentolamine on Plasma Noradrenaline and Angiotensin II

Plasma levels of noradrenaline were comparable among all the study groups (**Figure 4**), suggesting that neither fructose nor phentolamine altered the release of noradrenaline at this time point. Plasma angiotensin II levels were significantly elevated following 13 weeks of fructose feeding (**Figure 5**). The observed elevation of plasma angiotensin II in the fructose-fed rats and a recent finding of increased mRNA expression of angiotensin II receptor type I (AT₁) (Nyby MD, et al, 2007) in the hearts of fructose-fed rats inspired us to investigate the possible changes in cardiac AT₁ expression. Our result suggests neither fructose feeding nor phentolamine had any significant impact on AT₁ expression in the hearts (**Figure 6**).

3.3 The Effects of Fructose and Phentolamine on Cardiac Tissue Apoptosis

The fluorescent Hoechst dye taken into the cell nuclei produced a blue color under the microscope which revealed the morphology of cardiac cells. The color of cells

which had undergone the process of apoptosis appeared to be more intense compared to that of the healthy cells, as more dye molecules bound to their DNA fragments in the nuclei. Calculations of the average percentage apoptotic cell (apoptotic/total cells) suggested that neither fructose feeding nor phentolamine treatment had any significant effect in apoptosis of cardiac tissues (**Figure 7**).

3.4 The Effects of Fructose and Phentolamine on the Expression and Activities of β adrenergic receptors in the Heart and the Expression of these Receptors in the kidneys

The expression of β_1 adrenergic receptors in the heart could not be measured due to technical difficulties. The phosphorylation levels of protein kinase A (PKA), a downstream effector of β_1 adrenergic receptor activation, were similar among all the animal groups, suggesting the level of cardiac cyclic adenosine monophosphate (cAMP) was unchanged by either fructose or phentolamine treatment (**Figure 8**). The phosphorylation levels of phospholamban, a downstream effector of PKA and Ca^{2+} /calmodulin kinase II (CaMKII), at both serine 16 and threonine 17 were decreased significantly in all the phentolamine treated animals, which on the other hand were not affected by fructose feeding (**Figure 9**). Western blot result revealed a significant increase in cardiac β_2 adrenergic receptor expression in the fructose fed rats, which was normalized by phentolamine treatment. However, phentolamine had no effect on β_2 adrenergic receptor expression in the control animals (**Figure 10**). Cardiac β_3 adrenergic receptor expression was increased in the control rats treated with phentolamine; however,

such an increase was not observed in the fructose fed rats receiving the same treatment. 2-way ANOVA analysis suggests that there was an interaction between the diet and the drug in the expression of both β_2 and β_3 adrenergic receptors. (**Figure 11**).

In the kidneys, fructose feeding caused a decrease in the β_2 adrenergic receptor expression which was normalized by phentolamine treatment (**Figure 12**). Similarly, fructose feeding produced a decrease in the β_3 adrenergic receptor expression. In contrast, phentolamine treatment produced an increase in the β_3 adrenergic receptor expression in the treated animals; such an increase was enhanced to a significant level by fructose feeding (**Figure 13**). The PKA phosphorylation in the kidneys was significantly increased by both phentolamine treatment and fructose feeding, suggesting the cAMP levels might have been elevated by both treatments. However, the combination of fructose feeding and phentolamine treatment appeared to dampen the effect of phentolamine alone (**Figure 14**).

3.5 The Effects of Fructose and Phentolamine on Oxidative Stress

The fact that plasma 8-isoprostane levels were comparable among all groups suggests oxidative stress did not occur in this particular study (**Figure 15**). However, 8-isoprostane might not present the complete picture. The sum of nitrate and nitrite reflects the total production of NO in the circulation; therefore, plasma total nitrate & nitrite was chosen as a second marker to evaluate the state of oxidative stress, since oxidative stress has been linked to decreased production of NO (Tran et al., 2009a). The fact that the plasma total nitrate & nitrite was 7 fold higher in the fructose fed animals (**Figure 16**) suggests the total NO present in the circulation was significantly elevated by fructose

feeding. The measurement of eNOS and iNOS expressions in the heart and kidney was a means to search for a possible explanation of the above observation. The eNOS expression in the heart was only increased in the fructose fed animals treated with phentolamine while the expression of iNOS was only elevated in the control animals receiving phentolamine (**Figure 17**). On other hand, eNOS expression in the kidney was not affected by either fructose feeding or phentolamine treatment while the expression of iNOS was decreased by both the diet and the drug (**Figure 18**). Overall, the changes in the expressions of eNOS and iNOS in the heart and kidney could not provide a sufficient explanation to the observed increase in total NO production.

Figure 1: Effect of Phentolamine on Systolic Blood Pressure (Week 4 vs Week 13). Systolic blood pressures were significantly increased by fructose feeding following 13 weeks of study. This increase was prevented by chronic phentolamine treatment which, however, had no effects on animals receiving regular chow. Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs C, CP, and FP.

Table 1: Plasma Metabolic Parameters in Control and Fructose-Fed Rats following 4 Weeks and 13 Weeks of Study

Week 4	C	CP	F	FP
Plasma Cholesterol (mM)	1.8 $\pm$ 0.04	2.0 $\pm$ 0.12	2.7 $\pm$ 0.23*	3.0 $\pm$ 0.19*
Plasma Triglyceride (mM)	1.29 $\pm$ 0.11	1.45 $\pm$ 0.11	4.27 $\pm$ 0.26*	4.28 $\pm$ 0.19*
Plasma Glucose (ng/mL)	6.25 $\pm$ 0.14	6.89 $\pm$ 0.25#	7.47 $\pm$ 0.13#	8.3 $\pm$ 0.22#
Plasma Insulin (ng/mL)	1.05 $\pm$ 0.12	0.98 $\pm$ 0.08	1.63 $\pm$ 0.12*	1.97 $\pm$ 0.24*

Week 13	C	CP	F	FP
Plasma Cholesterol (mM)	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	3.1 $\pm$ 0.4*	4.0 $\pm$ 0.1*
Plasma Triglyceride (mM)	2.0 $\pm$ 0.1	1.6 $\pm$ 0.2	3.1 $\pm$ 0.2*	3.5 $\pm$ 0.2*
Plasma Glucose (ng/mL)	6.0 $\pm$ 0.3	6.3 $\pm$ 0.2	7.5 $\pm$ 0.3*	7.0 $\pm$ 0.2*
Plasma Insulin (ng/mL)	0.6 $\pm$ 0.1	0.5 $\pm$ 0.1	1.3 $\pm$ 0.1*	1.1 $\pm$ 0.1*

Note: Plasma levels of cholesterol, triglyceride, glucose and insulin were significantly elevated at an early stage (Week 4) in the fructose-fed rats and remained significantly higher than those in the control rats until termination (Week 13). In general, phentolamine treatment had no effects on the plasma levels of these four parameters throughout the course of this study. However, it slightly increased plasma glucose to a significant level at an early stage (Week 4) and such an effect was not observed at a later stage (Week 13). Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs C and CP, #p<0.05, different from control and all the other treatment groups.

Table 2: Body Weight of Control and Fructose-Fed Rats

Period	C	CP	F	FP
Week 1	264 $\pm$ 9	236 $\pm$ 6	243 $\pm$ 8	249 $\pm$ 9
Week 13	522 $\pm$ 9	477 $\pm$ 17	496 $\pm$ 19	541 $\pm$ 23

Note: Neither fructose feeding nor phentolamine had any effect on body weight throughout the study. Values expressed as mean $\pm$ SEM, n=6

Figure 2: Effect of Phentolamine on Plasma a) Insulin Response and AUC (inset) and b) Glucose Response and AUC (inset) during an OGTT in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding significantly increased the AUCs of both plasma insulin and glucose. In addition, plasma insulin levels were significantly increased by fructose feeding. Values expressed as mean $\pm$ SEM, $n=6$, * $p<0.05$ vs C and CP, # $p<0.05$ vs all C, F, and FP.

Figure 3: Effect of Phentolamine on Insulin Sensitivity Index Values in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding significantly decreased insulin sensitivity which was not altered by chronic phentolamine treatment. Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs C and CP.

Figure 4: Effect of Phentolamine on Plasma Noradrenaline Levels in Control and Fructose-Fed Rats following 13 Weeks of Study. Neither fructose feeding nor phentolamine treatment had any effect on plasma noradrenaline levels. Values expressed as mean $\pm$ SEM, n=6.

Figure 5: Effects of Phentolamine on Plasma Angiotensin II in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding significantly increased plasma angiotensin II levels which phentolamine treatment produced no effects on. Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs C and CP.

Figure 6: Representative Western Blot of Cardiac Angiotensin II Receptor Type I in Control and Fructose-Fed Rats following 13 Weeks of Study and Effects of Phentolamine on the Expression of Cardiac Angiotensin II Receptor Type I in Control and Fructose-Fed Rats following 13 Weeks of Study. Neither fructose feeding nor phentolamine treatment produced any effects on the expression of angiotensin II receptor type I. Values expressed as mean $\pm$ SEM, n=3.

Figure 7: Effect of Phentolamine on Apoptosis of Cardiac Tissues from Control and Fructose-Fed Rats following 13 Weeks of Study. Neither fructose feeding nor phentolamine treatment had any impact on cardiac tissue apoptosis. The hearts of 3 animals from each treatment group were randomly selected for this experiment. 12 random fields were selected from the heart from each animal in each group. All the % apoptosis values were pooled together within each group to express the overall % apoptosis in each group as mean $\pm$ SEM.

Figure 8: Representative Western Blot of Cardiac Phospho- and Total PKA in Control and Fructose-Fed Rats following 13 Weeks of Study and Effects of Phentolamine on the Cardiac PKA Phosphorylation in Control and Fructose-Fed Rats following 13 Weeks of Study. Neither fructose feeding nor phentolamine treatment produced any effect on the PKA phosphorylation levels. Values expressed as mean $\pm$ SEM, n=3

Figure 9a: Representative Western Blot of Phospho- Serine 16 and Total Phospholamban in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Phospholamban Phosphorylation at Serine 16 in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding did not produce any effect on phospholamban phosphorylation at serine 16 which was significantly reduced by phentolamine treatment. Values expressed as mean $\pm$ SEM, n=3.

Figure 9b: Representative Western Blot of Phospho- Threonine 17 and Total Phospholamban in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Phospholamban Phosphorylation at Threonine 17 in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding did not produce any effect on phospholamban phosphorylation at threonine 17 which was significantly reduced by phentolamine treatment. Values expressed as mean $\pm$ SEM, n=3

Figure 10: Representative Western Blot of Cardiac β_2 Adrenergic Receptors in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Expression of Cardiac β_2 Adrenergic Receptors in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding significantly increased the expression of the receptors which was normalized by chronic phentolamine. Values expressed as mean $\pm$ SEM, n=3, *p<0.05 vs, C, CP, and FP

Figure 11: Representative Western Blot of Cardiac β_3 Adrenergic Receptors in control and in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Expression of Cardiac β_3 Adrenergic Receptors in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding produced no effect on the receptor expressions. Phentolamine significantly increased the receptor expressions only in the control but not the fructose-fed rats. Values expressed as mean $\pm$ SEM, n=3, *p<0.05 vs, C, F, and FP.

Figure 12: Representative Western Blot of Renal β_2 adrenergic receptors in control and in Control and Fructose-Fed Rats following 13 Weeks of Study and Effects of Phentolamine on the Expression of Renal β_2 Adrenergic Receptors in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding significantly decreased the expression of the receptors which was normalized by chronic phentolamine. Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs, C, CP, and FP.

Figure 13: Representative Western Blot of Renal β_3 Adrenergic Receptors in Control and Fructose-Fed Rats following 13 Weeks of Study and Effects of Phentolamine on the Expression of Renal β_3 Adrenergic Receptors in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding significantly increased the expressions of the receptors. Phentolamine treatment significantly increased the receptor expressions only in the fructose-fed but not the control rats. Values expressed as mean $\pm$ SEM, $n=6$, * $p<0.05$ vs, C, CP, and F, # $p<0.05$ vs CP and CP.

Figure 14: Representative Western Blot of Renal Phospho- and Total PKA in Control and Fructose-Fed Rats following 13 Weeks of Study and Effects of Phentolamine on the Renal PKA Phosphorylation in Control and Fructose-Fed Rats following 13 Weeks of Study. PKA phosphorylation levels were significantly increased by both phentolamine treatment and fructose feeding. The combination of fructose feeding and phentolamine treatment dampened the effect of phentolamine alone. Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs C and CP, #p<0.05 vs C, F and FP.

Figure 15: Effect of Phentolamine on Plasma 8-Isoprostane in Control and Fructose-Fed Rats following 13 Weeks of Study. Neither fructose feeding nor phentolamine treatment had any effect on plasma 8-isoprostane levels. Values expressed as mean $\pm$ SEM, n=6.

Figure 16: Effects of Phentolamine on Plasma Total Nitrate/Nitrite in Control and Fructose-Fed Rats following 13 Weeks of Study. Fructose feeding significantly increased plasma angiotensin II levels which phentolamine treatment produced no effects on. Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs C

Figure 17a: Representative Western Blot of Cardiac eNOS in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Expression of Cardiac eNOS in Control and Fructose-Fed Rats following 13 Weeks of Study. eNOS expression in the heart was significantly increased in the fructose-fed rats treated with phentolamine. Values expressed as mean $\pm$ SEM, n=3, *p<0.05 vs C, CP and F

Figure 17b: Representative Western Blot of Cardiac iNOS in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Expression of Cardiac iNOS in Control and Fructose-Fed Rats following 13 Weeks of Study. Phentolamine only increased in the iNOS expression in the control rats receiving the treatment. This change was only significant if compared with the iNOS expression in control rats. Values expressed as mean $\pm$ SEM, n=3, [#]p<0.05 vs C.

Figure 18a: Representative Western Blot of Renal eNOS in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Expression of Renal eNOS in Control and Fructose-Fed Rats following 13 Weeks of Study. Neither fructose feeding nor phentolamine treatment had any effects on eNOS expressions in the kidneys. Values expressed as mean $\pm$ SEM, n=6.

Figure 18b: Representative Western Blot of Renal iNOS in Control and Fructose-Fed Rats following 13 Weeks of Study, and Effects of Phentolamine on the Expression of Renal iNOS in Control and Fructose-Fed Rats following 13 Weeks of Study. Both fructose feeding and phentolamine treatment decreased the iNOS expressions in the kidneys. There was no additive effect with the combination of both interventions. Values expressed as mean $\pm$ SEM, n=6, *p<0.05 vs C.

4. Discussion

4.1 General Overview

As a cluster of various risk factors including obesity, insulin resistance, hypertriglyceridemia, and hypertension, metabolic syndrome has been extensively studied in the past two decades. It has been suggested that individuals suffering from this syndrome have a much greater chance than others to develop cardiovascular diseases (Isomaa *et al.*, 2001; Lakka *et al.*, 2002; Malik *et al.*, 2004; Hunt *et al.*, 2004) and Type 2 diabetes (Hanson *et al.*, 2002; Lorenzo *et al.*, 2003; Laaksonen *et al.*, 2004). Research has been focused on seeking an ideal treatment for hypertension which can also alleviate metabolic disturbances such as hyperinsulinemia, hypertriglyceridemia, and increased free fatty acids (Alzamendi *et al.*, 2009). A variety of anti-hypertensive drugs developed for the treatment of essential hypertension have been found to control fructose-induced hypertension while remaining ineffective against the metabolic syndrome. Certain anti-hypertensive agents such as thiazide diuretics and conventional β blockers have been found to enhance insulin resistance in the patients with metabolic syndrome and increase the risk of new-onset diabetes (Lithell, 1991; Messerli *et al.*, 2004; Sarafids & Bakris, 2006a&b). In addition, β blockers have been found to promote weight gain (Messerli *et al.*, 2004) which can exacerbate the condition of the patients with this syndrome as abdominal obesity appears to be an important element in the development of adverse metabolic effects associated with antihypertensive medications (Cooper-DeHoff *et al.*, 2010).

Previous studies have shown that selective α_1 adrenergic receptor antagonists such as bunazosin (Kamide *et al.*, 2002) and prazosin (Tran, 2009) are able to prevent the increase in blood pressure in fructose-fed rats without affecting hyperinsulinemia and hypertriglyceridemia. Interestingly, yohimbine, a selective α_2 adrenergic receptor antagonist, has been found to produce no effects on the blood pressures in the fructose-fed rats (Mayer *et al.*, 2006) while a selective α_2 adrenergic receptor agonist, clonidine, prevented the blood pressure elevation induced by fructose feeding (Hwang IS *et al.*, 1987). To extend our understanding on the role of α adrenergic receptors in the development of elevated blood pressure in fructose-fed rats, a prevention study was designed to establish a pharmacological profile of phentolamine, a non-selective α adrenergic receptor antagonist, in fructose-fed rats. The objectives of the study, performed in both control and fructose-fed rats, were:

1. To investigate the ability of phentolamine to prevent the increase in blood pressure induced by fructose feeding and its possible effects on plasma levels of cholesterol, triglyceride, insulin and glucose levels.
2. To determine the effect of phentolamine on the plasma noradrenaline level.
3. To examine whether phentolamine would increase the plasma angiotensin II level which has been suggested to be linked to increased insulin resistance.
4. To investigate the effects of phentolamine on β adrenergic receptors expression and on β_1 adrenergic receptor-mediated apoptosis of cardiac myocytes.
5. Investigate the effects of phentolamine and fructose on the state of oxidative stress.

4.2 Fructose-Fed Rats: A Rodent Model of Metabolic Syndrome

In the past two decades, various studies have demonstrated that fructose feeding in rats induces hyperinsulinemia/insulin resistance, hypertriglyceridemia, and hypertension without affecting plasma glucose levels (Hwang *et al.*, 1987; Verma *et al.*, 1994; Katakam *et al.*, 1998; Galipeau *et al.*, 2001; Vasudevan *et al.*, 2005). It has been suggested that insulin resistance precedes hypertension (Katakam *et al.*, 1998; Tran *et al.*, 2009a), which is partially supported by the evidence that the use of metformin in preventing hyperinsulinemia and insulin resistance is also able to prevent the increase in systolic blood pressure (Verma *et al.*, 1994; Verma *et al.*, 2000; Vasudevan, 2009). Similarly, vanadium compounds which have been shown to enhance insulin sensitivity are able to prevent the blood pressure elevation induced by fructose feeding (Bhanot *et al.*, 1994; Bhanot *et al.*, 1995). Our results show that plasma insulin and triglyceride levels were significantly elevated as early as 4 weeks while systolic blood pressure was not increased until later, in this case 13 weeks of fructose feeding (**Figure 1, Table 1**), which agrees with the current literature. For the first time in our laboratory, we measured plasma cholesterol levels to provide additional information as to how fructose feeding affects plasma lipid contents besides inducing hypertriglyceridemia, since a recent study has found an increase in plasma ester cholesterol caused by fructose feeding (Nandhini *et al.*, 2002). Plasma cholesterol levels were shown to be significantly elevated by fructose feeding as early as 4 weeks (**Table 1**). A similar observation has been recorded by other researchers (Shahraki *et al.*, 2011). In the glycolytic cycle in the liver, fructose bypasses the step mediated by phosphofructokinase which is the rate-limiting enzyme in glucose metabolism (Elliot *et al.*, 2002; Vasdev *et al.*, 2004; Tran *et al.*, 2009a, Figure 19). It

Figure 19: Hepatic Metabolism of Fructose and Glucose (Tran *et al.*, 2009a)

has been suggested that high concentrations of fructose have shown to promote *de novo* lipogenesis (Schwarz *et al.*, 1993; Schwarz *et al.*, 1994), since fructose metabolism is not negatively regulated by ATP and citrate via phosphofructokinase allowing it to behave as a fruitful source of both glycerol-3-phosphate and acetyl-CoA for hepatic lipogenesis (**Figure 19**). This may partially explain the increases in plasma triglyceride and cholesterol levels (**Table 1**). The increases in plasma lipids have also been attributed to the fructose-induced secretion of apolipoproteins (mostly as B48-containing lipoprotein particles) which can enhance intestinal synthesis of free cholesterol, cholesterol ester, and triglyceride (Guo *et al.*, 2005). Vasdev *et al.* (2004) believe that in the liver fructose is converted to intermediate metabolites, such as glyceraldehyde, glyceraldehydes-3-phosphate and dihydroxyacetone phosphate, depleting cells of inorganic phosphate leading to inhibition of phosphofructokinase and subsequent decreased glucose

metabolism in the glycolytic pathway. This can lead to an increase in glucose metabolism through the polyol pathway (Dunlop, 2000, **Figure 20**) resulting in the formation of more fructose and a subsequent build-up of its intermediate metabolites. These intermediate metabolites can be further metabolized to methylglyoxal which inhibits the glycolytic pathway and subsequently leads to an increased production of aldehydes (Vasdev *et al.* 2004). Therefore, a high fructose diet can promote the production of excessive metabolic aldehydes. Reactive metabolic aldehydes are believed to be involved in the development of fructose-induced hypertension as an increase in tissue aldehydes has been found in fructose-fed rats (Vasdev *et al.*, 1998) and spontaneous hypertensive rats (Vasdev *et al.*, 1996), both of which are models of insulin resistance and hypertension. Therefore, fructose-induced hyperlipidemia may play an important role in the development of blood pressure elevation via increasing the production of reactive tissue aldehydes, in which methylglyoxal is believed to be an important mediator (Vasdev & Stuckless, 2010).

Figure 20: Polyol Pathway of Glucose Metabolism (Dunlop, 2000)

In contrast to the current findings, plasma glucose levels were found to be slightly but significantly elevated by fructose feeding as early as 4 weeks (**Table 1**). Similarly, two separate studies in our laboratory have previously demonstrated increased plasma glucose levels following 6 weeks of fructose feeding (Tran *et al.*, 2009b). Another prevention study on fructose-induced metabolic syndrome using a 10% (w/v) fructose solution (equivalent to a diet containing 48–57% fructose (Dai & McNeill, 1995) has also demonstrated similar results in 8 weeks (Shahraki *et al.*, 2011). The elevating effect of fructose feeding on plasma glucose was also observed in the glucose AUC in OGTT (Figure 2b), which has been reported early in two different series of experiments in our laboratory as well (Tran, 2009; Vasudevan, 2009). As discussed above, fructose can decrease glucose metabolism through the glycolytic pathway by inhibiting phosphofructokinase (Vasdev *et al.* 2004). It might be intuitive to speculate an increase in plasma glucose level upon chronic fructose feeding. However, Vasdev *et al.* (2004) also propose that high fructose can shunt glucose metabolism towards the polyol pathway which suggests the overall glucose metabolism might not be significantly decreased. Moreover, overwhelming evidence from the past two decades suggests no change in plasma glucose level by fructose feeding in animal models (Hwang *et al.*, 1987; Verma *et al.*, 1994; Katakam *et al.*, 1998; Galipeau *et al.*, 2001; Vasudevan *et al.*, 2005). It is difficult to explain the phenomenon of increased plasma glucose in this and other recent studies using the same animal model. However, the increase in plasma glucose in these studies recording such an increase was relatively small.

4.3 Effects of Phentolamine in Fructose-Induced Metabolic Disturbances and Elevated Blood Pressure

Therapeutic agents used in the treatment of essential hypertension, including thiazide diuretics, β blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB) and calcium channel blockers (CCB), have been used in treating hypertension in patients with metabolic syndrome. Clinical trial results suggest that these treatment options are beneficial in reducing hypertension and cardiovascular complications associated with the syndrome (Whaley-Connell *et al.*, 2005; Israili *et al.*, 2007; Makaryus *et al.*, 2009). These pharmacological agents focus on modifying the systems involved in increasing blood pressure, assuming certain dysfunctions of these systems. The antihypertensive and lipid-lowering treatment to prevent heart attack trial (ALLHAT) in 2002 and other recent clinical trials (Brown *et al.*, 2000; Black *et al.*, 2003) have proposed thiazide diuretics as the basis of antihypertensive therapy as they are comparatively effective in controlling blood pressure and reducing other cardiovascular complications while being more affordable than other antihypertensives. However, they have been suggested to worsen insulin resistance and increase the risk of developing Type 2 diabetes as compared to (ACE) inhibitors, ARBs, and CCBs. Similar to thiazide diuretics, β blockers have also been suggested to increase the risk of Type 2 diabetes (Palaniappan *et al.*, 2004).

Alpha adrenergic receptor antagonists (or α blockers) are also potential treatment candidates as they can produce antihypertensive effects by decreasing α adrenergic receptor-mediated vasoconstriction leading to a dilation of both resistance and

capacitance blood vessels (Katzung, 2007). Producing less reflex tachycardia than non-selective α blockers, selective α_1 blockers, such as prazosin and doxazosin, are preferred in the treatment of essential hypertension (Katzung, 2007). A recent clinical trial (ALLHAT, 2000) has compared the treatment outcomes of doxazosin and chlorthalidone (a thiazide diuretic), and suggested that the former is equally as effective as the latter in decreasing blood pressure and reducing the risk of other cardiovascular diseases in high-risk hypertensive patients. Bunazosin, another selective α_1 blocker, has been found to prevent the increase in systolic blood pressure in the fructose-fed rats (Kamide *et al.*, 2002). A recent study in our laboratory has demonstrated the effect of prazosin on attenuating the blood pressure elevation induced by fructose feeding (Tran 2009). In the present study, our results suggest phentolamine successfully prevented the fructose-induced elevation of systolic blood pressure in male Wistar rats (**Figure 1**). The anti-hypertensive effect of phentolamine can be largely attributed to the blockade of vascular α_1 receptor activities as similar results have been produced using selective α_1 blockers such as bunazosin (Kamide *et al.*, 2002) and prazosin (Tran 2009), suggesting that the development of elevated blood pressure may be attributed to an overactive sympathetic nervous system triggered by fructose feeding, which is in agreement with previous studies (Verma *et al.*, 1999; Mayer *et al.*, 2006; Mayer *et al.*, 2008). The terms “elevated blood pressure” and “hypertension” have been used interchangeably for the increase in systolic blood pressure in most of the studies using fructose-fed rats. However, a closer inspection of the data in most of these studies has revealed that the elevated systolic blood pressures seldom reach over 140 mmHg (Galipeau *et al.*, 2001; Kamide *et al.*, 2002; Vasdev *et al.* 2004; Tran *et al.*, 2009b), similar to what we observed in this study (**Figure**

1). Nevertheless, a minimum difference of 15 mmHg has always been seen all the studies conducted in our laboratory and by other researchers. In order to accurately relate the results of animal studies to their applications in human trials, we decided to use the term “elevated blood pressure” instead of “hypertension” to describe the increase in systolic blood pressure induced by fructose feeding throughout the discussion.

Similar to bunazosin (Kamide *et al.*, 2002) and prazosin (Tran 2009), phentolamine did not produce any significant changes in the fructose-induced elevated levels of plasma insulin with or without a high glucose challenge (**Table 1 & Figure 2a**). Furthermore, the decreased insulin sensitivity induced by fructose feeding was not prevented by phentolamine (**Figure 3**). These results appear to be puzzling since bunazosin (Suzuki *et al.*, 1992; Eriksson *et al.*, 1996) and prazosin (Swislocki *et al.*, 1989) were found to improve insulin sensitivity in hypertensive patients. Previous studies on fructose-fed rats have found moxonidine (Rosen *et al.*, 1997) and rilmenidine (Penicaud *et al.*, 1998), agonists of imidazoline receptors which lower blood pressure via central inhibition of the sympathetic nervous system (Head & Mayorov, 2006), are able to prevent insulin resistance in animal models. The inability of phentolamine and other α blockers to prevent insulin resistance in the fructose-fed rats suggests the insulin sensitivity of this particular animal model may not respond to the effect of α blockers or that the dose or treatment regimen of these agents used in these particular studies might not be sufficient to exert a significant effect on insulin resistance.

Interestingly, phentolamine produced a small but significant increase in plasma glucose levels in both the control and fructose-fed rats after 4 weeks of the study;

however, this effect was not observed at a later stage of the study (**Table 1**). A previous study in our laboratory also recorded similar findings following 9 weeks of prazosin treatment (Tran 2009). In both of these studies, such an effect was not reflected on the plasma glucose levels in an oral glucose tolerance test (OGTT, **Figure 2b**), which is consistent with previous findings from a study of bunazosin in the same animal model (Kamide *et al.*, 2002). It is unclear whether this might suggest an initial effect of α blockers on plasma glucose levels which disappears eventually as the duration of treatment exceeds a certain period of time. This speculation is unlikely since a recent study has found that long-term (12 months) treatment with doxazosin (α_1 blocker) in patients with Type 2 diabetes and hypertension significantly lowers fasting plasma glucose (Derosa *et al.*, 2005).

On the other hand, phentolamine produced no effect on plasma triglyceride levels (**Table 1**), which is similar to what was previously found when investigating the metabolic effects of prazosin in the same animal model (Tran 2009). An early study investigating the effect of doxazosin on lipoprotein lipases has also found no effect of doxazosin on plasma triglyceride level in cholesterol-fed rats (Jansen & Baggen, 1987). Interestingly, the results from human clinical trials have been mixed. Prazosin treatment has been found to produce no effect on plasma triglyceride level in one trial (Ferrara *et al.*, 1986) but a decrease in another (Swislocki *et al.*, 1989) in hypertensive patients. On the other hand, doxazosin therapy has been suggested to decrease plasma triglyceride level in hypertensive patients afflicted with either metabolic syndrome (Dell'Omo *et al.*, 2005) or Type 2 diabetes (Derosa *et al.*, 2005).

Similar to the results from an early study of prazosin in normal rats (Dall'Aglia *et al.*, 1989), phentolamine had no effect on plasma cholesterol levels in either control or fructose-fed rats (**Table 1**). Likewise, studies with doxazosin have showed no effect on plasma cholesterol levels in either normal or cholesterol-fed rats (Ontko & Woodside, 1989; Jansen & Baggen, 1987). However, evidence from clinic trials suggests that both prazosin (Ferrara *et al.*, 1986; Swislocki *et al.*, 1989) and doxazosin (Dell'Omo *et al.*, 2005; Derosa *et al.*, 2005) reduce total cholesterol level in hypertensive patients. Overall, the evidence suggests α blockers decrease plasma triglyceride and cholesterol in humans but not in rats.

4.4 Effect of Fructose Feeding and Phentolamine on Plasma Noradrenaline Level

Current literature has suggested that insulin resistance and the subsequent compensatory hyperinsulinemia induced by fructose feeding can lead to an increase activation of the sympathetic nervous system, resulting in an increase release of catecholamines (Tran *et al.*, 2009a; Abdulla *et al.*, 2011). Tran (2009) suggests that insulin has a direct stimulatory effect on the release of noradrenaline from sympathetic nerve endings, since both animals (Liang *et al.*, 1982; Tomiyama *et al.*, 1992) and humans (Anderson *et al.*, 1991; Berne *et al.*, 1992; Lembo *et al.*, 1992; Tack *et al.*, 1998) demonstrate elevated plasma noradrenaline levels following insulin administration. Recent studies in our laboratory have produced mixed results: plasma noradrenaline levels in male Wistar rats were significantly increased in one study (Tran 2009) but remained unchanged (Tran *et al.*, 2009b) in another in response to chronic fructose

feeding, although plasma insulin was significantly increased by fructose feeding in both studies. The present study demonstrates no effect of fructose on plasma noradrenaline level (**Figure 4**), even though the duration of fructose feeding was longer than previous studies (13 weeks vs 9 weeks). One challenge in analysis detecting a statistical significant difference in these experiments is that there is a large variation in individual values within each study groups. If the “insulin-stimulated noradrenaline release” hypothesis is true, plasma noradrenaline level should be increased in the face of chronic fructose feeding since the latter induces hyperinsulinemia. However, we are not certain about the exact role of hyperinsulinemia in affecting the plasma noradrenaline level and how the duration of fructose feeding and the extent of hyperinsulinemia might influence the end result. A study on bunazosin treatment in fructose-fed rats reveals an insignificant increase in urinary noradrenaline excretion induced by fructose-feeding (Kamide *et al.*, 2002). Since the plasma levels of noradrenaline are determined by its synthesis, release, metabolism and excretion, an increase in urine excretion, similar to what Kamide *et al.* suggest, might have occurred in this study and contributed to the absence of any change. It should be noted that noradrenaline was only measured at one time point in the study.

According to the current knowledge of phentolamine’s mechanism of action, phentolamine centrally blocks the inhibitory action of α_2 adrenergic receptors at presynaptic nerve terminal thereby disinhibiting the release of noradrenaline (Katzung 2007). Therefore, one would expect an increase in the plasma noradrenaline level in the control rats treated with phentolamine while the possible interaction of fructose feeding and phentolamine might make the effects of phentolamine in the fructose-fed rats difficult to predict. However, phentolamine produced no significant effect on the plasma

noradrenaline level in either the control or fructose-fed rats (**Figure 4**). It is possible that the dose used might not be high enough to cause a significant central blockade of α_2 adrenoceptor activity. It is also possible that chronic stimulation of α_2 adrenergic receptors has induced a receptor up-regulatory mechanism which counteracted the effect of phentolamine. It should be noted that noradrenaline was measured at only the termination time point. On the other hand, the absence of change in plasma noradrenaline level in the fructose-fed rats suggests a possible defect or dysfunction of α_2 receptors which renders their inability to respond to receptor blockade by any exogenous antagonist. This speculation is partially supported by evidence from a recent study which reveals a decreased activity of hypothalamic α_2 adrenoceptors in the fructose-fed rats (Mayer *et al.*, 2006). A recent study in our laboratory also provides indirect evidence to support such an assumption, as prazosin, a selective α_1 blocker which can indirectly enhance the interaction between α_2 adrenergic receptors and endogenous agonists, has been found to significantly decrease plasma noradrenaline levels in the fructose-fed rats (Tran 2009).

4.5 Effects of Fructose Feeding and Phentolamine on Plasma Angiotensin II

Plasma angiotensin II levels were significantly elevated following 13 weeks of fructose feeding (**Figure 5**). This is consistent with the previous findings from recent studies conducted in our and others' laboratories (Iyer *et al.*, 1996; Tran *et al.*, 2009b; Tran 2009). Our results and previous findings suggest that angiotensin II might play an important role in inducing the blood pressure elevation in the fructose fed animals since it is a potent vasoconstrictor. This assumption is supported by the existing evidence that

inhibition of the renin-angiotensin system with ACE inhibitors or ARBs prevents the fructose-induced elevated blood pressure (Iyer & Katovich, 1994; Navarro-Cid *et al.*, 1995; Higashiura *et al.*, 2000; Kamide *et al.*, 2002). Currently, we have limited knowledge about the cause of such a significant increase in plasma angiotensin II in fructose-fed rats. It is interesting to see no change in plasma noradrenaline in the presence of elevated angiotensin II in the circulation, since angiotensin II stimulates the release of noradrenaline from both sympathetic nerve terminal and adrenal medulla (Reid, 1992). Along with the decreased insulin sensitivity seen in the fructose fed animals (Figure 3), the observed increase in plasma angiotensin II levels in the fructose fed rats provides supportive evidence to the current literature which suggests angiotensin II is an important mediator of the development of insulin resistance (Iyer & Katovich, 1996a; Higashiura *et al.*, 2000; Uchida *et al.*, 2002; Tran *et al.*, 2009a; Olivares-Reyes *et al.*, 2009; Zhou *et al.*, 2009).

The observation that phentolamine had no effect on the plasma level of angiotensin II (**Figure 5**) suggests that phentolamine might have no or minimum impact on the production of renin in the kidneys, although it was hypothesized that phentolamine might increase the production of renin via indirectly enhanced activation of renal β_1 adrenergic receptors thereby increasing the release of angiotensin II into the circulation. To further confirm whether phentolamine had any effects on renal β_1 adrenergic receptor expression, we attempted to measure the receptor expression using Western Blotting. However, we were unable to achieve the goal due to technical difficulties (the primary antibodies did not work). Since renal β_1 adrenergic receptor activation leads to increased production of cyclic adenosine monophosphate (cAMP) and subsequent activation of

protein kinase A (PKA) (Boron & Boulpaep, 2003), we decided to evaluate renal β_1 adrenergic receptor activity by measuring PKA activation in the kidneys. PKA phosphorylation, which represents the activation of this signaling molecule, was significantly increased by phentolamine but decreased by fructose feeding (**Figure 14**). It is difficult to make any assumption on renal β_1 adrenergic receptor activity based on this result, as PKA activation is also mediated by activation of other proteins, such as dopamine receptor, glucagon receptor, etc (Boron & Boulpaep, 2003).

The observed fructose-induced elevation of plasma angiotensin II and recent findings of an increased expression of angiotensin II receptor type I (AT₁) in the hearts of fructose-fed rats (Iyer *et al.*, 1996; Nyby *et al.*, 2007) inspired us to investigate the possible changes in cardiac AT₁ expression. The result suggests neither fructose feeding nor phentolamine caused any significant change in AT₁ expression in the hearts (**Figure 6**). Recent evidence suggests that endothelial AT₁ receptors play an important role in contributing to the development of fructose-induced blood pressure elevation (Hsieh *et al.*, 2005; Nyby *et al.*, 2007). Furthermore, AT₁ receptor expression has also been found to be increased in aorta (Nyby *et al.*, 2007) and adipose tissues (Giacchetti *et al.*, 2000). Therefore, it is possible that AT₁ receptor expressions in the vasculature and adipose tissues might have been affected by fructose feeding or phentolamine. AT₁ receptors are also expressed in the kidneys (Bottari *et al.*, 1993) and adrenal glands (Aquitlera and Marusic, 1971), which stimulate renal sodium and water reabsorption when activated. Fructose feeding or phentolamine might also have an impact on the receptor expression in these tissues. An examination of the effects of fructose and phentolamine on AT₁ receptor expression in various tissues is merited in future investigations.

4.6 Effects of Phentolamine on β_1 Receptor-Mediated Apoptosis in the Heart and β Adrenergic Receptor Expressions

Phentolamine is known to cause tachycardia mediated by the baroreflex and/or indirect β_1 adrenergic receptor activation; it might induce arrhythmias if used in high doses (Katzung 2007). Since current evidence has suggested that chronic stimulation of β_1 adrenergic receptors in the heart appears linked to an increase in apoptotic cardiomyocytes (Communal *et al.*, 1999; Zaugg *et al.*, 2000; Li *et al.*, 2007) and chronic use of phentolamine can potentially lead to overstimulation of cardiac β_1 adrenergic receptors, cardiac tissue apoptosis was chosen as a marker to evaluate the extent of possible cardiac toxicity. The Hoechst staining technique is designed to examine cell morphology and detect cells that undergo the process of apoptosis featuring pyknotic nuclei, chromatin condensation and nuclear fragmentation (Simbulan-Rosenthal *et al.*, 1998). It has been used to evaluate apoptosis of cardiomyocytes (Malhotra & Brosius III, 1999). Examination of cardiac tissue morphology using Hoechst staining revealed no significant increase in apoptosis in the phentolamine-treated animals (**Figure 7**). This might suggest that the extent to which phentolamine indirectly enhanced the activation of cardiac β_1 adrenergic receptors was not pathological. Interestingly, fructose feeding did not have a significant detrimental impact on cardiac tissues (**Figure 7**), although fructose feeding has been found to promote cardiac pathology such as left ventricular hypertrophy (LVH) in which angiotensin II is suggested to play an important role (Kobayashi *et al.*, 1993; Kamide *et al.*, 2002). *In vitro* studies have found that angiotensin II can induce apoptosis of cardiomyocytes in both neonatal and adult rats (Cigola *et al.*, 1997; Kajstura *et al.*, 1997). It is quite surprising that the presence of a significant increase in plasma

angiotensin II (**Figure 5**) did not promote cardiac tissue apoptosis in this study. Perhaps this is partially due to the unchanged expression of AT₁ receptors in the heart (**Figure 6**). It has been suggested that direct inhibition of local renin-angiotensin system by an AT₁ receptor antagonist prevents the development of LVH (Kamide *et al.*, 2002). Therefore, the fact that AT₁ receptor expression was not altered by fructose feeding can in part explain the absence of increased apoptosis at the presence of a significant increase in plasma angiotensin. In addition, the absence of changes in plasma noradrenaline levels might also contribute to the lack of changes in apoptosis, since noradrenaline has been proposed to stimulate apoptosis of cardiomyocytes (Communal *et al.*, 1998).

To examine the effects of phentolamine on cardiac β receptors, the expression of the dominant β adrenergic receptors (β_1) was measured. Receptor activity was indirectly evaluated by measuring the phosphorylation of two downstream effectors, PKA and phospholamban (Xiao *et al.*, 2004). Unfortunately, due to the same technical difficulty we encountered when measuring the expression of β_1 adrenergic receptors in the kidneys, we were unable to measure β_1 adrenergic receptor expression in the heart using Western Blotting. The level of PKA phosphorylation was similar among all the animal groups (Figure 8). Based on the current knowledge of the classic “ β_1 adrenergic receptor-Gs-adnylyl cyclase (AC)-cAMP-PKA” signaling pathway (Xiao *et al.*, 2004; Xiao *et al.*, 2006), this result suggests that the level of cardiac cAMP was unchanged by either fructose or phentolamine treatment. However, this observation might not be a good indicator of β_1 adrenergic receptor activity, since cardiac cAMP levels are also regulated by other mechanisms, such as β_2 adrenergic receptor activation (Xiao *et al.*, 2004). Therefore, phospholamban was chosen as another means to evaluate β_1 adrenergic

receptor activities, since this small cardiac protein is activated via phosphorylation at its serine 16 (Vittone *et al.*, 1990; Vittone *et al.*, 1996) and threonine 17 amino acid (Hagemann *et al.*, 2000) by PKA and Ca^{2+} /calmodulin kinase II (CaMKII), respectively, upon β_1 adrenergic receptor activation in the heart. The phosphorylation levels at both serine 16 and threonine 17 were decreased significantly in both of the phentolamine treated groups (**Figure 9**), suggesting that chronic administration of phentolamine might have desensitized the receptors leading to a decrease in β_1 adrenergic receptor activity. The decreased phosphorylation at threonine 17 suggests a decrease in CaMKII activity. This may partially explain the absence of the predicted increase in cardiac apoptosis, as current evidence suggests a linkage between overstimulation of β_1 adrenergic receptors and cardiomyocyte apoptosis through activation of CaMKII (Zhu *et al.*, 2003). We attempted to measure the activity of CaMKII by measuring its phosphorylation level as CaMKII undergoes autophosphorylation to become activated (Yang & Schulman, 1999). However, our attempt in using Western Blotting failed due to a technical difficulty with the primary phospho- and total CaMKII antibodies. However, since previous studies suggest that the phosphorylation at serine 16 of PLN is a prerequisite for that at threonine 17 (Luo *et al.*, 1998; Mattiazzi *et al.*, 2005), the decreased phosphorylation at threonine 17 might be a consequence of that at serine 16. The phosphorylation level of phospholamban is also negatively regulated by protein phosphatase-1 (PP1) which is the main phosphatase that dephosphorylates phospholamban (MacDougall *et al.*, 1991; Steenaart *et al.*, 1992). The decreased phosphorylation at both serine 16 and threonine 17 might have been caused by an increase in PP1 activity.

Stimulation of β_2 adrenergic receptors appears to provide cardiac myocytes with protection from apoptosis (Communal *et al.*, 1999; Zaugg *et al.*, 2000; Zhu *et al.*, 2001). A significant increase in cardiac β_2 adrenergic receptor expression was observed in the fructose fed rats untreated with phentolamine (**Figure 10**). This may indicate a naturally evolved defense mechanism in which β_2 adrenergic receptors were involved in protecting the heart from the disturbances caused by fructose feeding. Although the exact anti-apoptotic mechanism of β_2 adrenergic receptors remains to be elucidated, recent studies suggest that β_2 adrenergic receptor-coupled G_i -proteins functionally localize concurrent β_1 adrenergic receptor-coupled G_s -mediated cAMP signaling cascade to weaken the propagation of downstream signaling to apoptosis, and mediate the $G_{\beta\gamma}$ -PI3K-Akt pathway to signal cell survival (**Figure 21**, Xiao 2001). Our result suggests that β_2 adrenergic receptor expression might have been increased to favor the G_i -coupling cascade in order to battle the cardiac detrimental effects caused by fructose-induced metabolic disturbances. This may partially contribute to the absence of a significant increase in cardiac apoptosis in the face of chronic fructose feeding (**Figure 7**). Phentolamine normalized β_2 adrenergic receptor expression in the fructose-fed rats, which is probably due to an indirect effect of phentolamine, since phentolamine slightly increased β_2 adrenergic receptor expression in the control animals (**Figure 10**). If the increased receptor expression in the fructose-fed rats was an adaptive mechanism of the heart in response to the fructose-induced elevated blood pressure, the absence of quantitative changes in β_2 adrenergic receptors in fructose-fed rats treated with phentolamine may well support such a hypothesis.

Figure 21: Signaling Pathway of β_2 Adrenoceptor-Dual Coupling to G_s and G_i Proteins in Cardiac Myocytes. Note: AR-adrenoceptor; G_s and G_i are stimulatory and inhibitor G proteins, respectively; PI3K-phosphoinositide 3-kinase; Akt-Protein Kinase B; PLB-phospholamban; TnI-Troponin I; PTX, pertussis toxin; β ARK-ct, a peptide inhibitor of $G_{\beta\gamma}$ signaling; LY, a PI3K inhibitor (Xiao, 2001)

Experimentally induced diabetes using streptozotocin has been found to increase the expression of β_3 adrenergic receptors in the rat hearts (Dincer *et al.*, 2001). On the other hand, β_3 adrenergic receptor expression has been found to increase in response to chronic β_1 or β_3 -adrenergic stimulation in rat cardiomyocytes (Ufer & Germack, 2009). Since fructose-induced metabolic syndrome promotes the onset of diabetes and long-term use of phentolamine can indirectly cause chronic activation of all β adrenergic receptors via increasing the interaction between noradrenaline and these receptors, we were curious whether fructose feeding or phentolamine had any effect on the expression of β_3 adrenergic receptors in the heart. The increase in cardiac β_3 adrenergic receptor expression in the control rats treated with phentolamine suggests that phentolamine might have caused a receptor up-regulation in these animals (**Figure 11**). This up-regulation

might be attributed to an enhanced interaction between β adrenergic receptors and noradrenaline by phentolamine, since the findings in recent studies suggests chronic β -adrenergic stimulation with either selective β_1 or β_3 (Ufer & Germack, 2009), or non-selective agonists (Germack & Dickenson, 2006) can up-regulate β_3 adrenergic receptor expression. On the other hand, fructose feeding had no effects on these receptors (**Figure 10**). The absence of an increase in the fructose-fed rats treated with phentolamine indicates that fructose feeding might have impaired the ability of these receptors to respond to phentolamine.

The expressions of β_2 and β_3 adrenergic receptors in the kidneys were also measured. Fructose feeding caused a decrease in β_2 adrenergic receptor expression which was normalized by phentolamine treatment (**Figure 12**). In contrast, fructose feeding had a minimum effect on β_3 adrenergic receptor expression. However, phentolamine treatment produced an increase in the β_3 adrenergic receptor expression in the treated animals; such an increase was enhanced to a significant level by fructose feeding (**Figure 13**). Since the exact physiological functions of β_2 and β_3 adrenergic receptors in the kidney remain to be uncovered, currently we do not know the physiological significance of these changes.

Overall, the Western blot data of receptor proteins of interest suggest that the difference in changes in β adrenergic receptor subtype expressions in response to fructose feeding and phentolamine treatment is tissue specific. It should be noted that the data presented in **Figure 6-11** are only considered as preliminary since the N number was 3 and there were technical difficulties associated with the experiments. It is recommended that the experiments be repeated in the future investigations.

4.7 Effects of Phentolamine on Oxidative Stress

Increased oxidative stress has been suggested to be a contributor of the pathological development in fructose fed rats (Tran *et al.*, 2009a). It has been proposed to play a role in the development of insulin resistance (Eriksson, 2007) and elevated blood pressure (de Champlain *et al.*, 2004). Plasma 8-isoprostane was used as an indicator of oxidative stress in this study, since it has been suggested to be a marker of free radical-induced injury in certain oxidative stress related diseases (Greco *et al.*, 2000; Praticò *et al.*, 2001) and it has been found to be elevated by fructose feeding (Nyby *et al.*, 2007). Plasma levels of 8-isoprostane were similar among the four groups (**Figure 15**), suggesting neither fructose feeding nor phentolamine had any effect on this particular indicator of oxidative stress. However, 8-isoprostane might not present the complete picture. Oxidative stress and abnormal nitric oxide (NO) activity have been proposed to be a link to the development of elevated blood pressure (Wilcox, 2005). In addition, oxidative stress has been linked to a decrease in NO production (Tran *et al.*, 2009a). Therefore, plasma total nitrate/nitrite was chosen as a second marker to evaluate the state of oxidative stress since the sum of nitrate and nitrite reflects the total production of NO in the circulation (Sun *et al.*, 2003). The fact that the levels of plasma total nitrate/nitrite were 7 fold higher in the fructose fed animals (**Figure 16**) suggests the total amount of NO present in the circulation was significantly elevated by fructose feeding. Similarly, plasma total nitrate/nitrite was also found to be significantly elevated following 8 weeks of fructose feeding in Sprague Dawley rats (Wang *et al.*, 2007). This might appear surprising as plasma NO level has been suggested to normally be diminished in fructose fed rats as a result of either decreased activity of endothelial nitric oxide synthase (eNOS)

or impaired release of NO, a sign of endothelial dysfunction (Tran *et al.*, 2009a). However, increased expressions of eNOS and inducible nitric oxide synthase (iNOS) in the kidneys have been found in the spontaneous hypertensive rats (SHRs), an animal model of hypertension (Welch *et al.*, 1999; Kumar *et al.*, 2005). Kumar *et al* (2005) suggest that excessive NO production causes nitritative damage and oxidative stress. Due to the contradiction in existing evidence, it is difficult to pinpoint the physiological significance of such a dramatic increase in total NO production. To investigate the source of the increase in plasma NO, eNOS and iNOS expressions were measured in both the hearts and kidneys. The overall changes in eNOS and iNOS expressions in the heart and kidney (**Figure 17, 18**) cannot provide a sufficient explanation for the observed elevation of plasma NO. However, eNOS and iNOS are abundantly expressed in a variety of other tissues which were not investigated in this study. Fructose feeding has been found to produce tissue-specific effects on eNOS and iNOS expressions in rats. In skeletal muscles, fructose feeding has been found to produce no effects on either eNOS or iNOS (Koshinaka *et al.*, 2004). An increase in both eNOS and iNOS expression has been found in aortic tissues (Wang *et al.*, 2007). The elevated circulating NO levels observed in this study might be attributed to the sum of changes in the expressions of NOSs in all the NOS-expressive tissues.

5. Conclusions and Future Experiments

5.1 Conclusion

1. A variety of anti-hypertensive agents have been investigated for the purpose of treating hypertension associated with metabolic syndrome and many have been tested in both animal experiments and human clinical trials. In this study, we examined the effect of phentolamine on fructose-induced elevated blood pressure and found that phentolamine was able to prevent the blood pressure elevation in the fructose-fed rats without affecting the plasma levels of cholesterol, triglyceride, insulin and glucose. Furthermore, insulin sensitivity which was decreased by fructose feeding was not affected by phentolamine.
2. The development of elevated blood pressure in fructose-fed rats might involve multiple mechanisms which may be interconnected with one another. Sympathetic nervous system and renin-angiotensin system were investigated by examining the changes in plasma levels of noradrenaline and angiotensin II, respectively. Fructose significantly increased plasma angiotensin II levels while having no effect on plasma noradrenaline. On the other hand, phentolamine did not affect on either noradrenaline or angiotensin II.
3. Chronic treatment of phentolamine, although suggested to possibly cause cardiac chronotropic disturbances, did not increase apoptosis of cardiac tissues. The absence of changes in cardiac tissue apoptosis was somewhat unexpected as chronic stimulation of β adrenoceptors in the heart is suggested to be linked to increased

apoptosis of cardiomyocytes and phentolamine can indirectly enhance the activation of these receptors. The effects of phentolamine on β adrenoceptors in the heart and kidney appeared to be tissue-specific. Due to technical difficulties and small N numbers in cardiac tissue sample replicates, the data should be considered as preliminary and future experiments are required.

4. The state of oxidative stress, a proposed mechanism involved in the development of fructose-induced elevated blood pressure, was investigated by measuring the changes in plasma levels of 8-isoprostane and total nitrate/nitrite. Fructose feeding had no effect on plasma 8-isoprostane while significantly elevating plasma nitrate/nitrite, suggesting total production of NO in the plasma was increased. We attempted to trace the source causing such a dramatic increase in plasma NO production by measuring the expressions of eNOS and iNOS in the heart and kidney. Unfortunately, the changes in eNOS and iNOS expressions in both tissues could not provide a sufficient explanation of the observed increase in NO production, suggesting possible changes in NOS expressions in other tissues might have contributed to our observation. Phentolamine had no effect on either indicator of oxidative stress.

5.2 Future Experiments in Fructose-Fed Rats in the Upcoming Investigation

1. In order to verify the contradictory findings of changes in plasma noradrenaline level, we have decided to monitor the changes in plasma noradrenaline level by measuring it at multiple time points throughout the study.
2. To further investigate the role of NO in oxidative stress associated with this animal model, we have decided to measure plasma NO level at multiple time points throughout the study and examine the possible changes in eNOS and iNOS expressions in a variety of tissues including blood vessels, heart, skeletal muscles, kidneys, etc.
3. To confirm the findings of changes in the expression of β_2 and β_3 receptors and investigate the possible changes in β_1 receptors in the heart, we will increase the N number of samples and refine our Western Blot technique.
4. Uric acid has been suggested to be involved in the development of elevated blood pressure in fructose-fed rats (Nakagawa *et al.*, 2006) since increased level of uric acid has been found in these animals (Nakagawa *et al.*, 2005). We will monitor plasma uric acid at multiple time point to determine whether an increase in uric acid triggers an elevation in systolic blood pressure induced by fructose feeding.

Reference

ALLHAT Collaborative Research Group [no authors listed], “Major cardiovascular events in hypertensive patients randomized to doxazosin vs chlorthalidone: the antihypertensive and lipid-lowering treatment to prevent heart attack trial (ALLHAT)”, **JAMA** 283(15):1967-75, 2000

ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group [no authors listed], “Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker vs. diuretic”, **JAMA** 288:2981–2997, 2002 [published corrections appear in **JAMA** 289:178, 2003 and 291:2196, 2004]

Abdulla MH, Sattar MA, Johns EJ, “The Relation between Fructose-Induced Metabolic Syndrome and Altered Renal Haemodynamic and Excretory Function in the Rat”, **International Journal of Nephrology** Volume 2011, Article ID 934659, 17 pages, 2011

Alberti KG, Zimmet P, Shaw J, “The metabolic syndrome- a new worldwide definition”, **Lancet** 336: 1059-1062, 2005

Alzamendi A, Giovambattista A, Raschia A, Madrid V, Gaillard RC, Rebolledo O, Gagliardino JJ, and Spinedi E, “Fructose-rich diet-induced abdominal adipose tissue endocrine dysfunction in normal male rats”, **Endocrine** 35(2): 227-232, 2009

Anderson EA, Hoffman RP, Balon TW, Sinkey CA, Mark AL, “Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans”, **J Clin Invest** 87: 2246-2252, 1991

Anh D and Marine JE, “Beta Blockers as Anti-Arrhythmic Agents”, **Heart Failure Reviews** 9(2): 139 -147, 2004

Anitha Nandhini AT, Balakrishnan SD, Anuradha CV, “Taurine improves lipid profile in rats fed a high fructose-diet”, **Nutrition Research** 22: 343–354, 2002

Aquitlera G, Marusic ET, “Role of the renin angiotensin system on the biosynthesis of aldosterone”, **Endocrinology** 89: 1524-1529, 1971

Bahadir O, Uzunlulu M, Oguz, and Bahadir MA, “Effects of telmisartan and losartan on insulin resistance in hypertensive patients with metabolic syndrome”, **Hypertens Res** 30: 49-53, 2007

Balkau B, Charles MA, “Comment on the provisional report from the WHO consultation”, European Group for the Study of Insulin Resistance (EGIR). **Diabet Med** 16: 442-443, 1999

Bayer AL, Heidkamp MC, Patel N, Porter M, Engman S, Samarel AM, “alterations in protein kinase C isoenzyme expression and autophosphorylation during the progression of pressure overload-induced left ventricular hypertrophy”, **Mol Cell Biochem** 242(1-2): 145-152, 2003

Behr-Roussel D, Oudot A, Caisey S, Coz OL, Gorny D, Bernabe J, Wayman C, Alexandre L, Giuliano FA, “Daily treatment with sildenafil reverses endothelial dysfunction and oxidative stress in an animal model of insulin resistance”, **Eur Urol** 53:1272–128, 2008

Berne C, Fagius J, Pollare T, Hjendahl P, “The sympathetic response to euglycaemic hyperinsulinaemia. Evidence from microelectrode nerve recordings in healthy subjects”, **Diabetologia** 35: 873-879, 1992.

Bhanot S, McNeill JH, Bryer-Ash M, “Vanadyl sulfate prevents fructose-induced hyperinsulinemia and hypertension in rats”, **Hypertens** 23(3): 308-312, 1994

Bhanot S, Michoulas A, McNeill JH, “Antihypertensive effects of vanadium compounds in hyperinsulinemic, hypertensive rats”, **Mol Cell Biochem** 153(1-2): 205-209, 1995

Bisognano JD, “Myocardial-directed overexpression of the human β_1 -adrenergic receptor in transgenic mice”, **J. Mol. Cell. Cardiol** 32: 817-830, 2000

Black HR, Elliott WJ, Grandits G, Grambsch P, Lucente T, White WB, Neaton JD, Grimm RH Jr, Hansson L, Lacourciere Y, Muller J, Sleight P, Weber MA, Williams G, Wittes J, Zanchetti A, Anders RJ, CONVINCE Research Group, “Principal results of the Controlled Onset Verapamil Investigation of Cardiovascular End Points (CONVINCE) trial”, **JAMA** 289:2073-2082, 2003

Boron WF and Boulpaep EL, **Medical Physiology: A Cellular and Molecular Approach**, Philadelphia, PA : W.B. Saunders, 2003

Bottari SP, de Gasparo M, Steckelings UM, Levens NR, “Angiotensin II receptor subtypes: characterization, signalling mechanisms, and possible physiological implications”, **Front Neuroendocrinol** 14: 123-171, 1993

Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, Pickard T, Roden R, Bristow MR, Sabbah HN, Mizrahi JL, Gromo G, King GL, Vlahos CJ, "Increased protein kinase C activity and expression of Ca²⁺-sensitive isoforms in the failing human heart", **Circulation** 99(3): 384-391, 1999

Bray GA, Nielsen SJ, Popkin BM, "Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity", **Am J Clin Nutr** 79: 537–543, 2004

Bristow MR, "β-adrenergic receptor blockade in chronic heart failure", **Circulation** 101:558-69, 2000

Brown MJ, Palmer CR, Castaigne A, de Leeuw PW, Mancia G, Rosenthal T, Ruilope LM, "Morbidity and mortality in patients randomised to double-blind treatment with a long-acting calcium-channel blocker or diuretic in the International Nifedipine GITS study: Intervention as a Goal in Hypertension Treatment (INSIGHT)" **Lancet** 356:366-372, 2000 [published correction appears in Lancet. 356:514, 2000]

Cigola E, Kajstura J, Li B, Meggs LG, Anversa P, "Angiotensin II activates programmed myocyte cell death in vitro", **Exp Cell Res** 231(2):363-71, 1997

Communal C, Singh K, Pimentel DR, Colucci WS, "Norepinephrine Stimulates Apoptosis in Adult Rat Ventricular Myocytes by Activation of the β-Adrenergic Pathway", **Circulation** 98:1329-1334, 1998

Communal C, Singh K, Sawyer DB, Colucci WS, "Opposing effects of β₁- and β₂-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G-protein", **Circulation** 100: 2210-2212, 1999

Cooper-DeHoff RM, Wen S, Beitelshes AL, Zineh I, Gums JG, Turner ST, Gong Y, Hall K, Parekh Vishal, Chapman AB, Boerwinkel E, Johnson JA, "Impact of abdominal obesity on incidence of adverse metabolic effects associated with antihypertensive medications", **Hypertens** 55: 61-68, 2010

Cosenzi A, Bernobich E, Plazzotta N, Seculin P, Bellini G, "Bosentan reduces blood pressure and the target-organ damage induced by a high-fructose diet in rats", **J Hypertens** 17:1843–1848, 1999

Dai S, McNeill JH, "Fructose-induced hypertension in rats is concentration- and duration-dependent", **J Pharmacol Toxicol Methods** 33:101–107, 1995

Dall'Aglio E Helen Chang H, Reaven GM, “Disparate effects of prazosin and propranolol on lipid metabolism in a rat model”, **Am J Med** 76(2) part 1:85-88, 1984

de Champlain J, Wu R, Girouard H, Karas M, El Midaoui A, Laplante MA, Wu L, “Oxidative stress in hypertension”, **Clin Exp Hypertens** 26:593–601,2004

Dell'Omo G, Penno G, Pucci L, Pellegrini G, Scotti A, Del Prato S *et al.*, “The vascular effects of doxazosin in hypertension complicated by metabolic syndrome”, **Coron Artery Dis** 16: 67-73, 2005

Dennison BA, Rockwell HL, Baker SL, “Excess fruit juice consumption by preschool-aged children is associated with short stature and obesity”, **Pediatrics** 99: 15–22, 1997

Derosa G, Cicero AF, Gaddi A, Mugellini A, Ciccarelli L, Fogari R, “Effects of doxazosin and irbesartan on blood pressure and metabolic control in patients with type 2 diabetes and hypertension”, **J Cardiovasc Pharmacol** 45: 599-604, 2005

Dincer UD, Bidasee KR, Guiner S, Tay A, Ozcelikay AT, Altan VM, “The effect of diabetes on expression of β_1 -, β_2 -, β_3 -adrenoreceptors in rat hearts”, **Diabetes** 50: 455-461, 2001

Dinh DT, Frauman AG, Johnston CI, Fabiani ME, “Angiotensin receptors: distribution, signaling and function”, **Clin Sci (Lond)** 100: 481-492, 2001

Dunlop M, “Aldose reductase and the role of the polyol pathway in diabetic nephropathy”, **Kidney International** 58: S3–S12, 2000

Einhorn D, Reaven GM, Cobin RH, Ford E, Ganda OP, Handelsman Y *et al.*, “American College of Endocrinology position statement on the insulin resistance syndrome”, **Endocr Pract** 9: 237-252, 2003

Elliott SS, Keim NL, Stern JS, Teff K, and Havel PJ, “Fructose, weight gain, and the insulin resistance syndrome”, **Am J Clin Nutr** 76 (5): 911–922, 2002

Eriksson JW, “Metabolic stress in insulin’s target cells leads to ROS accumulation—a hypothetical common pathway causing insulin resistance”, **FEBS Lett** 581:3734–3742, 2007

Eriksson JW, Jansson PA, Foley K, Lithell H. “Insulin sensitivity following treatment with the alpha 1-blocker bunazosin retard and the beta 1-blocker atenolol in hypertensive non-insulin-dependent diabetes mellitus patients”, **J Hypertens** 14: 1469-1475, 1996

Ervin RB, “Prevalence of metabolic syndrome among adults 20 years of age and over, by sex, age, race and ethnicity, and body mass index: United States, 2003–2006”, **National Health Statistics Reports** 13, 2009

Faeh D, Minehira K, Schwarz JM, Periasamy R, Park S, Tappy L, “Effect of fructose overfeeding and fish oil administration on hepatic de novo lipogenesis and insulin sensitivity in healthy men”, **Diabetes** 54: 1907-1913, 2005

Ferrara LA, Marotta T, Rubba P, De Simone B, Leccia G, Soro S, Mancini M, “Effects of alpha-adrenergic and beta-adrenergic receptor blockade on lipid metabolism”, **Am J Med** 80:104-108, 1986

Ford ES, Giles WH, Mokdad AH, “Increasing prevalence of the metabolic syndrome among U.S. adults”, **Diabetes Care** 27: 2444–2449, 2004

Galipeau D, Arikawa E, Sekirov I, McNeill JH, “Chronic thromboxane synthase inhibition prevents fructose-induced hypertension”, **Hypertension** 38:872–876, 2001

Galipeau D, Verma S, McNeill JH, “Female rats are protected against fructose-induced changes in metabolism and blood pressure”, **Am J Physiol Heart Circ Physiol** 283: H2478–H2484, 2002a

Galipeau DM, Yao L, McNeill JH, “Relationship among hyperinsulinemia, insulin resistance, and hypertension is dependent on sex”, **Am J Physiol Heart Circ Physiol** 283:H562–H567, 2002b

Germack R and Dickenson JM, “Induction of beta3-adrenergic receptor functional expression following chronic stimulation with noradrenaline in neonatal rat cardiomyocytes”, **J Pharmacol Exp Ther** 316(1):392-402, 2006

Greco A, Minghetti L, Levi G, “Isoprostanes, novel markers of oxidative injury, help understanding the pathogenesis of neurodegenerative diseases”, **Neurochem Res** 25(9/10), 1357-1364, 2000

Grundy SM, Hansen B, Smith Jr SC, Cleeman JI, Kahn RA; American Heart Association; National Heart, Lung, and Blood Institute; American Diabetes Association, “Clinical management of metabolic syndrome: report of the American Heart Association/National Heart, Lung, and Blood Institute/American Diabetes Association conference on scientific issues related to management”, **Circulation** 109:551–556, 2004

Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith Jr SC, Spertus JA, Costa F, “Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement”, **Circulation** 112: 2735-2752, 2005

Guo Q, Avramoglu RK, Adeli K, “Intestinal assembly and secretion of highly dense/lipid-poor apolipoprotein B48-containing lipoprotein particles in the fasting state: evidence for induction by insulin resistance and exogenous fatty acids”, **Metabolism** 54(5):689-97, 2005

Hagemann D, Kusehel M, Kuramochi T, Zhu WZ, Chen HP, Xiao RP, “Frequency-encoding Thr17 phospholamban phosphorylation is independent of Ser16 phosphorylation in cardiac myocytes”, **J Biol Chem** 275(29): 22532-22536, 2000

Hanson RL, Imperatore G, Bennett PH, Knowler WC, “Components of the ‘‘metabolic syndrome’’ and incidence of type 2 diabetes”, **Diabetes** 51:3120–3127, 2002

Head GA and Mayorov DN, “Imidazoline Receptors, Novel Agents and Therapeutic Potential”, **Cardiovasc Hematol Agents Med Chem** 4(1): 17–32, 2006

Higashiura K, Ura N, Takada T, Li Y, Torii T, Togashi N, Takada M, Takizawa H, Shimamoto K, “The effects of an angiotensin-converting enzyme inhibitor and an angiotensin II receptor antagonist on insulin resistance in fructose-fed rats”, **Am J Hypertens** 13:290–297, 2000

Hsieh PS, Huang WC, “Neonatal chemical sympathectomy attenuates fructose induced hypertriglyceridemia and hypertension in rats”, **Chin J Physiol** 266: E717- E724, 2001

Hsieh PS, Tai YH, Loh CH, Shih KC, Cheng WT, Chu CH, “Functional interaction of AT1 and AT2 receptors in fructose-induced insulin resistance and hypertension in rats”, **Metabolism** 54:157–164, 2005

Hunt KJ, Resendez RG, Williams K, Haffner SM, Stern MP, “National Cholesterol Education Program versus World Health Organization metabolic syndrome in relation to all-cause and cardiovascular mortality in the San Antonio Heart Study”, **Circulation** 110:1251–1257, 2004

Hwang IS, Ho H, Hoffman BB, Reaven GM, “Fructose-induced insulin resistance and hypertension in rats”, **Hypertension** 10: 512–516, 1987

Hwang IS, Huang WC, Wu JN, Shian LR, Reaven GM, “Effect of fructose-induced hypertension on the rennin-angiotensin-aldosterone system and atrial natriuretic factor”, **Am J Hypertens**. 2: 424-427, 1989

Imura O, Shimamoto K, Matsuda K, Masuda A, Takizawa H, Higashiura K, Miyazaki Y, Hirata A, Ura N, Nakagawa M, “Effects of angiotensin receptor antagonist and angiotensin converting enzyme inhibitor on insulin sensitivity in fructose-fed hypertensive rats and essential hypertensives”, **Am J Hypertens** 8:353–357, 1995

Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR, Groop L, “Cardiovascular morbidity and mortality associated with the metabolic syndrome”, **Diabetes Care** 24:683–689, 2001

Israili ZH, Lyoussi B, Hernández-Hernández R, Velasco M, “Metabolic syndrome: treatment of hypertensive patients”, **Am J Ther** 14:386–402, 2007

Iyer SN and Katovich MJ, “Effect of chronic losartan potassium treatment on fructose-induced hypertension”, **Life Sci** 55: PL139–PL144, 1994

Iyer SN; Katovich MJ, Raizada MK, “Changes in angiotensin AT1 receptor density during hypertension in fructose-fed rats”, **Adv Exp Med Biol** 396:49–58, 1996

Iyer SN and Katovich MJ, “Effect of acute and chronic losartan treatment on glucose tolerance and insulin sensitivity in fructose-fed rats”, **Am J hypertens** 9: 662-668, 1996a

Iyer SN and Katovich MJ, “Vascular reactivity to phenylephrine and angiotensin II in hypertensive rats associated with insulin resistance”, **Clin Exp Hypertens** 18: 227–242, 1996b

Jansen H, Baggen RG, “Effects of doxazosin and propranolol administration on lipoprotein lipases in cholesterol-fed rats”, **J Cardiovasc Pharmacol** 10 Suppl 9: S16-20, 1987

Johnson RK and Frary C, “Choose beverages and foods to moderate your intake of sugars: The 2000 Dietary Guidelines for Americans-What’s All the Fuss About?”, **J Nutr** 131(10): 2766S-2771S, 2001

Juan CC, Fang VS, Hsu YP, Huang YJ, Hsia DB, Yu PC, Kwok CF, Ho LT, “Overexpression of vascular endothelin-1 and endothelin-A receptors in a fructose-induced hypertensive rat model”, **J Hypertens** 16:1775–1782, 1998

Kajstura J, Cigola E, Malhotra A, Li P, Cheng W, Meggs LG, Anversa P, “Angiotensin II induces apoptosis of adult ventricular myocytes in vitro”, **J Mol Cell Cardiol** 29(3):859-70, 1997

Kakita T, Hasegawa K, Iwai-Kanai E, Adachi S, Morimoto T, Wada H, Kawamura T, Yanazume T, Sasayama S, “Calcineurin pathway is required for endothelin-1-mediated protection against oxidant stress-induced apoptosis in cardiac myocytes”, **Circ Res** 88:1239–1246, 2001

Kamide K, Rakugi H, Higaki J, Okamura A, Nagai M, Moriguchi K, Ohishi M, Satoh N, Tuck ML, Ogihara T, “The renin-angiotensin and adrenergic nervous system in cardiac hypertrophy in fructose-fed rats”, **Am J Hypertens** 15:66–71, 2002

Katakam PV, Ujhelyi MR, Hoenig ME, Miller AW, “Endothelial dysfunction precedes hypertension in diet-induced insulin resistance”, **Am J Physiol** 275:R788–R792, 1998

Katzung BG, **Basic and Clinical Pharmacology**, 10th Edition, McGraw-Hill Companies, 2007

Kobayashi R, Nagano M, Nakamura F, Higaki J, Fujioka Y, Ikegami H, Mikami H, Kawaguchi N, Onishi S, Ogihara T, “Role of angiotensin II in high fructose-induced left ventricular hypertrophy in rats”, **Hypertension** 21:1051–1055, 1993

Koshinaka K, Oshida Y, Han YQ, Ohsawa I, Sato Y, “The Effect of Nitric Oxide Synthase Inhibitor on Improved Insulin Action by Pioglitazone in High-Fructose-Fed Rats”, **Metabolism** 53(1): 22-27, 2004

Kumar U, Chen J, Sapoznikhov V, Canteros G, White BH and Sidhu A, "Overexpression of inducible nitric oxide synthase in the kidney of the spontaneously hypertensive rat", **Clin Exp Hypertens** 1: 17-31, 2005

Laaksonen DE, Lakka HM, Niskanen LK, Kaplan GA, Salonen JT, Lakka TA, “Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study”, **Am J Epidemiol** 156:1070–1077, 2002

Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J, Salonen JT, “The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men”, **JAMA** 288: 2709–2716, 2002

Li WM, Gan RT, Wang X, Wu S, Shen JX, Xiu CH, “The effects of combined beta 1 adrenergic receptor antagonist and beta 2 adrenergic receptor agonist therapy on cardiac function and myocardial apoptosis in heart failure rats” (article in Chinese), **Zhonghua Xin Xue Guan Bing Za Zhi** 35 (7): 615-619, 2007

Liang C, Doherty JU, Faillace R, Maekawa K, Arnold S, Gavras H, Hood WB, “Insulin infusion in conscious dogs: Effects on systemic and coronary hemodynamics, regional blood flows, and plasma catecholamines”, **J Clin Invest** 69: 1321-1336, 1982

Lithell HO, “Effect of antihypertensive drugs on insulin, glucose, and lipid metabolism”, **Diabetes Care** 14(3): 203-209, 1991

Lorenzo C, Okoloise M, Williams K, Stern MP, Haffner SM, “The metabolic syndrome as predictor of type 2 diabetes: the San Antonio heart study”, **Diabetes Care** 26:3153–3159, 2003

Ludwig DS, Peterson KE, Gortmaker SL, “Relation between consumption of sugar-sweetened drinks and childhood obesity: a prospective, observational analysis”, **Lancet** 357: 505–508, 2001

Luo W, Chu G, Sato Y, Zhou Z, Kadambi VJ, Kranias EG, “Transgenic approaches to define the functional role of dual site phospholamban phosphorylation”, **J Biol Chem** 273:4734–4739, 1998

MacDougall LK, Jones LR, Cohen P, “Identification of the major protein phosphatases in mammalian cardiac muscle which dephosphorylate phospholamban”, **Eur J Biochem** 196(3): 725-734, 1991

Makaryus AN, Akhrass P, McFarlane SI, “Treatment of hypertension in metabolic syndrome: Implications of recent clinical trials”, **Curr Diab Rep** 9:229-237, 2009

Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR, Williams GR , “Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults”, **Circulation** 110:1245–1250, 2004

Matsuda M and DeFronzo RA, “Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp”, **Diabetes Care** 22: 1462–1470, 1999

Mattiazzi A, Mundiña-Weilenmann C, Chu G, Vittone L, Kranias EG, “Role of phospholamban phosphorylation on Thr¹⁷ in cardiac physiological and pathological conditions”, **Cardiovasc Res** 68(3): 366-375, 2005

Mattiazzi A, Mundiña-Weilenmann C, Vittone L, Said M, Kranias EG, “The importance of the Thr¹⁷ residue of phospholamban as a phosphorylation site under physiological and pathological conditions”, **Braz J Med Biol Res** 39(5): 563-572, 2006

Mayer MA, Hocht C, Opezzo J AW, Peredo HA, Navacchia D, Taira CA, Fernandez BE, Puyo AM, “Role of hypothalamic α_2 -adrenoceptor activity in fructose-induced hypertension”, **Clin and Exp Pharmacol and Physiol** 33: 904-909, 2006

Mayer MA, Hocht C, Gironacci M, , Opezzo J AW Taira CA, Fernandez BE, Puyo AM, “High fructose diet increases anterior hypothalamic alpha 2-adrenoceptors responsiveness”, **Neuroscience Letters** 423: 128-132, 2007

Mayer MA, Hocht C, Gironacci M, , Opezzo J AW Taira CA, Fernandez BE, Puyo AM, “Hypothalamic angiotensinergic-noradrenergic systems interaction in fructose induced hypertension”, **Regulatory Peptides** 146, 2008

Messerli FH, Grossman E, Leonetti G, “Antihypertensive therapy and new onset diabetes”, **J Hypertens** 22:1845-1847, 2002

Metra M, Nodari S, D’Aloia A, Bontempi L, Boldi E, Cas LD, “A rationale for the use of beta-blockers as standard treatment for heart failure”, **Am Heart J** 139:511-21, 2000

Miller AW, Hoenig ME, Ujhelyi MR, “Mechanisms of impaired endothelial function associated with insulin resistance”, **J Cardiovasc Pharmacol Ther** 3:125–134, 1998

Mokdad AH, Bowman BA, Ford ES, Vinicor F, Marks JS, Koplan JP, “The continuing epidemics of obesity and diabetes in the United States”, **JAMA** 286: 1195–1200, 2001

Nakagawa T, Tuttle KR, Short RA, Johnson RJ, “Hypothesis: fructose-induced hyperuricemia as a causal mechanism for the epidemic of the metabolic syndrome”, **Nat Clin Pract Nephrol** 1:80–86, 2005

Nakagawa T, Hu H, Zharikov S, Tuttle KR, Short RA, Glushakova O, Ouyang X, Feig DI, Block ER, Herrera-Acosta J, Patel JM, Johnson RJ, “A causal role for uric acid in fructose-induced metabolic syndrome”, **Am J Physiol Renal Physiol** 290:F625–F631, 2006

Navarro-Cid J, Maeso R, Perez-Vizcaino F, Cachofeiro V, Ruilope LM, Tamargo J, Lahera V, “Effects of losartan on blood pressure, metabolic alterations, and vascular reactivity in the fructose-induced hypertensive rat”, **Hypertension** 26:1074–1078, 1995

Neumann J, “Altered phosphatase activity in heart failure, influence on Ca^{2+} movement”, **Basic Res Cardiol** 97: I91-95, 2002

Nyby MD, Abedi K, Smutko V, Eslami P, Tuck ML, “Vascular angiotensin type I receptor expression is associated with vascular dysfunction, oxidative stress and inflammation in fructose-fed rats”, **Hypertens Res** 30: 451-457, 2007

Ontko JA and Woodside WF, “Influence of doxazosin on lipid transport in rats and hamsters”, **J Cardiovasc Pharmacol** 13 Suppl 2:S31-6, 1989

Palaniappan L, Carnethon MR, Wang Y, Hanley AJG, Fortmann SP, Haffner SM, Wagenknecht L, “Predictors of the incident metabolic syndrome in adults: the Insulin Resistance Atherosclerosis Study”, **Diabetes Care** 27:788–793, 2004

Park OJ, Cesar D, Faix D, Wu K, Shackleton CH, Hellerstein MK, “Mechanisms of fructose-induced hypertriglyceridaemia in the rat: activation of hepatic pyruvate dehydrogenase through inhibition of pyruvate dehydrogenase kinase”, **Biochem J** 282 (3): 753—757, 1992

Parks EJ, and Hellerstein MK, “Diarbohydrate-induced hypertriacylglycerolemia: historical perspective and review of biological mechanisms”, **Am J Clin Nutr** 71(2): 412–433, 2000

Praticò D, Lawson JA, Rokach, J, FitzGerald GA, “The isoprostanes in biology and medicine”, **Trends Endocrinol Metab** 12(6) 243-247, 2001

Penicaud L, Berthault MF, Morin J, Dubar M, Ktorza A, Ferre P, “Rilmenidine normalizes fructose-induced insulin resistance and hypertension in rats”, **J Hypertens Suppl** 16:S45-49, 1998

Putnam JJ, Allshouse JE, “Food consumption, prices, and expenditures, 1970–97”, Washington, DC: Economic Research Service, US Department of Agriculture, 1999

Reaven GM, “Role of insulin resistance in human disease”, **Diabetes** 37: 1595–1607, 1988

Reid IA, “Interactions between ANG II, sympathetic nervous system, and baroreceptor reflexes in regulation of blood pressure”, **Am J Physiol** 262: E763-778, 1992

Rosen P, Ohly P, Gleichmann H, “Experimental benefit of moxonidine on glucose metabolism and insulin secretion in the fructose-fed rat”, **J Hypertens Suppl** 15(1):S31-8, 1997

Saito S, Hiroi Y, Zou YZ, Aikawa R, Toko H, Shibasaki F, Yazaki Y, Nagai R, Komuro I, “ β -Adrenergic pathway induces apoptosis through calcineurin activation in cardiac myocytes”, **J Biol Chem** 275(44): 34528-34533, 2000

Sarafids PA & Bakris GL, “Antihypertensive treatment with beta-blockers and the spectrum of glycaemic control”, **Q J M** 99:431-436, 2006a

Sarafids PA & Bakris GL, “Antihypertensive Therapy and the risk of new-onset diabetes”, **Diabetes Care** 29(5): 1167-1169, 2006b

Schulze MB, Manson JE, Ludwig DS, Colditz GA, Stampfer MJ, Willett WC, Hu FB, “Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women”, **JAMA** 292: 927–934, 2004

Schwarz JM, Neese RA, Schakleton C, Hellerstein MK, “De novo lipogenesis during fasting and oral fructose ingestion in lean and obese hyperinsulinemic subjects”, **Diabetes** 42(suppl):A39 (abstr), 1993

Schwarz JM, Neese RA, Turner SM, Nguyen C, Hellerstein MK, “Effect of fructose ingestion on glucose production (GP) and de novo lipogenesis (DNL) in normal and hyperinsulinemic obese humans”, **Diabetes** 43(suppl):52A (abstr), 1994

Grundy SM, Brewer Jr HB, Cleeman JI, Smith Jr Sidney C, Lenfant C, “Definition of Metabolic Syndrome”, **Arterioscler Thromb Vasc Biol** 24: e13-e18, 2004

Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith Jr SC, Spertus JA, “Diagnosis and Management of the Metabolic Syndrome”, **Circulation** 112: 2735-2752, 2005

Shahraki MR, Harati M, and Shahraki AR, “Prevention of high fructose-induced metabolic syndrome in male Wistar rats by aqueous extract of *tamarindus indica* seed”, **Acta Medica Iranica** 49(5): 277-283, 2011

Song D, Arikawa E, Galipeau D, Battell M, McNeill JH, “Androgens are necessary for the development of fructose-induced hypertension,” **Hypertension** 43:667–672, 2004

Song D, Arikawa E, Galipeau DM, Yeh JN, Battell ML, Yuen VG, McNeill JH, “Chronic estrogen treatment modifies insulin-induced insulin resistance and hypertension in ovariectomized rats”, **Am J Hypertens** 18:1189–1194, 2005

Steenart NA, Ganim JR, Di Salvo J, Kranias EG, “The phospholamban phosphatase associated with cardiac sarcoplasmic reticulum is a type 1 enzyme”, **Arch Biochem Biophys** 293(1): 17-24, 1992

Sun J, Zhang X, Broderick M, Fein H, “Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay”, **Sensors** 3:276-284, 2003

Suzuki M, Hirose J, Asakura Y, Sato A, Kageyama A, Harano Y et al, “Insulin insensitivity in nonobese, nondiabetic essential hypertension and its improvement by an alpha 1-blocker (bunazosin)”, **Am J Hypertens** 5: 869-874, 1992

Swislocki AL, Hoffman BB, Sheu WH, Chen YD, Reaven GM, “Effect of prazosin treatment on carbohydrate and lipoprotein metabolism in patients with hypertension”, **Am J Med** 86: 14-18, 1989

Tack Cees JJ, Lenders Jacques WM, Willemsen JJ, van Druten Johannes AM, Thien T, Lutterman JA, Smits P, “Insulin stimulates epinephrine release under euglycemic conditions in humans”, **Metabolism** 47(3):243-249, 1998

Takagawa Y, Berger ME, Tuck ML, Golub MS, “Impaired endothelial alpha-2 adrenergic receptor-mediated vascular relaxation in the fructose fed rats”, **Hypertens Res** 25: 197-202, 2002

Tay A, Ozcelikay AT, Altan VM, “Effects of L-arginine on blood pressure and metabolic changes in fructose-hypertensive rats”, **Am J Hypertens** 15:72–77, 2002

“Tracking Heart Disease and Stroke in Canada”, Heart and Stroke Foundation of Canada Statistics, 2009

Tomiya H, Kushiro T, Abeta H, Kurumatani H, Taguchi H, Kuga N et al. Blood pressure response to hyperinsulinemia in salt-sensitive and salt-resistant rats. **Hypertension** 20:596-600, 1992

Tran LT, Yuen Violet, McNeill JH, “The fructose-fed rat: a review on the mechanisms of fructose-induced insulin resistance and hypertension”, **Mol Cell Biochem** 332:145–159, 2009a

Tran LT, MacLeod KM, McNeill JH, “Endothelin-1 modulates angiotensin II in the development of hypertension in fructose-fed rats”, **Mol Cell Biochem** 325:89–97, 2009b

Tran LT, “The Mechanism of Metabolic Syndrome” (Ph.D Thesis), Faculty of Pharmaceutical Sciences, the University of British Columbia, 2009

Thorburn A *et al.*, “Fructose-induced in vivo insulin resistance and elevated plasma triglyceride levels in rats”, **Am J Clin Nutr** 49: 1155-1163, 1989

Uchida T, Okumura K, Ito T, Kamiya H, Nishimoto Y, Yamada M, Tomida T, Matsui H, Hayakawa T, “Quinapril treatment restores the vasodilator action of insulin in fructose hypertensive rats”, **Clin Exp Pharmacol Physiol** 29:381–385, 2002

Ufer C and Germack R, “Cross-regulation between β_1 - and β_3 -adrenoceptors following chronic β -adrenergic stimulation in neonatal rat cardiomyocytes”, **Br J Pharmacol** 158(1): 300–313, 2009

Vasdev S, Mian T, Ford CA, Longerich L, Parai S, “Role of endogenous aldehydes in spontaneously hypertensive and disulfiram-induced hypertensive rats”, **Nutr Metab Cardiovasc Dis** 6: 130–140, 1996

Vasdev S, Ford CA, Longerich L, Gadag V, Wadhawan S, “Role of aldehydes in fructose induced hypertension”, **Mol Cell Biochem** 181: 1–9, 1998

Vasdev S, Longerich L, and Gill V, “Prevention of fructose-induced hypertension by dietary vitamins”, **Clin Biochem** 37(1): 1–9, 2004

Vasdev S and Stuckless J, “Role of methylglyoxal in essential hypertension”, **Int J Angiol** 19(2):e58-e65, 2010

Vasudevan H, Xiang H, McNeill JH, “Differential regulation of insulin resistance and hypertension by sex hormones in fructose-fed male rats”, **Am J Physiol Heart Circ Physiol** 289: H1335–H1342, 2005

Vasudevan Harish, “Testosterone-dependent vascular arachidonic acid metabolism in the regulation of insulin resistance and blood pressure”, (Ph.D Thesis), Faculty of Pharmaceutical Sciences, the University of British Columbia, 2009

Verma S, Bhanot S, McNeill JH, “Antihypertensive effects of metformin in fructose-fed hyperinsulinemic, hypertensive rats”, **J Pharmacol Exp Ther** 271:1334–1337, 1994

Verma S, Bhanot S, McNeill JH, “Effect of chronic endothelin blockade in hyperinsulinemic hypertensive rats”, **Am J Physiol** 269: H2017–H2021, 1995

Verma S, Skarsgard P, Bhanot S, Yao L, Laher I, McNeill JH, “Reactivity of mesenteric arteries from fructose hypertensive rats to endothelin-1”, **Am J Hypertens** 10:1010–1019, 1997

Verma S, Bhanot S, McNeill JH, “Sympathectomy prevents fructose-induced hyperinsulinemia and hypertension”, **Eur J Pharmacol** 373:R1–R4, 1999

Verma S, Yao L, Domont AS, McNeill JH, “Metformin treatment corrects vascular insulin resistance in hypertension”, **J Hypertens** 18(10): 1145-1150, 2000

Vittone L, Mundiña C, Chiappe de Cingolani G et al, “cAMP and calcium dependent mechanisms of phospholamban phosphorylation in intact hearts”, **Am J Physiol** 258:H318-H325, 1990

Vittone L, Mundiña-Weilenmann C, Ortale M, de Cingolani GC, Mattiazzi A, “Immunodetection of phosphorylation sites gives new insights into the mechanisms underlying phospholamban phosphorylation in the intact heart”, **J Biol Chem** 271: 33561-33567, 1996

Wajchenber BL, Malerbi DA, Rocha MS, Lerario AC, Santomauro AT, “Syndrome X: a syndrome of insulin resistance”, Epidemiological and clinical evidence. **Diabetes Metab Rev** 10: 19-29, 1994

Wang W, Zhang HY, Gao Hui, Kubo H, Berretta RM, Chen XW, Houser SR, “ β_1 -adrenergic receptor activation induces mouse cardiac myocyte death through both L-type calcium channel-dependent and independent pathways”, **Am J Physiol Heart Circ Physiol** (in press), 2010

Wang J, Liu X, Sentex E, Takeda N, Dhalla NS, “Increased expression of protein kinase C isoforms in heart failure due to myocardial infarction”, **Am J Physiol Heart Circ Physiol** 284(6): H2277-87, 2003

Wang X, Hattori Y, Satoh H, Iwata C, Banba N, Monden T, Uchida K, Kamikawa Y, Kasai K, “Tetrahydrobiopterin prevents endothelial dysfunction and restores adiponectin levels in rats”, **Euro J of Pharma** 555: 48–53, 2007

Welch WJ, Tojo A, Lee JU, Kang DG, Schnackenberg CG, Wilcox CS, “Nitric oxide synthase in the JGA of the SHR: expression and role in tubuloglomerular feedback”, **Am J Physiol** 277(1 Pt2): F130-138, 1999

Whaley-Connell A, Palmer J, Sowers JR, “Risk stratification and treatment options for patients with hypertension with metabolic syndrome and prediabetes”, **Adv Stud Med** 5(10C):S1011-S1018, 2005

Wilcox CS, “Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension?”, **Am J Physiol Regul Integr Comp Physiol** 289: R913-935, 2005

Wild SH, Byrne CD, “The global burden of the metabolic syndrome and its consequences for diabetes and cardiovascular disease”, In: Byrne CD, Wild SH, eds. *The metabolic syndrome*. Chichester, England: John Wiley: 1-41, 2005

Xiao RP, “ β -Adrenergic Signaling in the Heart: Dual Coupling of the β_2 -Adrenergic Receptor to G_s and G_i Proteins”, **Science’s STKE** 2001(104):re15, 2001

Xiao RP, Zhu W, Zheng M, Cao C, Zhang Y, Lakatta EG, Han Q, “Subtype-specific α_1 - and β_1 -adrenoceptor signaling in the heart”, **Trends in Pharma Sci** 27(6): 330-337, 2006

Yang E and Schulman H, "Structural examination of autoregulation of multifunctional calcium/calmodulin-dependent protein kinase II", **J Biol Chem** 274 (37): 26199–26208, 1999

Zaugg M, Xu W, Lucchinetti E, Shafiq SA, Jamali NZ, Siddiqui MAQ, “ β -adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes”, **Circulation** 102: 344-350, 2000

Zhou MS, Schulman IH, Raij L, “Role of angiotensin II and oxidative stress in vascular insulin resistance linked to hypertension”, **Am J Physiol Heart Circ Physiol** 296: H833-H839, 2009

Zhu WZ, Wang SQ, Chakir K, Yang D, Zhang T, Brown JH, Devic Eric, Kobilka BK, Cheng H, Xiao RP, “Linkage of β_1 -adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca^{2+} /calmodulin kinase II”, **J Clin. Invest** 111: 617- 625, 2003

Zhu, WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP, “Dual modulation of cell survival and cell death by β_2 -adrenergic signaling in adult mouse cardiac myocytes”, **Proc. Natl. Acad. Sci. U. S. A.** **98**:1607–1612, 2001