

**TESTOSTERONE-DEPENDENT VASCULAR ARACHIDONIC ACID
METABOLISM IN THE REGULATION OF INSULIN RESISTANCE
AND BLOOD PRESSURE**

by

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ABSTRACT

Insulin resistance (IR) and elevated blood pressure (BP) are two key features of the metabolic syndrome, which play an important role in the development of secondary cardiovascular complications. Feeding rats a diet rich in sugars such as fructose induces IR followed by vascular abnormalities and elevation in BP. Insulin resistance impairs the fine balance between endothelial vasoconstrictors and vasorelaxants, which results in endothelial dysfunction (ED). Differences in sex hormones play an important role in the development of insulin resistance and blood pressure as testosterone is essential for the development of ED and the increase in BP. Testosterone regulates the cyclooxygenase and Cyp4A-catalyzed metabolites of arachidonic acid, which have been implicated in vascular homeostasis. However, their regulation by insulin is unclear. We hypothesized that **In the presence of testosterone insulin resistance favors increased synthesis of vasoconstrictor metabolites of arachidonic acid, which contributes to the development of endothelial dysfunction and subsequent elevation of blood pressure.**

Intact and/or gonadectomized male Wistar rats were fed for 9-12 weeks with fructose following which changes in blood pressures and vascular reactivity were determined. Treatment with testosterone restored the elevated blood pressure in gonadectomized rats. In addition, fructose-feeding induced insulin resistance in intact rats, which was ameliorated by inhibiting the androgen receptor or 20-HETE synthesis. Insulin resistance increased Cyp4A1, 2/3 expression in the superior mesenteric artery (SMA), which was decreased by blocking testosterone function. Finally, the increase in BP and Cyp4A1, 2/3 were prevented by treating rats for 2 weeks with the Cyp4A inhibitor HET0016. The role of IR in arachidonate metabolism was evaluated by treatment with metformin (500 mg/kg) for 10 weeks.

Prevention of insulin resistance prevented both endothelial dysfunction and the increase in BP. Further, inhibition of Cyp4A by DDMS improved endothelial relaxation in the SMA of only intact and untreated FFR but not the gonadectomized and metformin-treated groups. Similar effects were observed upon blocking COX with indomethacin. IR increased the participation of COX-2, which was testosterone-dependent in both aorta and SMA. In conclusion, in the presence of testosterone, IR induces vascular complications by altering arachidonic acid metabolism to increase vasoconstrictor levels.

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LIST OF ABBREVIATIONS

ABT	1-aminobenzotriazole
ACh	Acetylcholine
ANOVA	Analysis of variance
APA	Apamin
AT-II	Angiotensin-II
AUC	Area under the Curve
BP	Blood Pressure
C	Intact/Sham-operated and normal chow-fed
$[Ca^{2+}]_i$	Intracellular calcium
K_{Ca}	Calcium-sensitive potassium channel
CA	Control chow-fed and treated with ABT (Cyp inhibitor)
CF	Control chow-fed and treated with flutamide (anti-androgen)
CM	Control chow-fed and treated with metformin
COX	Cyclooxygenase
CT	Control chow-fed and treated with HET0016
CTX	Charybdotoxin
CVD	Cardiovascular Disease
CYP4A	Cytochrome P450 4A (Arachidonic acid hydroxylase)

DDMS	N-methylsulfonyl-12, 12-dibromododec-11-enamide (Cyp4A inhibitor)
EC ₅₀	Molar concentration of an agonist, which produces 50% of the maximum possible response for that agonist.
ED ₇₀	Molar concentration of an agonist, which produces 70% of the maximum possible response for that agonist.
EDHF	Endothelium derived hyperpolarization factor
EET	Epoxy eicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
F	Intact/Sham-operated and high (60%) fructose chow-fed rat
FA	Fructose-fed rat treated with ABT (Cyp inhibitor)
FF	Fructose-fed rat treated with flutamide (anti-androgen)
FFR	Fructose fed Rat
FM	Fructose-fed rat treated with metformin (insulin sensitizer)
FT	Fructose-fed rat treated with HET0016 (Cyp4A inhibitor)
G	Gonadectomized and normal chow-fed
GF	Gonadectomized and high fructose chow-fed
GFM	Gonadectomized and high fructose chow-fed treated with metformin

GFT	Gonadectomized high fructose chow-fed plus testosterone
GM	Gonadectomized and normal chow-fed treated with metformin
GT	Gonadectomized normal chow-fed and testosterone treated
HDL	High Density Lipoprotein
HERS	Heart and Estrogen/progestin replacement study
HET0016	N-(4-Butyl-2-methylphenyl)-N ⁰ -hydroxyformamidine-Selective Cyp4A inhibitor
20-HETE	20-hydroxyeicosatetraenoic acid
i.p.	Intraperitoneal
IR	Insulin resistance
ISI	Insulin sensitivity Index
KCl	Potassium Chloride
MAP	Mean arterial pressure
mRNA	Messenger Ribonucleic acid
L-NAME	N ⁰ -nitro-L-arginine methyl ester hydrochloride
NO	Nitric Oxide
NIH	National Institutes of Health
NS-398	N-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide-COX- 2 selective inhibitor

OGTT	Oral Glucose Tolerance Test
PCOS	Polycystic Ovary Syndrome
pD ₂	Negative logarithm of the EC ₅₀ value
PE	Phenylephrine
PGI ₂	Prostacyclin
PKB	Protein Kinase B
PLA ₂	Phospholipase A ₂
RAS	Renin angiotensin system
RIA	Radioimmunoassay assay
R _{max}	Percent maximal response by a tissue to a given agonist
RT-PCR	Reverse transcriptase polymerase chain reaction
s.c.	Subcutaneous
SC-560	5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)- 1H-pyrazole-COX-1 selective inhibitor
SD	Sprague Dawley
SEM	Standard error of the mean
SHR	Spontaneously Hypertensive rat
TG	Triglyceride
TXA ₂	Thromboxane A ₂

VSM	Vascular smooth muscle
WHO	World Health Organization
WKY	Wistar Kyoto rat

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**DEDICATED TO
MY PARENTS**

1. INTRODUCTION

1.1 The Metabolic Syndrome

Insulin resistance (IR) is a relatively new concept in our understanding of diabetes. While diabetes has been known for centuries, the first cases of insulin resistance (IR) were reported in the 1930-40s (Himsworth, 1934; Himsworth, 1938). Resistance to insulin and associated hyperinsulinemia play a key role in the way the body utilizes substrates to produce energy. Thus attenuated insulin action impairs the physiological breakdown of glucose, increases lipolysis and increases the dependence on fatty acids and triglycerides (Randle, 1998), which in turn releases by-products that in the long run may harm the body. Furthermore high density lipoprotein (HDL), the “good cholesterol”, levels are decreased while levels of low density lipoprotein (LDL) also called “bad cholesterol” are elevated. One of the key symptoms associated with the metabolic syndrome is the increase in blood pressure and elevated risk of developing cardiovascular diseases (CVD) (Cornier *et al.*, 2008). The concomitant occurrence of these symptoms along with obesity and insulin resistance constitutes the metabolic syndrome. However insulin resistance is also reported in non-obese subjects (Cornier *et al.*, 2008) suggesting the involvement of a genetic or neuroendocrine component. On the other hand, a high carbohydrate and/or fat diet, lack of exercise and stress are environmental factors which also play a key role in the metabolic syndrome (Adamo *et al.*, 2008; Bianchi *et al.*, 2008; Tamashiro *et al.*, 2006). Of the criteria set by various institutions to define metabolic syndrome, the international diabetes federation (IDF) in 2006 has stated that the presence of two or more of the following phenotypes in addition to central obesity are essential to be suffering from the metabolic syndrome. These are fasting plasma glucose higher than 100 mg/dl or 5.56 mmol/L; plasma triglycerides greater than 150

mg/dl or 8.33 mmol/L and blood pressure greater than 130/85 mm Hg. These are very similar to the criteria set by the NECP-ATPIII guidelines (2001) (Cornier *et al.*, 2008). In summary, the metabolic syndrome reflects impaired metabolic homeostasis and is suggested to be the precursor to Type 2 diabetes. The risk of developing Type 2 diabetes is up to five times higher in individuals suffering from the metabolic syndrome. Over the last 2 decades, the metabolic syndrome and its associated complications have been linked to high morbidity and mortality. Initially a first world phenomenon, insulin-associated disorders are on the rise in developing and industrialized countries such as China and India (Misra *et al.*, 2008), where a sharp change in diet and lifestyle has been observed. The National Institutes of Health (NIH) stated (2001) that the contribution of the metabolic syndrome was of an equal magnitude to that of cigarette smoking in the development of cardiovascular disease.

1.2 Insulin resistance

Although identified in the mid-twentieth century (Himsworth, 1934; Himsworth, 1938), most of the key findings regarding insulin resistance, metabolic syndrome and their secondary complications have been reported in the last 2-3 decades. To date, no factor or pathway has been implicated as the sole inducer of insulin resistance. Instead it is a combination of genetic and environmental factors that are responsible for its development. To maintain euglycemia, insulin resistance is accompanied by compensatory hyperinsulinemia (DeFronzo, 2004). However it is unclear whether hyperinsulinemia follows insulin resistance or vice versa as patients with insulinoma-induced hyperinsulinemia are not necessarily insulin resistant (Reaven *et al.*, 1996). Over time, as insulin resistance increases, the pancreas is unable to keep up with the increased demand, which results in loss of both insulin

production and function. The diabetic state is finally achieved by a relative state of hypoinsulinemia and consequent hyperglycemia. The major target organs where insulin resistance has been extensively studied are skeletal muscle, liver and adipose tissue. In the past decade, cardiac insulin resistance has also been reported in which the lack of insulin action impairs energetics and substrate utilization in the heart and coronary vasculature (Bertrand *et al.*, 2008). In addition, insulin resistance also impairs the equilibrium between the various vasoactive agents involved in controlling blood pressure.

1.3 Insulin resistance and blood pressure

Insulin resistance and hypertension are two of the major factors contributing to the metabolic syndrome. Although Type 2 diabetes is associated with various secondary complications, the role of insulin resistance in increasing the blood pressure and its involvement in the induction of various cardiovascular diseases has attracted significant attention. Taken together with the available clinical evidence, a close interrelationship exists between insulin resistance and cardiovascular disease. This was first hypothesized by Reaven when he suggested that metabolic impairments involved in inducing IR are directly related to the development of hypertension (Reaven, 1988). This hypothesis was attractive because it helped to explain the apparent inability of conventional antihypertensive drugs to decrease the incidence of coronary ischemic events, since these drugs may worsen rather than improve insulin action (Goyal, 1999; Lind *et al.*, 1994; Pollare *et al.*, 1989). On the other hand, treatment with insulin sensitizers such as metformin ameliorated insulin resistance and normalized the blood pressure suggesting hypertension to be induced by insulin resistance and not the other way round (Verma *et al.*, 1994a; Verma *et al.*, 1994b; Verma *et al.*, 2000). Similar associations

between insulin resistance, blood pressure and altered vascular reactivity have been reported in various genetic or diet induced models of hypertension or metabolic syndrome. These include genetic models such as the spontaneously hypertensive rat (SHR) (Verma *et al.*, 1994b), Zucker rats (Song *et al.*, 2006) and diet-induced models such as rats fed on high fat (Gomez-Perez *et al.*, 2008) or carbohydrate diets (Vasudevan *et al.*, 2006; Verma *et al.*, 1994a). An important factor observed in insulin resistant rats (Verma *et al.*, 1999) and humans (Penesova *et al.*, 2008) is increased sympathetic discharge, which plays an important role in inducing hypertension. However, it is unclear clinically whether insulin resistance or hyperinsulinemia initiates this increase in sympathetic nervous function. Studies in hyperinsulinemic patients show increased sympathetic nervous system (SNS) activity (Bunag *et al.*, 1991), although the patients were not insulin resistant (Facchini *et al.*, 1996; Reaven *et al.*, 1996; Scherrer *et al.*, 1996). Previous studies from our laboratory have demonstrated a reduction in blood pressure in fructose-fed rats upon blocking the sympathetic activity by chemical sympathectomy (Verma *et al.*, 1999). Decreasing sympathetic outflow using rilmenidine also prevented hypertension (Penicaud *et al.*, 1998). This is supported by unpublished data from our laboratory (Tran *et al.* 2009) where blocking the α_1 -adrenoceptor by prazosin prevents the increase in blood pressure. The association between insulin resistance and blood pressure has been reviewed in detail by Sharma and McNeill (Sharma *et al.*, 2006a; Sharma *et al.*, 2006b; Sharma *et al.*, 2006c; Sharma *et al.*, 2006d).

1.4 Insulin resistance and altered vascular reactivity

In addition to regulating the body's energy procurement, insulin also influences the production and regulation of various vasoactive agents. Through the PI3K/AKT pathway, insulin induces endothelial nitric oxide synthase (eNOS) expression, which catalyzes the production of nitric oxide (Sowers, 2004). Insulin induced-endothelial nitric oxide plays a key role in skeletal muscle glucose uptake and in the endothelium-dependent regulation of vascular tone (Baron *et al.*, 1997; Steinberg *et al.*, 1994). Elevating the eNOS expression in fructose-fed rats restores insulin sensitivity and reduces hypertension (Zhao *et al.*, 2009). Briefly, the endothelium is the innermost single layer of cells in the blood vessel, which regulates vascular homeostasis and responses to various endogenous agents. The synthesis or functions of several vasorelaxants and vasoconstrictors occurs in the endothelium in response to any stimulus. Upon increase in the endothelial $[Ca^{+2}]_i$, eNOS-mediated nitric oxide is generated in the endothelium. This endothelial nitric oxide, through activation of cyclic GMP decreases calcium signaling inside the vascular smooth muscle $[Ca^{+2}]_i$ which decreases vascular tone. In disease states involving high oxidative stress such as diabetes and insulin resistance (Holvoet, 2008; Maritim *et al.*, 2003), endothelial relaxation is impaired (Vasudevan, 2005) along with activation of endothelium-independent vasoactive agents. Endothelium derived hyperpolarizing factor (EDHF) is the other endothelial vasorelaxant, whose function is insulin dependent. Both diabetes and insulin resistance attenuate EDHF-dependent vasorelaxation (Fukao *et al.*, 1997; Iida *et al.*, 2001; Katakam *et al.*, 1999). The effects of insulin resistance on eNOS and EDHF will be discussed in section 1.61.

Insulin also influences several other endothelial vasoactive agents such as endothelin-1 (ET-1), which is one of the most potent vasoconstrictors reported. ET-1 acts on its

receptors (ET-A and ET-B) to produce vasoconstriction (Potenza *et al.*, 2005). Nitric oxide and endothelin-1 counteract each other's actions to maintain equilibrium (Sarafidis *et al.*, 2007). Following insulin resistance, when IRS-1 signaling is attenuated, insulin acts as a vasoconstrictor through increased effects of ET-1 and ET-A receptor upregulation (Elgebaly *et al.*, 2008; Miller *et al.*, 2002). Insulin resistance is also associated with increased renin angiotensin system (RAS) activity. Various groups including our laboratory (Tran *et al.*, 2009) have demonstrated increased angiotensin-II (A-II) levels and AT-1 receptor activity. Conversely, A-II has also been suggested to interfere with insulin signaling and induce insulin resistance (Olivares-Reyes *et al.*, 2008; Sowers, 2004), although additional evidence is needed to validate this claim. As alterations in these agents are mirrored by changes in the blood pressure, endothelial function is a key determinant of blood pressure.

1.5 Animal models of metabolic syndrome

1. *Spontaneously hypertensive rat*: The spontaneously hypertensive rat is a genetic model for studying hypertension. Interestingly it was also extensively used to study the association between insulin resistance, impaired glucose metabolism and hypertension. In comparison with its genetic control, the Wistar-Kyoto rat (WKY), insulin signaling is impaired at multiple levels including the insulin receptor substrate 1 (IRS 1) in the skeletal muscle (Kahn *et al.*, 1992), heart (Morisco *et al.*, 2000) and vasculature (Sugita *et al.*, 2004). Additionally other pathways such as stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Sugita *et al.*, 2004), PI3K/AKT and eNOS (Iaccarino *et al.*, 2004; Zecchin *et al.*, 2003) and glycogen synthase (Farrace *et al.*, 1995) are also affected in SHR. Various pathways associated with both insulin resistance and blood pressure are upregulated in the SHR such as

the renin-angiotensin system. Blocking the angiotensin 2 type 1 receptor (AT1) reversed the insulin resistance in the skeletal muscle and reduced blood pressure (Rizzoni *et al.*, 2008). Treating SHR with insulin sensitizers and insulin enhancers such as metformin (Verma *et al.*, 1994b) and vanadium (Bhanot *et al.*, 1994a) respectively also decreased both insulin resistance and hypertension. However other studies contradict these findings as 7-week normal chow-fed SHR did not develop hyperinsulinemia and hypertriglyceridemia. Imbibing a 12% sucrose solution for 7 weeks modestly increased the blood pressure but not plasma insulin or triglycerides, which can be compared with the metabolic effects observed subsequent to drinking a 5 or 10% fructose solution for 12 weeks (Dai *et al.*, 1995). Thus the development of insulin resistance in SHR seems to be inconsistent and varies with diet and age (Oron-Herman *et al.*, 2008).

2. *Fructose-fed hypertensive rat*: Fructose is a monosaccharide obtained from fruits and is also a component of sucrose. It is used commercially as a sweetening substitute (fructose corn syrup) for glucose and sucrose in the preparation of desserts, condiments and carbonated beverages (Elliott *et al.*, 2002). First reported in 1987 (Hwang *et al.*, 1987). feeding rats a high carbohydrate (fructose, 60%) diet for 8-10 weeks results in a model of acquired systolic hypertension, which is accompanied by insulin resistance and hyperinsulinemia. Similar to observations with fructose, symptoms of insulin resistance and hypertension have been reported in Sprague Dawley (SD) rats fed with high amounts of sucrose (Hulman *et al.*, 1994) or glucose (Reaven *et al.*, 1991), but fructose-feeding has been shown to be the most effective in inducing characteristics of the metabolic syndrome. This concept is reflected in humans, where consumption of high amounts of fructose in the diet increases the risk of

dyslipidemia (Parks *et al.*, 2000; Swanson *et al.*, 1992), obesity (Elliott *et al.*, 2002), insulin resistance and heart disease in susceptible individuals (Vasdev *et al.*, 2004). High amounts of fructose are consumed in the form of sweetened beverages, where fructose corn syrup is used as a cheaper sweetening substitute to sucrose (Bray *et al.*, 2004). As these beverages possess a high glycemic index, they are suggested to alter satiety and contribute to the development of obesity (Harrington, 2008; Wylie-Rosett *et al.*, 2004). Similar to humans, fructose-fed animals also show elevated triglyceride levels (Galipeau, 2001; Vasudevan, 2005). In previous studies from our laboratory, body weights and plasma glucose levels were unchanged at the time of termination (7-10 weeks) (Galipeau, 2001; Vasudevan, 2005). Furthermore there is also evidence that long-term feeding in rats with carbohydrate (sucrose) does produce obesity and hyperglycemia (Pierce *et al.*, 1989). The model mirrors the early sequelae observed in humans associated with insulin resistance, such as endothelial dysfunction (Vasudevan *et al.*, 2006) and hypertension (Galipeau *et al.*, 2001; Verma *et al.*, 1994a). Development of IR and subsequent hypertension however depends on the strain of the animal, the concentration of fructose used and the duration of exposure to fructose (Dai *et al.*, 1995). Our lab has previously demonstrated hypertension in Sprague Dawley (SD) rats, which had been fed with 66% fructose for 3 weeks (Verma *et al.*, 1994a). On the other hand, Wistar rats needed to be maintained on the fructose diet for as long as 4-7 weeks prior to detection of hypertension (Galipeau *et al.*, 2001). This model provided the initial evidence which was instrumental in establishing the causal nature of the link between IR and hypertension (Hwang *et al.*, 1987; Reaven *et al.*, 1988; Reaven *et al.*, 1989).

Though unclear in humans with regard to its mechanism of action, fructose may be involved in inducing insulin resistance by decreasing insulin secretion from the pancreas in

response to a high fructose meal, as fructose, unlike glucose, does not stimulate insulin secretion from the pancreatic islet cells (Curry, 1989; Elliott *et al.*, 2002). This may lead to a state of hyperglycemia and impaired insulin-dependent glucose metabolism. The second hypothesis deals with an early reduction in circulating leptin levels associated with lower insulin levels subsequent to chronic high fructose feeding. In addition to insulin resistance, chronic fructose feeding has also been suggested to induce leptin resistance (Shapiro *et al.*, 2008). Compensatory hyperinsulinemia and hyperleptinemia may also induce insulin resistance following fructose-induced perturbances in the endocrine system (Elliott *et al.*, 2002; Huang *et al.*, 2004; Teff *et al.*, 2004). Further byproducts of fructose metabolism such as methyl glyoxal have also been suggested to attenuate insulin sensitivity by increasing oxidative stress (Jia *et al.*, 2007; Vasdev *et al.*, 2004; Wang *et al.*, 2006). Although fructose-fed rats are not obese we have observed increased visceral fat deposits in them (unpublished data), which is similar to reports from other groups (Stanhope *et al.*, 2008). Fructose has been shown to induce insulin resistance in the liver (Elliott *et al.*, 2002; Le *et al.*, 2006b; Vasdev *et al.*, 2004), skeletal muscle (Hyakukoku *et al.*, 2003; Le *et al.*, 2006a; Li *et al.*, 2008) and adipose tissue (Jia *et al.*, 2007). Alzamendi and co-workers (2009) have demonstrated an increase in abdominal adipose tissue mass and size along with hyperleptinemia and attenuation in insulin receptor substrate (IRS 1 & 2) signaling in rats treated for 3 weeks with fructose in drinking water. Similarly fructose, by itself or as a component of sucrose, impairs IRS-1 and phosphoinositide-3-kinase (PI3K) in the rat skeletal muscle (Hyakukoku *et al.*, 2003) in addition to impairing mitochondrial respiration (Lambert *et al.*, 2003). In the liver, in high amounts, fructose bypasses metabolism by hepatic phosphofructokinase (PFK) in the glycolytic cycle. Phosphofructokinase is the rate-limiting enzyme in glucose metabolism

(Vasdev *et al.*, 2004). The metabolites of fructose block PFK activity and thus decrease hepatic glucose production resulting in insulin resistance (Vasdev *et al.*, 2004). Further, high fructose feeding has been shown to increase the rate of *de novo* lipogenesis as compared to glucose (Elliott *et al.*, 2002). This effect may explain the development of hypertriglyceridemia in fructose-fed rats. The elevation in the blood pressures subsequent to fructose feeding is up to 15-30 mm Hg compared to normal chow-fed rats. We therefore believe that the fructose-fed rat model is an excellent tool for investigating the mechanisms linking insulin resistance and hypertension in the context of the metabolic syndrome.

Insulin resistance induced by high fructose diet is involved in the development of hypertension. This statement is supported by studies in which hypertension in rats was reduced following normalization of hyperinsulinemia and insulin sensitivity by treating with metformin (Verma *et al.*, 1994a; Verma *et al.*, 2000). The improvement in insulin sensitivity and hypertension observed were, however, reversible in fructose-fed rats subsequent to restoration of the hyperinsulinemic state (Bhanot *et al.*, 1995). Studies have been reported with promising results using other insulin sensitizing agents such as thiazolidinediones (Lee *et al.*, 1994). Similarly, insulin enhancing agents such as vanadium have been shown to prevent the development of hypertension in fructose fed rats (FFR) (Bhanot *et al.*, 1994b).

1.6 The superior mesenteric artery

Our laboratory has worked on changes in vascular reactivity in the superior mesenteric arteries. The classification of the superior mesenteric artery (SMA) as a conduit vessel is based largely on data obtained from anesthetized animals, which may not be representative of the true *in vivo* situation. *In vivo* measurements of blood flow through the mesenteric artery

bed in conscious rats have revealed that the feed arteries can be just as active in flow control as the microvasculature, and are therefore true resistance arteries (Christensen *et al.*, 1993). Mesenteric arteries contribute substantially to vascular resistance in conscious rats, (Fenger-Gron *et al.*, 1997) Intestinal blood flow is controlled by both feed arteries and the microcirculatory resistance vessels in freely moving rats. Further, there is evidence that in both anesthetized and conscious animals, resistance control can be exerted by either the proximal arteries or the microvasculature of the mesenteric bed depending on the condition that provokes the control (Christensen *et al.*, 2001). We therefore feel that the SMA is an appropriate artery to use in our study because, based on the above studies, it is a true resistance artery, and, importantly, affords sufficient tissue for protein and mRNA estimation.

1.7 Mechanisms linking insulin resistance to hypertension

1.7.1 *Insulin resistance and endothelial vasodilators*

Defects in endothelial function have been shown to play a role in the altered responses to insulin in the development of IR-induced hypertension (Baron, 1999; Goodfellow *et al.*, 1996; Katakam *et al.*, 1998; Miller *et al.*, 1998; Pinkney *et al.*, 1997). We have previously reported that endothelium-dependent relaxation to ACh is depressed in the mesenteric arteries from insulin resistant rats (Verma *et al.*, 1996; Verma *et al.*, 1997b; Verma *et al.*, 2000). This attenuation was mainly due to a decrease in the endothelial NO and EDHF-dependent relaxation pathways (Vasudevan *et al.*, 2006). Similar results have been reported in high-fat and high sucrose-fed rats (Bourgoin *et al.*, 2008). Thus it is possible that

following IR, the synthesis or release of endothelial vasodilators may be diminished, resulting in exaggerated responses of the vascular smooth muscle (VSM) to endogenous vasoconstrictors. Alternatively, IR may attenuate sensitivity to vasodilators, thus enabling vasoconstrictors to elicit increased responses. In addition, unchanged vasoconstrictor responses to phenylephrine in insulin resistant rats (Vasudevan *et al.*, 2006) support our hypothesis that it is the impaired relaxation that contributes to vascular abnormalities and increased blood pressure; not increased contraction.

As previously mentioned in section 1.3, insulin relaxes blood vessels under physiological conditions via the release of NO formed by the action of the constitutive endothelial nitric oxide synthase (eNOS) (Baron, 1994). Insulin also induces endothelium-dependent vasoconstrictors (Frank *et al.*, 1993), which are counteracted by endothelial NO in states of normoinsulinemia (Eringa *et al.*, 2002). Following insulin resistance and hyperinsulinemia, insulin-induced vasodilation is impaired due to attenuated NO function (Verma *et al.*, 1996). This results in the unmasking of insulin-induced vasoconstriction due to increased endothelin-1 action (vascular effects of endothelin-1 are described in following section) (Schroeder *et al.*, 1999). Indeed, our laboratory has previously examined this hypothesis and shown that arteries from insulin-resistant rats are refractory to insulin-induced vasodilation (Verma *et al.*, 1997a). We have also demonstrated that endothelial NO is the major relaxant in superior mesenteric arteries (Vasudevan *et al.*, 2006).

In addition to NO, two other important endothelium-derived vasorelaxing factors have been characterized, which are the endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI₂). Endothelium-derived hyperpolarizing factor is an important vasodilator and plays a key role in relaxing the resistance vasculature (Shimokawa *et al.*, 1996).

Although the exact identity of EDHF is currently debated, the majority of studies support one or more of the epoxyeicosatrienoic acids (EETs), which are cytochrome P450 (Cyp450)-catalyzed byproducts of arachidonic acid (Figure 1.1). Arachidonic acid epoxygenases belonging to Cyp2C family of enzymes are suggested to be EDHF synthases (Fisslthaler *et al.*, 1999; Fleming, 2004). Several other candidates, namely anandamide (Randall *et al.*, 1996; Randall *et al.*, 1997), H₂O₂ (Matoba *et al.*, 2000) and K⁺ ion (Edwards *et al.*, 1998) have been characterized in different vascular beds as the putative EDHF. Therefore, the nature of EDHF appears to be regiospecific with respect to the vasculature. Also unclear is whether the hyperpolarization is due to a simple electrochemical coupling facilitated by the gap junction between the endothelium and vascular smooth muscle (VSM) (Fleming, 2004). Irrespective of its chemical nature, EDHF activates the endothelial calcium sensitive potassium channels (K_{Ca} channels) in response to changes in endothelial intracellular [Ca²⁺]_i levels (Ungvari *et al.*, 2002). The efflux of K⁺ thus induced, hyperpolarizes the endothelium and subsequently the VSM resulting in relaxation (Fleming, 2004). K_{Ca} function has been shown to be inactivated during insulin resistance (IR) (Dimitropoulou *et al.*, 2002; Katakam *et al.*, 1998; Takagawa *et al.*, 2001), thus disrupting endothelial hyperpolarization. Additionally, IR decreases Cyp2C expression and responses to EETs (Dimitropoulou *et al.*, 2002) in the rat mesenteric arteries (Katakam *et al.*, 2000). Therefore defects in the production of EDHF or EDHF-evoked K_{Ca} function may lead to increased responses to vasoconstrictors. The third vasodilator, prostacyclin is produced by the action of cyclooxygenase enzyme (COX) on arachidonic acid. However, most reports do not suggest a significant role for PGI₂ in relaxing mesenteric arteries (Chauhan *et al.*, 2003; Katakam *et al.*, 1999).

Cytochrome P450 (Cyp) is one of the most versatile enzyme systems in nature, which is responsible for catalyzing several processes such as metabolism, detoxification and biosynthesis of endogenous or foreign organic molecules. Its active site includes a heme ion bound to a protoporphyrin moiety (Shaik *et al.*, 2005). In humans Cyp's are concentrated in the liver and mediate a variety of oxidative reactions to detoxify or activate drug. However a few Cyp isoforms, 2C, 2J, 4A and 4F; have been identified in the cardiovascular and renal systems. These enzymes also called monooxygenases, are responsible for adding a single oxygen atom to arachidonic acid to form stereoselective vasodilator epoxide (EETs) and vasoconstrictor hydroxide (HETEs) derivatives (Roman, 2002).

1.7.2 *Insulin resistance and endothelial vasoconstrictors*

Equally important in the pathogenesis of hypertension in several etiologically distinct models of hypertension are endothelial vasoconstrictors. Endothelin-1 (ET-1) is a 21 amino-acid peptide primarily produced in the endothelial cells, which produces a sustained increase in vascular tone (Schiffrin *et al.*, 1998). Insulin stimulates the production and release of ET-1 as well as the expression of its receptor, which promotes vasoconstriction (Ferri *et al.*, 1996; Frank *et al.*, 1993; Hu *et al.*, 1993; Oliver *et al.*, 1991). ET-1 has been shown to be involved in elevating the blood pressure (BP) in a number of experimental models of hypertension such as the stroke-prone SHR, Dahl rats and angiotensin II-infused rats (Schiffrin, 1998). ET-1 also contributes to the rise in BP in fructose hypertensive rats (FHR), as treatment with the endothelin receptor antagonist bosentan prevented hypertension in this model (Cosenzi *et al.*, 1999; Verma *et al.*, 1995). Our laboratory has previously shown that the vascular ET-1 content is higher in FHR as compared to normal chow-fed rats (Verma *et al.*, 1995), in

addition to altered reactivity of the mesenteric arteries to ET-1 (Verma *et al.*, 1997b). An increase in the expressions of both ET-1 peptide and its ET_A receptor subtype have also been demonstrated in FHR (Juan *et al.*, 1998; Miller *et al.*, 2002). ET-1 has been shown to interact with other vasoconstrictor systems such as COX-2 and thromboxane A₂. Jiang *et al.* (2007) have shown increased aortic COX-2 expression and plasma TXA₂ in fructose-fed rats, which was prevented by treatment with bosentan (Jiang *et al.*, 2007). Recent reports from our laboratory have also shown crosstalk between ET-1 and the renin-angiotensin system (RAS) (Tran *et al.*, 2009), thus suggesting that not one, but a network of pathways are involved in inducing IR-associated vascular complications. However, it is unclear whether these pathways are unidirectional or mutually influence each other.

1.7.3 Arachidonic acid in vascular function

Arachidonic acid is a 20-carbon long fatty acid produced by the breakdown of membrane phospholipids by phospholipase A₂ (PLA₂) and phospholipases C and D (PLC and PLD) following an inflammatory stimulus (Bogatcheva *et al.*, 2005). In the endothelium, arachidonic acid is metabolized by various enzyme systems to yield vasoactive prostanoids and eicosanoids.

Metabolites of arachidonic acid constitute the second major class of endothelial vasoconstrictors. In addition to the formation of relaxing factors, endothelial arachidonic acid is also converted to potential vasopressor agents as shown in Figure 1.1 (Sarkis *et al.*, 2004) by various enzyme systems. These are:

1.7.3.1 Cyclooxygenase (COX)

Cyclooxygenase plays an important role in maintaining vascular tone. Cyclooxygenase breaks down arachidonic acid to release various vasoactive prostanoids, which influence both vascular contraction and relaxation (Figure A). Of the 2 isoforms, COX-1 and COX-2, characterized in the vasculature, COX-2 has been implicated to a greater degree in influencing vascular tone. Selective inhibition of COX-2 has been shown to attenuate responses to PE in the aortas of spontaneously hypertensive rats (Alvarez *et al.*, 2007). Insulin resistance, which is associated with chronic oxidative stress (Song *et al.*, 2005), upregulates several vasoconstrictor systems such as thromboxane A2 (Galipeau *et al.*, 2001), the renin-angiotensin system (Puyo *et al.*, 2007; Shinozaki *et al.*, 2004) and endothelin-1 (Miller *et al.*, 2002). Interestingly cyclooxygenase-2 (COX-2) is also upregulated in these conditions (El Midaoui *et al.*, 2002; Jiang *et al.*, 2007). A recent report has demonstrated that selective inhibition of COX-2 can prevent insulin resistance and thus other associated complications (Hsieh *et al.*, 2008). In addition, COX-2 plays an important role in the vasculature secondary to insulin resistance. Previous studies from our laboratory have demonstrated elevated levels of COX-2 mRNA (Song *et al.*, 2004) and protein (Jiang *et al.*, 2007) in the aortas of fructose-fed rats. COX-2 also provides the intermediate required for synthesizing thromboxane A2 (TXA2), which was elevated following insulin resistance (Galipeau *et al.*, 2001).

Cyclooxygenase may be a mediator of agonist-induced vasoconstriction. This is demonstrated by its involvement in the attenuated responses to PE following angiotensin receptor blockade (Alvarez *et al.*, 2007) thereby suggesting crosstalk between these two systems. In addition, separate studies have reported altered prostanoid levels in the

mesenteric bed of fructose-fed rats in the presence of angiotensin 2 and norepinephrine (Puyo *et al.*, 2007). Cyclooxygenase is also a downstream target of endothelin-1, which has been implicated in mediating cardiovascular complications secondary to insulin resistance (Jiang *et al.*, 2007; Sugiyama *et al.*, 2004). Thus it may be possible that in an insulin resistant milieu, isoform-specific or non-specific COX-dependent mechanisms may contribute to agonist-induced vasoconstriction. Currently, there are no functional data to demonstrate the specific contributions of individual COX isoforms in maintaining vascular tone.

1.7.3.2 Thromboxane A2 (TXA2)

Renal and/or vascular production of TXA2 is increased in various hypertensive animal models including SHR (Ishimitsu *et al.*, 1988a; Ishimitsu *et al.*, 1988b) and Dahl salt-sensitive rats (Yamashita *et al.*, 1988). Our laboratory has previously shown that hypertension in FHR is accompanied by an increase in plasma TXB2 levels, which is the stable metabolite of TXA2. Treatment with the TXA2 synthase inhibitor dazmegrel prevented the increase in BP. It was therefore suggested that hypertension in insulin resistant rats could be dependent on TXA2 synthesis (Galipeau *et al.*, 2001). The mechanism by which TXA2 becomes elevated is not fully known.

1.7.3.3 Arachidonic acid ω -hydroxylases

While epoxygenation of arachidonic acid produces EETs, hydroxylation at various positions results in the formation of hydroxyeicosatetranoic acids. Of these, 20-HETE is the most extensively studied HETE analog. It is produced by the action of Cyp4A and 4F on arachidonic acid in the vasculature (Figure A). The Cyp4A family of enzymes is also known

as ω -hydroxylases i.e. they hydroxylate the carbon at the terminal ω or $\omega-1$ position of saturated and unsaturated fatty acids (Kroetz *et al.*, 2005). Of the 13 genes identified in rats, Cyp4A1, A2 and A3 are the most extensively studied enzymes. These are expressed in the kidney, liver, mesenteric and cerebral vasculatures (Roman, 2002; Schwartzman *et al.*, 1996). Cyp4A expression is affected by various drugs such as clofibrate (Aoyama *et al.*, 1990) and PPAR agonists (Ishizuka *et al.*, 2003). Owing to a high degree of homology between the isoforms, isoform-selective antibodies are not available. Most reports have measured plasma or tissue levels of 20-HETE as an indicator of Cyp4A function. Isoform-specific changes have been reported using RT-PCR (Ito *et al.*, 1998; Wang *et al.*, 2001; Yamaguchi *et al.*, 2002).

20-HETE is a vasoconstrictor in various beds such as renal, cerebral and mesenteric vasculature (Roman, 2002). On the other hand, 20-HETE is a vasodilator in the pulmonary vasculature (Morin *et al.*, 2007; Zhu *et al.*, 2002). Several Cyp4A isoforms have been reported to catalyze 20-HETE synthesis. These are Cyp4A1 and 4A8. Cyp4A2/3 catalyzes the synthesis of both 20-HETE and EETs (Ito *et al.*, 1998; Wang *et al.*, 2001; Yamaguchi *et al.*, 2002). As the high sequence homology between the Cyp4A isoforms limits the development of isoform-specific antibodies, most studies on 20-HETE are based on gas/liquid chromatography-mass spectrometry analysis (GC/LC-MS). 20-HETE constricts a variety of blood vessels present in the renal, mesenteric and skeletal muscle beds. It blocks K_{Ca} channels, which mediates hyperpolarization (Miyata *et al.*, 2005). Recently Cheng *et al.* (Cheng *et al.*, 2008) have reported that 20-HETE uncouples eNOS in endothelial cell cultures. The attenuation of endothelial relaxation and subsequent increase in intracellular calcium in the vascular smooth muscle may explain the contribution of 20-HETE to impaired

relaxation. Several endogenous vasoconstrictors such as norepinephrine and angiotensin-2, whose responses are altered in insulin resistance, recruit 20-HETE as a downstream target. However the role of 20-HETE in insulin resistance is unclear. The effects of insulin on Cyp4A activity and thereby 20-HETE synthesis vary depending on the organ. Diabetes increases Cyp4A activity in the liver (Barnett *et al.*, 1993; Benter *et al.*, 2005b) while it is reduced in the kidney (Chen *et al.*, 2008). Studies on high fat fed rats show decreased 20-HETE levels in the kidney despite obesity and an increase in blood pressure (Laffer *et al.*, 2004; Wang *et al.*, 2003). Although a couple of studies in human obese subjects attempted to study the association between circulating insulin and 20-HETE levels, the findings are inconclusive (Ward *et al.*, 2006). 20-HETE plays a major role in altering vascular reactivity and increasing blood pressure in SHR (Schwartzman *et al.*, 1996; Wang *et al.*, 2001). Similarly, elevated 20-HETE has also been reported in other models of hypertension such as stroke-prone SHR (Dunn *et al.*, 2008) and androgen infused rats (Nakagawa *et al.*, 2003).

1.7.4 The renin angiotensin system (RAS): The RAS plays a very important role in regulating the renal and vascular arms of blood pressure along with maintaining renal electrolyte balance. Angiotensin-II impairs peripheral, cardiac and vascular insulin-dependent substrate metabolism and energy production by interfering with the downstream signaling of insulin receptor substrate (IRS)-1 (Giani *et al.*, 2009). Inhibition of IRS-1 prevents the activation of PI3K/Akt, which is essential for producing endothelial nitric oxide and metabolizing glucose (Olivares-Reyes *et al.*, 2008). Our laboratory has recently demonstrated increased plasma AII levels in fructose-fed rats (Tran *et al.*, 2009). In addition, insulin resistance also upregulates plasma angiotensin 2 levels (Tran *et al.*, 2009) and the

angiotensin II Type 1 (AT-1) receptor (Tran *et al.*, 2009; Zhou *et al.*, 2009). Treatment with agents such as ramipril (Yavuz *et al.*, 2003), angiotensin 1-7 or losartan (Bahadir *et al.*, 2007) has been shown to improve insulin sensitivity and decrease the blood pressure in both humans and experimental models. As a result, the RAS is a key player in the metabolic syndrome. Furthermore, the RAS has also been implicated in the induction of cardiovascular tissue fibrosis and vascular remodeling by activating matrix metalloproteinases (MMPs) 2 and 9 (Brassard *et al.*, 2005; Guo *et al.*, 2008; Tsuruda *et al.*, 2004). Although separate studies have implicated the RAS (Rizzoni *et al.*, 2008) or MMP pathways (Boden *et al.*, 2008; Miksztowicz *et al.*, 2008) in IR-associated vascular complications, direct evidence linking the 2 pathways is unavailable. Angiotensin II constricts blood vessels by recruiting downstream pathways such as COX-2 (Alvarez *et al.*, 2007) and 20-HETE (Croft *et al.*, 2000). Similar to AII, inhibition of IRS-1 has also been reported with other IR-related mediators such as tumor necrosis factor alpha (TNF- α) (Liang *et al.*, 2008) and fatty acids (Anderwald *et al.*, 2007; Silveira *et al.*, 2008), suggesting high oxidative stress and a proinflammatory environment are associated with attenuated insulin action.

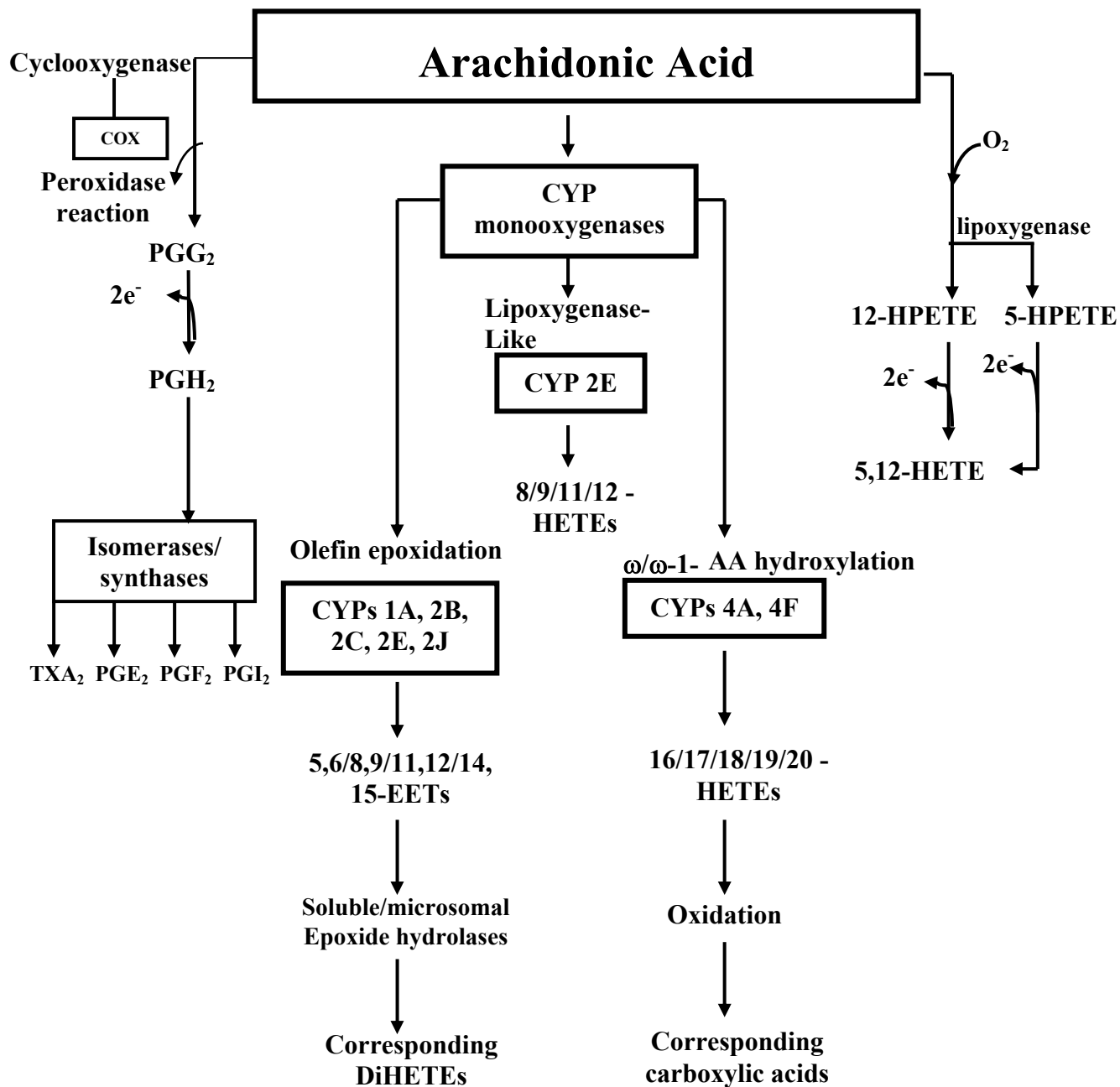


Figure 1.1 Schematic representation of the major pathways involved in the metabolism of arachidonic acid. The figure is adapted from the review by Sarkis and Roman (Sarkis *et al.*, 2004).

1.8 Role of gender and sex hormones in insulin resistance and hypertension

In humans, while cardiovascular disease (CVD) affects both sexes, the risk of developing hypertension and other CVD is approximately doubled in males as compared to age-matched females according to a study conducted by the World Health Organization (Gorodeski *et al.*, 1999). The difference in ratio is observed until the fourth and fifth decades, where the risk of CVD in women starts rising and nearly reaches a 1:1 ratio with males by the seventh decade of life. The time indicated coincides with the development of menopause in women accompanied by reduced levels of female sex hormones, particularly estrogen. As a result, treatment with estrogen post menopause was thought to be an effective therapeutic strategy in reducing mortality due to CVD (Baker *et al.*, 2003). Similar differences are observed between both adult and adolescent (Li *et al.*, 2009) men and women in susceptibility to the metabolic syndrome including insulin resistance and hypertension. This difference in susceptibility could be due to the genetic and gender-dependent differences in anatomical fat distribution. Thus while females show more subcutaneous fat, males have greater abdominal fat content, which reflects functional levels of gonadal hormones. Although the role of gender in adipose redistribution is unclear, increased abdominal fat has been reported in both humans (Le *et al.*, 2006a) and rats (Alzamendi *et al.*, 2009; de Moura *et al.*, 2008) fed with fructose. Fructose feeding caused a redistribution of fat and an increase in visceral fat. Thus estrogen was suggested to play a protective role in preventing CVD. However major clinical studies such as the Women's Health Initiative (WHI) (Wassertheil-Smoller *et al.*, 2003) or the HERS (Grady *et al.*, 2002; Hulley *et al.*, 1998) trials reported no improvement in the post menopausal incidences of thrombosis and heart disease in women treated with estrogen alone or in combination with progestins or no hormone treatment. Interestingly recent reports

suggest oversight or lack of understanding of parameters critical to designing these studies, which has led to renewed interest in the metabolic and cardiovascular effects of estrogen. Our laboratory has previously reported that gonadal estrogen protected both male and female rats from developing insulin resistance and hyperinsulinemia (Galipeau *et al.*, 2002a; Galipeau, 2001). However, the blood pressure in female rats was similar to controls, whereas in males treated with estrogen, systolic blood pressure was greater than control but less than fructose-fed rats (Vasudevan *et al.*, 2005). Only in the gonadectomized rats were blood pressure values similar to controls despite the development of insulin resistance (Vasudevan *et al.*, 2006; Vasudevan *et al.*, 2005). Estrogen treatment prevented insulin resistance in both intact and gonadectomized rats without affecting the blood pressure. Estrogen and testosterone protected and permitted respectively the development of insulin resistance in Wistar rats fed a high fat diet (Gomez-Perez *et al.*, 2008). Clinically both insulin resistance and hypertension are observed in polycystic ovary syndrome where testosterone is relatively higher following the loss of estrogen (Escobar Morreale, 2008). In summary, we suggest that more than the presence of estrogen, the absence of testosterone plays a key role in regulating blood pressure secondary to insulin resistance.

1.9 Effects of testosterone on hypertension

The male sex hormone testosterone and its effects on the cardiovascular system has been an interesting area of research. As estrogen appeared to prevent the development of cardiovascular disease in insulin resistant rats, the question arose as to whether testosterone was involved in promoting hypertension. To date, relatively little is known regarding the influence of androgens on blood pressure and CVD. Clinical studies have been unable to

clarify whether testosterone promotes or prevents the induction of CVD. Most clinical studies advocate testosterone therapy to improve insulin sensitivity and reduce secondary complications (La Vignera *et al.*, 2008). In addition, most of these studies are controlled for the gender variable. Studies such as analysis of the Rancho Bernardo study involving men between ages 30 and 79 have suggested a higher risk of coronary artery disease (CAD), myocardial infarction and hypertension in males with lower circulating levels of testosterone (Hughes *et al.*, 1989; Jaffe *et al.*, 1996; Jones *et al.*, 2004b; Kalin *et al.*, 1990; Khaw *et al.*, 1988; Liu *et al.*, 2003). However this claim is unresolved, as males have been found to have higher blood pressure as compared to age-matched premenopausal women. Similarly higher testosterone levels have been associated with insulin resistance in males with alopecia (Gonzalez-Gonzalez *et al.*, 2008). On the other hand, studies in animals indicate a strong correlation between the presence of testosterone *in vivo* and the development of hypertension. Removal of the testes in male rats has been shown to reduce blood pressure, which was restored by replacing testosterone *in vivo*. Indeed, isolated arteries from gonadectomized rats exhibited decreased pressor responses to various vasoconstrictors (Gonzales *et al.*, 2004; Stallone, 1994). Blocking the testosterone receptor in spontaneously hypertensive rats (SHR) using flutamide (Reckelhoff *et al.*, 1999) resulted in decreased blood pressure, in addition to increased vasorelaxation *in vitro* (Ba *et al.*, 2002); thus indicating a genomic effect of testosterone receptor activation in facilitating the development of hypertension. Furthermore, gonadectomy resulted in a reduction in the renal angiotensinogen and renin mRNA levels of SHR, which were restored by testosterone implantation (Chen *et al.*, 1992). Testosterone *in vivo* also influences the expression and functions of other vasoconstrictor pathways such as COX-2, TXA2 and 20-HETE. The effects of testosterone

on 20-HETE synthesis will be discussed in detail in section 1.9.3. Thus experimental evidence suggests a potential role for testosterone in the development of hypertension.

However, testosterone also relaxes blood vessels by its non-genomic actions. Reports on isolated mesenteric arteries (Tep-areenan *et al.*, 2002) and aorta (Tep-areenan *et al.*, 2003) have demonstrated that incubation with testosterone produces endothelium-dependent relaxation whereas in coronary arteries the relaxation is by direct calcium antagonism in the smooth muscle (Jones *et al.*, 2004a).

1.10 Role of testosterone in insulin resistance, blood pressure and vascular reactivity

The effects of gender and sex hormones on the interrelationship between insulin sensitivity and hypertension were unknown until recently. Our laboratory has demonstrated that the sex hormones estrogen and testosterone are protective and permissive, respectively to the induction of hypertension. Thus male fructose-fed rats developed insulin resistance, hyperinsulinemia and hypertriglyceridemia along with elevated blood pressure which did not occur in females (Galipeau *et al.*, 2002b). The loss of testosterone by gonadectomy prevented endothelial dysfunction and elevation in blood pressure although insulin resistance was unchanged (Song *et al.*, 2004; Vasudevan *et al.*, 2006). In addition, estrogen improved insulin sensitivity in both intact and gonadectomized rats but did not affect blood pressure in gonadectomized rats (Vasudevan *et al.*, 2005). Similar studies on the association between testosterone and blood pressure have been conducted on sucrose-treated rats by Banos' group using intact and gonadectomized male rats treated with 30% sucrose in the drinking water for 24 weeks (Banos *et al.*, 2005; Perez-Torres *et al.*, 2008; Torres *et al.*, 2007). Thus

testosterone is essential for the alterations in vascular function and blood pressure. However it needs to be confirmed if replacing testosterone would restore the increase in blood pressure in FFR. Testosterone has been strongly associated with the development of insulin resistance and hypertension in women with polycystic ovary syndrome (PCOS) (Hart *et al.*, 2004). This disease is associated with the loss of ovarian hormones and affects premenopausal women during their reproductive years. Further, such patients are hyperandrogenic and oligomenorrhic (Hart *et al.*, 2004). Recent reports have made a strong case for the induction of endothelial dysfunction following PCOS, which has been associated with hyperandrogenism (Orio *et al.*, 2004; Paradisi *et al.*, 2001; Paradisi *et al.*, 2003). In postmenopausal women, due to the loss of estrogen, a relative hyperandrogenic state along with elevated risk of IR and hypertension was observed (Rexrode *et al.*, 2003; Van Beek *et al.*, 2004). Similar hyperandrogenic states have been reported in pregnant women with preeclampsia (Laivuori *et al.*, 1998). In combination with other reports, our studies indicate a differential regulation of insulin resistance by estrogen and testosterone (Vasudevan *et al.*, 2005). In fructose-fed rats testosterone levels are unchanged compared to controls suggesting that the mere presence of testosterone is sufficient to induce secondary complications (Vasudevan *et al.*, 2006). This led us to investigate the testosterone-dependent vasoactive pathway(s) that are activated following insulin resistance, which induce endothelial dysfunction and increase blood pressure. Some of these pathways are discussed below.

1.10.1 The renin-angiotensin system

The importance of testosterone in RAS function has been studied in several models of hypertension such as the SHR, Dahl SS rats and the fructose-fed rats. In the SHR, loss of

testosterone prevented the RAS-mediated increase in blood pressure. Other studies have shown a gender-dependent effect of AII on the central nervous system and sympathetic outflow (Xue *et al.*, 2007). While separate studies have demonstrated interactions between AII, COX-2, 20-HETE and norepinephrine, their functions seem to be directly or indirectly regulated by the presence of testosterone. This suggests a potential interrelationship between these systems, which merits further investigation.

In addition testosterone also regulates the synthesis of several vasoactive metabolites of arachidonic acid. These are:

1.10.2 Prostanoids/Cyclooxygenase-2

Fructose feeding elevated the gene expression of the inducible cyclooxygenase isoform, COX-2 in the mesenteric arteries and the thoracic aorta, which was normalized by gonadectomy (Song *et al.*, 2004). This indicated that testosterone may affect the development of hypertension in hyperinsulinemic/insulin resistant animals via the COX pathway and thus arachidonate metabolism (Song *et al.*, 2004). Some studies have also reported the involvement of COX in inducing insulin resistance (Hsieh *et al.*, 2008), which was normalized by treating with celecoxib. However some questions remain unanswered. Although fructose increased aortic COX 2 mRNA expression, the effects of testosterone on the individual contributions of COX 1 and 2 in mediating vasoconstrictor responses to endogenous vasoconstrictors such as norepinephrine need to be investigated. Thromboxane A2 may be another potential target. Although Higashiura *et al* and other groups suggest a testosterone-dependent upregulation in the TXA2 receptor density in the coronary and aortic smooth muscle cells (Higashiura *et al.*, 1997; Matsuda *et al.*, 1995), Jiang et al did not find

any changes in the TXA2 receptor expression between the aortas of control and fructose-fed rats (Jiang *et al.*, 2007). Additional studies are needed to resolve this issue.

1.10.3 Cyp4A/20-HETE

As discussed in section 1.42, Cyp4A/20-HETE pathway is the most investigated among Cyp4A catalyzed metabolites. Reports have suggested the involvement of various Cyp4A isoforms such as 4A1, 2/3 and 8 in rat while in mice Cyp4A11 and 4A14 have been reported (Holla *et al.*, 2001; Roman, 2002). In SHR, 20-HETE levels are elevated in the presence of testosterone and gonadectomy reverses this increase (Ishizuka *et al.*, 2004). Also rats infused with dihydrotestosterone show increased 20-HETE levels (Nakagawa *et al.*, 2003). One of the earliest findings that demonstrated the influence of testosterone was in Cyp4A14 knock-out mice where Cyp4A 11 expression and 20-HETE levels were elevated. Gonadectomy decreased 20-HETE levels, which was restored by testosterone replacement (Holla *et al.*, 2001). Although its role in insulin resistance is unclear, 20-HETE is implicated as a downstream target of A-II (Croft *et al.*, 2000), which is increased in insulin resistance. We also found that in vitro inhibition of 20-HETE synthesis by 1-aminobenzotriazole (ABT) improved the relaxation to acetylcholine in intact but not gonadectomized fructose-fed rats (Vasudevan, 2005). This suggests that the loss of insulin action may be the stimulus needed to decrease endothelial function and induce a concomitant increase in 20-HETE. Taken together, Cyp4A/20-HETE may be a key pathway, which is recruited in the presence of testosterone to induce endothelial dysfunction and increase blood pressure secondary to insulin resistance.

2. OVERALL RATIONALE, HYPOTHESES AND OBJECTIVES

2.1 Rationale

The present thesis extends our previous work where we demonstrated that (1) Estrogen prevents, while testosterone promotes, the development of endothelial dysfunction and hypertension secondary to insulin resistance (Vasudevan, 2005). (2) The mere presence of testosterone rather than the presence or absence of estrogen plays a key role in the development of these complications. (3) Endothelial dysfunction arises due to IR-induced impairment in NO and EDHF-mediated relaxation, (4) Preliminary evidence suggesting that prohypertensive arachidonic acid metabolites may be involved in inducing endothelial dysfunction.

Based on the current literature and previous data from our laboratory we can summarize the following:

Insulin resistance impairs endothelial function by impairing NO and EDHF-mediated relaxation, which plays an important role in elevating blood pressure. The mere presence of testosterone is essential and sufficient to promote these processes. However, we needed to confirm the role of testosterone in promoting these complications. We also needed to confirm if the effects of testosterone were mediated through the androgen receptor or through non-genomic pathways. Interestingly insulin sensitivity does not influence circulating testosterone and vice versa. Thus the question arose “Is/are there a testosterone dependent pathway(s) that is/are upregulated secondary to testosterone? If so do they act individually or regulate one another? Although several testosterone-dependent pathways have been studied in the cardiovascular and renovascular systems, such as COX, RAS and 20-HETE, their contribution to basal or agonist-induced vasoreactivity is unclear in states of insulin

resistance. In addition, several vasoactive metabolites that are testosterone dependent are products of arachidonic acid metabolism. These agents such as TXA2 and 20-HETE and their interactions with NO and EDHF have been well documented in various vascular beds. Furthermore they are downstream of the ET-1 and RAS pathways, which implicates them as key targets in preventing endothelial dysfunction and other associated complications.

2.2 Hypotheses

We hypothesize that: **(1) Insulin resistance induces a shift in the equilibrium between the vasoactive metabolites of arachidonic acid, which contributes to the development of endothelial dysfunction and subsequent elevation of blood pressure and (2) Testosterone is required *in vivo* for effecting this switch towards increased vasoconstrictor function.**

2.3 Objectives

In order to test these hypotheses we asked the following questions:

1. **Does testosterone replacement in gonadectomized fructose-fed rats restore the hypertensive state secondary to insulin resistance?** We investigated whether testosterone replacement therapy in gonadectomized fructose-fed male rats will restore the state of hypertension as observed in fructose-fed rats with intact testes.
2. **How does testosterone increase the blood pressure in fructose-fed rats? Is it mediated through the androgen receptor? Is it by stimulating 20-hydroxyeicosatetranoic acid (20-HETE) synthesis?** We investigated the role of the

androgen-receptor and Cyp4A in the development of high blood pressure secondary to insulin resistance.

3. **Does 20-HETE indeed play a role in the induction of endothelial dysfunction and hypertension secondary to fructose feeding?** Using a Cyp4A selective inhibitor we investigated the involvement of 20-HETE in the development of endothelial dysfunction and high blood pressure secondary to insulin resistance.
4. **Does insulin resistance, by itself, deregulate the synthesis and actions of vasoactive metabolites of arachidonic acid? If so, how does the presence of testosterone contribute to this deregulation?** We attempted to validate the role of insulin resistance in the development of endothelial dysfunction and high blood pressure. Secondly, we studied the changes in individual vasoactive agents in the presence of insulin resistance. Finally we determined whether these changes were dependent or independent of testosterone.
5. **What is the role of cyclooxygenase in phenylephrine (PE)-induced vascular contraction? If involved, is it isoform-specific?** We investigated the specific contributions of cyclooxygenase 1 and 2 in the pressor responses to PE in the aorta and superior mesenteric arteries. We also measured the changes in the expression of various proteins such as COX-2 and Cyp4A1, 2/3 in these tissues.

3. MATERIALS AND METHODS

3.1 Animals

In all our studies, 6 week old male Wistar rats used. They were shipped from Charles River, Montreal, Canada. In studies 3.4.1, 3.4.4 and 3.4.5, half of the rats were surgically gonadectomized at the age of 5 weeks. The age-matched rats with intact testes underwent a sham surgery to eliminate any potential effects of the surgery. Following arrival, the rats were allowed to acclimatize in the animal care facility at the Faculty of Pharmaceutical Sciences, UBC and cared for as per the guidelines given by the Canadian Council for Animal Care (CCAC). The study protocol was pre-approved by the university animal care centre (Animal care Certificate enclosed in Appendix). When the rats were 7-weeks old, basal blood pressure recordings were obtained using the tail-cuff method (3.5.2). prior to fructose feeding, blood was collected for measuring various basal plasma parameters. Unless mentioned otherwise, the rats were allowed *ad libitum* access to food and water.

3.2 Drugs

Some of the drugs used in our in vivo/in vitro studies are described below. The other drugs are well characterized molecules both in terms of their chemistry and therapeutic/inhibitory values. They have been extensively tested and reviewed.

1. 1-aminobenzotriazole (ABT): A suicide substrate for Cyp enzymes, ABT is one of the standard compounds used in drug metabolism studies. It covalently inhibits the heme prosthetic group of the Cyp enzyme subsequent to its activation via formation of a benzyne adduct (Linder *et al.*, 2009; Ortiz de Montellano *et al.*, 1981). However ABT is an isoform

non-selective inhibitor of Cyp. Until the development of Cyp4A selective inhibitors and in recent studies, ABT has been the drug of choice for studying the role of Cyp4A and 20-HETE in hypertension (Benter *et al.*, 2005a; Chabova *et al.*, 2007). In isolated superior mesenteric arteries from fructose-fed rats, incubation with ABT (2×10^{-4} M) improved the relaxation to ACh. However this phenomenon was observed only in vessels from fructose-fed rats with intact testes. Tissues from gonadectomized rats did not respond to ABT, which suggested that the involvement of 20-HETE was testosterone-dependent (Vasudevan, 2005).

2. HET0016: N-(4-Butyl-2-methylphenyl)-N0-hydroxyformamidine (HET0016) is the most potent and selective inhibitor of 20-HETE synthesis. Its IC₅₀ values for inhibiting 20-HETE synthesis in human and rat renal microsomes were less than 12 nM and ~40 nM respectively (Miyata *et al.*, 2001). HET0016 inhibited COX and other Cyp isoforms such as 2C9, 3A4 and 2D6 in micromolar to millimolar concentrations, thus indicating a high selectivity towards Cyp4A. In contrast, other Cyp inhibitors such as 1-ABT and 17-ODYA exhibited micromolar IC₅₀ values for both Cyp4A and Cyp2C (Miyata *et al.*, 2001; Sato *et al.*, 2001). Studies on recombinant individual Cyp4A isoforms have also shown that HET0016 is a selective, noncompetitive and irreversible Cyp4A inhibitor (Seki *et al.*, 2005). Inhibition of 20-HETE synthesis by HET0016 has been shown to prevent angiogenesis in HUVEC and U251 human glioblastoma cancer cells (Chen *et al.*, 2005) and ameliorate cerebral infarcts and vascular function (Dunn *et al.*, 2008). However, in the kidney inhibition of 20-HETE has been shown to promote salt sensitive hypertension (Hoagland *et al.*, 2003).

HET0016 has a short half life in the body which necessitates a minimum dosing of twice a day. The drug is suspended in a 20% warm lecithin suspension by sonication and injected i.p.

3. DDMS: N-methylsulfonyl-12, 12-dibromododec-11-enamide (DDMS) is a Cyp4A inhibitor with an IC_{50} value of 2 μ M, which although better than ABT, is relatively less potent compared to HET0016. However DDMS was selective for Cyp4A as its IC_{50} value for Cyp2C inhibition was 60 μ M (Wang *et al.*, 1998).

4. NS-398: N-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide (NS-398) is a COX-2 selective inhibitor. Although not as selective as several classical NSAIDs, NS-398 exhibits high selectivity towards ovine COX-2 ($IC_{50} \sim 4 \mu$ M) compared to COX-1 (IC_{50} 100 μ M) (Futaki *et al.*, 1994). NS-398 showed a 260-fold selectivity in inhibiting recombinant COX-2 over COX-1 (Ouellet *et al.*, 1995).

5. SC-560: 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560) is an orally active diaryl heterocyclic drug. It is a potent and selective inhibitor of COX-1 with IC_{50} value of 9 nM as compared to 6.3 μ M for COX-2 (Smith *et al.*, 1998).

6. Metformin: Metformin has been extensively used over the last few decades to improve insulin sensitivity in both experimental and clinical conditions. The pharmacology of metformin has been extensively reviewed by Correia *et al.* (Correia *et al.*, 2008). An insulin sensitizer of the biguanide class, metformin has recently attracted attention due to its positive effects on AMP-activated kinase (AMPK), which acts as a fuel gauge in hyperglycemic states to activate insulin release (Musi *et al.*, 2002; Zhou *et al.*, 2001). Further, metformin has also been shown to improve glucose tolerance by non-AMPK-

dependent mechanisms such as activation of the MAP-kinase pathway (Saeedi *et al.*, 2008). We used a 500 mg/kg/day dose in the drinking water as this has been previously shown in our laboratory to prevent insulin resistance and associated secondary complications (Verma *et al.*, 1994b; Verma *et al.*, 2000).

3.3 Reagents

The 60% fructose diet was obtained from Teklad laboratory diets, Madison, WI. Potassium chloride, phenylephrine, acetylcholine, NS-398, SC-560, indomethacin, charybdotoxin, apamin and 1-aminobenzotriazole were purchased from Sigma. DDMS was purchased from Cayman Chemicals. Metformin hydrochloride and HET0016 were gift samples kindly provided by Apotex Inc. Toronto and Taisho Pharma, Japan respectively. Buffer chemicals unless specified were obtained from Sigma. Phenylephrine hydrochloride, acetylcholine chloride and L-NAME were dissolved in Krebs Ringer buffer while CTX and apamin were dissolved in freshly distilled water. NS-398 and SC-560 were dissolved in dimethylsulfoxide and 20 µl solution was added to each tissue.

3.4 Studies conducted

3.4.1 Effects of testosterone replacement on blood pressure in fructose-fed rats

The study design and experimental protocol in study A are reproduced from Vasudevan et al. 2006 (Vasudevan et al., 2006) – used with permission.

To confirm that the presence of testosterone was indeed responsible for the development of hypertension, we studied the changes in blood pressure in fructose-fed rats subsequent to exogenous provision of testosterone. Sham-operated and gonadectomized male

Wistar rats were divided into 6 experimental groups, sham-operated chow-fed control (C; n=4), sham-operated fructose-fed (F; n=5), gonadectomized normal chow-fed (G; n=4), gonadectomized-fructose-fed (GF; n=4), gonadectomized normal chow-fed and testosterone-treated (GT; n=4) and gonadectomized-fructose-fed and testosterone-treated (GFT; n=5). The rats were started on the fructose-enriched diet. To extend our previous findings, which demonstrate the role of testosterone in the development of hypertension (Song *et al.*, 2004; Vasudevan, 2005), we determined the changes in insulin sensitivity and blood pressure upon reintroduction of testosterone. Following 5 weeks of normal chow or fructose feeding, 9 of the gonadectomized male rats (GT and GFT) were injected intraperitoneally with 10 mg/kg/day testosterone propionate (UnivetTM suspension). The dose was based on previous literature reports where using testosterone propionate provided physiological levels of testosterone (Khalid *et al.*, 2002). The rats were injected daily for 5-6 weeks. Blood was withdrawn after seven days of treatment for measuring testosterone. After 5 weeks of treatment, i.e. study week 10, the blood pressure was measured and a truncated oral glucose tolerance test was performed the following week. Briefly, blood was collected at time points of 0, 10, 30 and 90 minutes subsequent to glucose ingestion. The animals were terminated and blood was collected by cardiac puncture for analysis of plasma testosterone.

VASUDEVAN, H., NAGAREDDY, P.R. & MCNEILL, J.H. (2006). Gonadectomy prevents endothelial dysfunction in fructose-fed male rats, a factor contributing to the development of hypertension. *Am J Physiol Heart Circ Physiol*, **291**, H3058-64 (*Used with Permission*).

3.4.2 Role of insulin resistance and testosterone on Cyp4A expression

The objectives of this study were: (1) To ascertain whether testosterone-dependent increase in blood pressure following insulin resistance is mediated by the androgen receptor. (2) If Cyp4A expression is affected by insulin resistance or testosterone or both.

Seventy-two 6-week old male Wistar rats (210-240 gms) were divided into six groups of 12 rats each: control (C), fructose-fed (F), control + flutamide (anti-androgen) (CF), fructose + flutamide (FF), control + 1-aminobenzotriazole (ABT; Cyp4A inhibitor) (CA) and fructose + ABT (FA). Subsequent to measuring their baseline systolic blood pressure, the animals were kept on high fructose diet or normal rat chow for 9 weeks. At the end of 9 weeks blood pressure was measured again and two groups, CF and FF were injected with a suspension of flutamide in castor oil (8 mg/kg/day, s.c.) (Reckelhoff *et al.*, 1999), while another 2 groups, CA and FA, were treated with 25 mg/kg/day i.p. ABT for 3 weeks each (Llinas *et al.*, 2004). Following treatment, blood pressure was measured. The rats were subjected to an oral glucose tolerance test, subsequent to which they were sacrificed. Blood was collected by cardiac puncture into polypropylene tubes containing 100 IU heparin and centrifuged at 4500 rpm, 4°C for 25 minutes to obtain plasma for measuring testosterone and 20-HETE.

3.4.3 Effect of selective Cyp4A inhibition on insulin sensitivity and blood pressure

As ABT is a non-selective Cyp inhibitor, we needed to obtain direct evidence with a more potent and Cyp4A selective inhibitor. We used HET0016, which is a Cyp4A selective inhibitor of 20-HETE synthesis. Thirty-two rats were divided into 4 groups (n=8 per group), namely control normal chow-fed (C), control fructose-fed (F), control treated (CT) and

fructose-fed and treated (FT). Rats were fed with the normal or the fructose-rich chow for 9-11 weeks to render them insulin resistant. Following 8 weeks of fructose feeding, rats in groups CT and FT were treated with HET0016 (10 mg/kg i.p.; daily) (Blanton *et al.*, 2006; Hoagland *et al.*, 2003) for 2 weeks until termination. At the end of 9 weeks (post 1 week of HET0016 treatment), induction of insulin resistance was confirmed by the oral glucose tolerance test. Prior to feeding fructose and at the end of 9 weeks, systolic blood pressure was recorded. Rats were sacrificed and blood was collected by cardiac puncture for measuring 20-HETE and other eicosanoids. The mesenteric bed was isolated, cleaned and frozen for determining changes in Cyp4A protein expression.

3.4.4 Role of insulin resistance and testosterone in vascular arachidonic acid metabolism and vascular reactivity

We were interested in studying androgen-dependent changes in arachidonate metabolism following insulin resistance. Understanding this phenomenon in terms of Cyp4A expression and 20-HETE functions may enable us to shed light on the possible chain of biochemical events, which occur subsequent to IR and influence the pathogenesis of hypertension.

3.4.4.1 Experimental Design

Six-week old male Wistar rats were divided into 8 groups: control (C; n=8), fructose-fed (F; n=8), control-metformin treated (CM; n=8), fructose-metformin (FM; n=8), gonadectomized (G; n=8), gonadectomized fructose-fed (GF; n=8), gonadectomized-metformin (GM; n=8) and gonadectomized fructose-fed + metformin (GFM; n=8). After measuring their baseline

systolic blood pressure, the animals were kept on the fructose diet or normal rat chow for 10 weeks. Four groups, CM, FM, GM and GFM were treated with the insulin sensitizer, metformin (500 mg/kg/day, p.o.) (Verma *et al.*, 2000) for 10 weeks. Metformin, obtained as a gift sample from Apotex Inc., was administered in the drinking water. Blood pressure and insulin sensitivity were measured in addition to changes in daily fluid intake and body weights. The rats were sacrificed and blood was collected. The superior mesenteric arteries were isolated, cleaned of connective tissue, and cut into 3 mm ring segments. Tissues were mounted between two stainless steel hooks in an organ bath and connected to a force transducer. Tissues were bathed in warmed, oxygenated (37°C, 95% O₂) modified Krebs-Ringer buffer. Changes in tension were recorded with a Grass polygraph chart recorder.

3.4.4.2 Vascular reactivity studies

Subsequent to the challenge with 40 mM KCl and assessment of endothelial integrity, dose response curves to increasing concentrations of phenylephrine (PE) were generated. Following precontraction of the tissues with the ED₇₀ dose of PE, relaxation responses to graded doses of acetylcholine (ACh) were obtained in the presence and absence of the following drugs: N^o-nitro-L-arginine methyl ester (L-NAME; 10⁻⁶ M), indomethacin (10⁻⁵ M) and a combination of charybdotoxin (CTX 10⁻⁸ M) plus apamin (0.25 x 10⁻⁶ M). Together, CTX and apamin induce total blockade of the K_{Ca} resulting in a complete loss of EDHF action (Coleman *et al.*, 2004). Further, the tissues were also incubated with DDMS (10⁻⁵ M), a selective Cyp4A blocker. This was to confirm our previous results using 1-ABT.

Although several studies have reported the concentration for L-NAME to be 10^{-5} M, our previous experience showed that 10^{-5} M or higher concentrations of L-NAME completely inhibited the endothelium-dependent relaxation in the SMA.

3.4.5 Role of testosterone and cyclooxygenase 1 and 2 in mediating alpha-adrenoceptor-stimulated vasoconstriction

Our objectives in this study were: (1) To examine the effect of COX-inhibition on endothelium-dependent relaxation. (2) To identify the individual contributions of COX-1 and COX-2 to PE-induced vasoconstriction in the SMA and aorta of normal and fructose-fed rats and (3) To determine whether testosterone affects the effects of COX in regulating vascular reactivity.

3.4.5.1 Experimental Design

Rats were divided into 4 groups, sham-operated normal chow-fed control (C; n=8), sham-operated fructose-fed (F; n=8), gonadectomized normal chow-fed (G; n=8) and gonadectomized-fructose-fed (GF; n=8). Prior to initiation of the fructose diet, blood was collected from the rats following a 5-hour fast for measuring glucose, insulin and testosterone.

Blood pressure was measured at 0 and 9 weeks of fructose-feeding as mentioned in studies A-E. At the end of 9 weeks of fructose feeding, an oral glucose tolerance test was performed on the rats as previously described below in 3.1. At termination, the rats were euthanized by a single injection of 60 mg/kg i.p. pentobarbital (EuthanylTM) followed by opening of the chest cavity. Blood was collected to obtain plasma. The superior mesenteric artery (SMA)

and thoracic aorta were isolated to determine changes in vascular reactivity and protein expression.

3.4.5.2 Vascular reactivity studies

Tissues were assessed for changes in contraction to phenylephrine (PE) (10^{-9} - 10^{-4} mol/L), in the presence of the following drugs: (1) A selective COX-2 inhibitor (NS-398; 10^{-6} M); (2) A selective COX-1 inhibitor (SC-560; 10^{-5} M) and (3) A non-selective COX inhibitor (indomethacin; 10^{-5} M). These drugs have been individually discussed in detail in section 3.2. Our aim was to individually inhibit COX-2 and COX-1 followed by total blockade with indomethacin to provide an insight about the role played by each isoform in vascular reactivity.

Briefly, following the challenge with 40 mM KCl and confirmation of endothelial integrity, a basal cumulative response curve to PE was obtained. Post equilibration, the tissues were incubated with NS-398, SC-560 and indomethacin (in that order) for 20 minutes each. Responses to PE were taken after each treatment. The responses to PE were compared between sham-operated and gonadectomized fructose-fed animals and rats maintained on standard lab diet. At the end of the experiment, the tissues were blotted onto KimWipes® and weighed. Responses to PE are reported as mg/sq. mm in the SMA and as percentage of maximum KCl contraction in the aorta.

3.5 General methodology

Techniques 3.5.1-3.5.4 are discussed in brief. For additional details please refer (Vasudevan, 2005)

3.5.1 Assessment of insulin resistance/sensitivity

At the end of 9 weeks of fructose feeding, an oral glucose tolerance test (OGTT) was performed to assess changes in insulin sensitivity and glucose clearance in rats. Following an overnight fast (16 hours) rats were orally gavaged with a 1g/kg glucose (40% solution). Blood samples were withdrawn from the tail vein at 0, 10, 20, 30, 60 and 90 minutes respectively after the glucose load. The blood was centrifuged in a 4°C Beckmann tabletop centrifuge (Beckmann Allegra 21R) at 14000 rpm for 25 minutes. Once spun, the plasma was collected and frozen at -80°C.

Insulin sensitivity index (ISI) was calculated as per the formula given by DeFronzo and Matsuda (Matsuda *et al.*, 1999), which is:

$$\text{ISI} = \frac{100}{\text{Square root of } [(FPG \times FPI) \times (MPG \times MPI)]}$$

Samples for measurement of fasting plasma glucose (FPG) and insulin (FPI) values were taken just before glucose administration (after fasting for 16 hours), while mean plasma glucose (MPG) and insulin (MPI), were calculated as the mean values of all the time points considered in the test i.e. time 0 (before glucose gavage) to 90 minutes (after glucose gavage). Among all methods used, the values obtained by the oral glucose tolerance test

(OGTT) offered the best correlation with values from the euglycemic hyperinsulinemic clamp,(Matsuda *et al.*, 1999) which is highly regarded as the standard for measuring insulin sensitivity.

3.5.2 Measurement of systolic blood pressure

Animals were preconditioned to the procedures prior to actual measurements. Systolic blood pressure was measured in conscious rats using the indirect non-invasive tail-cuff method as previously described (Bunag, 1973; Galipeau, 2001). Briefly, the rats were placed in plexiglas rodent restrainers of suitable sizes, following which they were allowed to calm down within the restrainer. They were placed in a chamber, where the temperature was maintained at 24°C. The tail was inserted into an inflatable cuff, containing a photoelectric sensor. The sensor was connected to a multi-sensor manual scanner (Model 65-120) and blood pressure amplifier, attached to an analog/digital recorder (Model 179) from IITC Life Sciences Inc. (Woodland Hills, Ca). Cuffs were inflated to a pressure of 150 mm Hg. Upon gradual deflation of the cuff; the reappearance of pulsations was detected by the sensor, and recorded as the systolic blood pressure. A minimum of 5 readings were recorded for each rat at a given time point in the studies.

3.5.3 Vascular reactivity in isolated blood vessels

Changes in vascular reactivity were determined in the same animals that were used in the studies explained in (2). The treatment protocols with different drugs will be described separately. All rats were prepared for experiments on the isolated blood vessel using similar procedures. The rats were anaesthetized using a high dose (60 mg/kg) of pentobarbital

(SomnotolTM/EuthanylTM) administered intraperitoneally. Upon loss of foot and blink reflexes, the abdomen was opened and the superior mesenteric artery (SMA) and/or thoracic aorta were excised. The tissues were placed in oxygenated ice-cold Krebs Ringer solution, the composition of which in mM was NaCl (118), KCl (4.7), CaCl₂ (2.5), MgSO₄ (1.2), KH₂PO₄ (1.2), NaHCO₃ (25), Glucose (11.1), and EDTA (0.026). Prior to and after excision, the tissues were cleaned in Krebs Ringer and rendered free of any surrounding connective tissue and blood. After cleaning, the tissues were cut into 3-4 mm long rings, which were mounted onto thin steel hooks and then attached to glass rods. The mounted tissues were then suspended on stainless steel hooks into a glass bath containing 20 ml of Krebs Ringer buffer gassed with 95% O₂ and 5% CO₂ and maintained at 37°C. Each tissue ring was placed under a resting tension of 2g and equilibrated for 45 minutes with periodic washings and readjustment of tension. Changes in tension were recorded by means of a pressure transducer attached to the stainless steel hooks, which was in turn connected to a Grass polygraph (Model 79D). After completion of the study, the tissues were lightly blotted with Kim wipesTM and the length and wet weights determined. For the SMA cross-sectional area (CSA) of each tissue was calculated as follows:

$$\text{CSA (g/mm}^2\text{)} = \frac{\text{weight (mg)}}{\text{length (mm) x density (mg/mm}^3\text{)}}$$

The density was assumed to be 1.04 mg/mm³. Tension responses were either expressed as percentage relaxation to acetylcholine in tissues precontracted by a ED₇₀ dose of phenylephrine (%) or as tension normalized to CSA (g/mm²). Since only contractile

responses to phenylephrine were determined in the aorta, the data was expressed as percentage of to maximal contraction to potassium chloride-induced (% KCl). This is an accepted baseline as KCl-induced contraction is not mediated by any receptor. Tissue sensitivity to agonist was calculated in terms of pD_2 ($-\log EC_{50}$ - molar concentration of an agonist, which produces 50% of the maximum possible response for that agonist). The percent maximal response for each agonist was determined by the R_{max} value. Both pD_2 and R_{max} were calculated by nonlinear regression analysis of the concentration response curves.(Galipeau *et al.*, 2002a; Galipeau *et al.*, 2002b)

3.5.4 Biochemical analyses

Following euthanasia by an overdose (60 mg/kg i.p.) of pentobarbital, blood was collected from rats by cardiac puncture into polypropylene tubes containing 100 IU heparin. All other blood samples obtained during the studies were collected from tail vein into 350 μ l heparinized tubes. Samples were centrifuged in a desktop microcentrifuge (25 minutes 14000 rpm for tail vein samples and 4500 rpm for samples from cardiac puncture, 4°C Beckmann Allegra 21R) to separate the plasma. Plasma was aspirated out and frozen at -80°C . In study E, blood was collected upon termination by cardiac puncture in tubes containing a mixture of 2% ethylenediaminetetraacetic acid (EDTA) and 0.04M indomethacin (COX inhibitor), which was as per the recommendations in the manufacturer's (Amersham) protocol for estimation of thromboxane A_2 . The remaining plasma was collected in 100 IU heparin containing tubes for measuring 20-HETE. Upon centrifugation at 4500 rpm at 4°C for 25 minutes, the plasma was stored separately at -80°C until assayed. Plasma glucose was

estimated with an automatic Beckman glucose analyzer II. Plasma insulin was measured using commercially available radioimmunoassay kits from Linco Diagnostics Inc, USA, while testosterone were measured in the plasma using RIA kits from MP Biomedicals, USA.

3.5.5 Thromboxane A2

Using an enzyme immunoassay kit (EIA) (Amersham/GE) TXB2-the stable metabolite of TXA2 was measured in the plasma as previously reported (Galipeau *et al.*, 2001). The prostanoids were extracted from the plasma using a solid phase column as specified in the instructions accompanying the kit. The prostanoids were eluted using methyl formate, dried under nitrogen and reconstituted in assay buffer according to instructions. This sample was used in the EIA.

3.5.6 Western blotting

Changes in the protein levels of COX-2 and Cyp4A (arachidonic acid ω -hydroxylase) in the tissues were evaluated by western blotting in studies 3.4.2, 3.4.3 and 3.4.5. Protein expressions were determined in the SMA in 3.4.2 and 3.4.5; the aorta in 3.4.5 and the mesenteric bed in 3.4.3 respectively. Based on previous literature, rabbit polyclonal antibodies against COX-2 (Abcam, MA and Cell Signaling, Danvers, MA) and Cyp4A1, 2/3 were used in our studies (Abcam, MA and Acris, Herford, Germany). Briefly protein lysates were prepared by homogenizing tissues in RIPA buffer.

3.5.6.1 Sample preparation

Protein homogenates were centrifuged at 14000 rpm for 15 minutes. The supernatant was collected for protein estimation using Bradford reagent (1:4 dilution). Extinction was measured at 595 nm and concentrations were obtained by extrapolating absorbance values to a standard curve. The protein concentration was standardized to 0.5-1 µg/µl with suitable volumes of RIPA buffer and a constant volume of sample loading buffer. The mixture was heated at 95°C for 5 minutes in a heating block for denaturing the proteins and stored at -80°C until used.

3.5.6.2 Electrophoresis and Western Blotting

30 µl of sample/group was loaded onto 1 mm thick polyacrylamide gels (PAGE) and the proteins were separated by electrophoresis. The proteins were then transferred onto PVDF membranes. Following a 1-hour blocking with 5% non-fat milk in TBS-T (tris buffered saline containing 0.001% Tween-20), the membranes were incubated overnight at 4°C with 1:5000 COX-2 antibody or 1: 3000 Cyp4A1, 2/3 in BSA (bovine serum albumin-fraction 5, Sigma/Roche). The membranes were then washed in TBS-T (3x7 mins) and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1: 10,000 in 5% non-fat milk) at room temperature for 1 hour. Following washings with TBS-T, the membranes were developed in ECL reagent (ECL reagent A&B; GE Life Sciences) and exposed onto X-ray films to obtain bands. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping control as its expression is unchanged by most disease states. A monoclonal mouse antibody (1:2000) in 5% BSA was used to determine GAPDH expression. The remaining procedures were same as above.

3.5.6.3 Immunoprecipitation

As the initial western blots were inconclusive due to the high noise and non-specific binding of the Abcam antibodies, we switched to the Cell Signaling and Acris antibodies and measured protein expression in immunoprecipitated samples. 500 μl of $1\mu\text{g}/\mu\text{l}$ aliquots of protein lysates were prepared with RIPA buffer to which $1.1\ \mu\text{l}$ of antibody was added and rotated overnight at 4°C . The next day $20\ \mu\text{l}$ of protein A sepharose slurry was added and rotated for 1.5 hours at 4°C . The suspension was centrifuged and washed twice with RIPA buffer. After discarding the supernatant, the final pellet was resuspended in $60\ \mu\text{l}$ of resuspension buffer. $20\ \mu\text{l}$ of sample loading buffer was added and boiled at 95°C for 5 minutes. The samples thus obtained were run on polyacrylamide gels and protein bands visualized as described in 6b.

3.5.6.4 Analysis

The bands were scanned and changes in their densities were obtained using ImageJTM software. In each film, a corresponding blank of the same area as the sample was taken. Sample band densities were normalized to their corresponding GAPDH band density to obtain a ratio. The IgG bands observed at 44 kD were used as loading controls. For the data obtained from immunoprecipitated samples, These values were in turn expressed relative to control (C) i.e. assuming each C value to be 1.

3.6 Statistical Analyses

All data are presented as mean \pm the standard error of the mean (SEM). In studies involving multiple time points, intergroup comparisons of the dependent variables in each *in-vivo* study (glucose, plasma insulin, etc.) were performed by general linear model analysis of variance (GLM-ANOVA). The GLM ANOVA was also used to compare individual data points of the OGTT and concentration response curves. For comparing insulin sensitivity indices (ISI), pD_2 , AUC and maximum response (R_{Max}), one-way ANOVA was used. Mean values were considered significant at $P < 0.05$. The Newman-Keuls multiple comparison test was used for post hoc comparisons upon detection of difference in the means. All statistical analyses were performed using the Number cruncher statistical system (NCSS) software package.

4. RESULTS

4.1 Effects of exogenous testosterone replacement on blood pressure in fructose-fed rats

The results in study A are reproduced from Vasudevan et al. 2006 (Vasudevan et al., 2006) – used with permission

By replacing testosterone in the body we intended to confirm the involvement of testosterone in the development of increased blood pressure following insulin resistance. Fructose feeding did not affect the body weights of either the gonadectomized or the sham-operated rats (478 ± 18 gm; C vs. 490 ± 22 gm; F). Treatment with testosterone did not affect the body weights in the fructose-fed rats (437 ± 9 gm; GF vs. 452 ± 20 gm; GFT). Feeding and drinking patterns were uniform in all the groups.

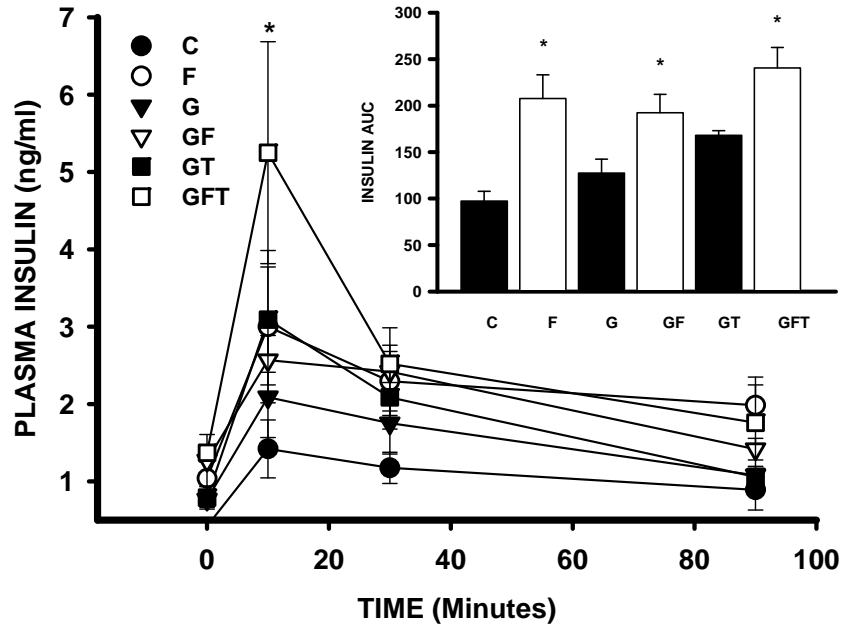
Changes in glucose and insulin values subsequent to glucose challenge (Figure 4.1A-B) were consistent with previous studies (Song *et al.*, 2004; Vasudevan *et al.*, 2005) and the comparison of ISI values calculated revealed attenuation in insulin sensitivity in the fructose-fed rats (Figure 4.1C). Treatment with testosterone (GFT) did not affect the insulin sensitivity in gonadectomized fructose-fed rats.

Prior to the start of testosterone injection, after 5 weeks of fructose feeding; there was no significant rise in the blood pressure. At study week 10, (5 weeks post-injection) the testosterone-treated gonadectomized fructose-fed rats (GFT) showed an increase in BP (133 ± 2 mm Hg) while the control (C) and gonadectomized untreated groups (G, GF & GT) were normotensive. The values of blood pressure in the GFT rats were comparable with those from F (Figure 4.2). Thus testosterone treatment negated the positive effects of gonadectomy on the blood pressure.

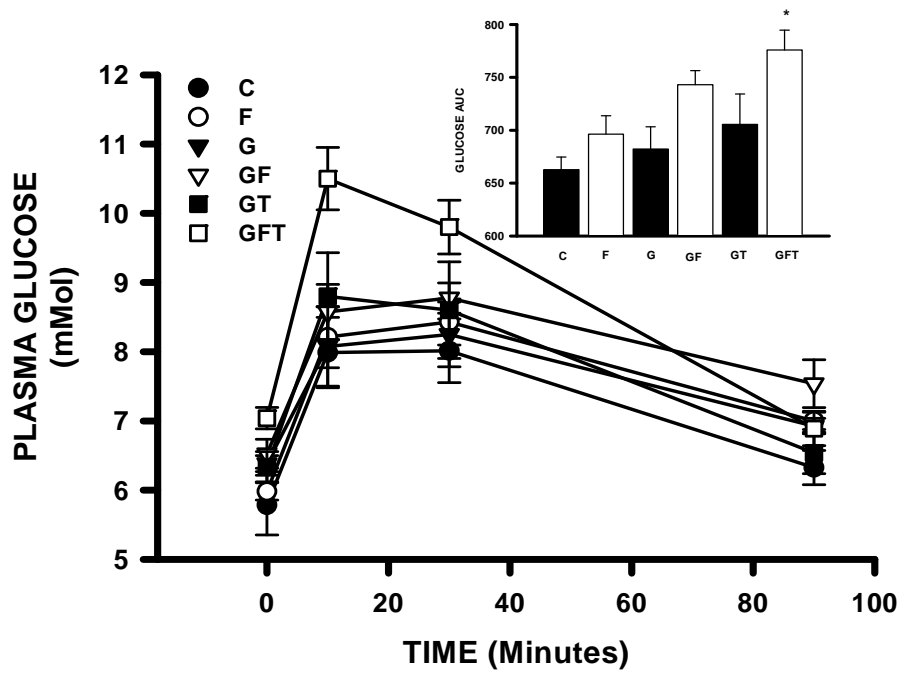
Testosterone was undetectable in gonadectomized animals. In addition the difference in diet did not affect plasma testosterone levels (Figure 4.3). In the rats injected with (GFT), testosterone levels at the end of 1 week post-treatment were 2.3 ± 1.0 ng/ml. At termination the levels were as high as 12 ± 6 ng/ml, which was not statistically different as compared to the group receiving normal chow and injected with testosterone (GT).

VASUDEVAN, H., NAGAREDDY, P.R. & MCNEILL, J.H. (2006). Gonadectomy prevents endothelial dysfunction in fructose-fed male rats, a factor contributing to the development of hypertension. *Am J Physiol Heart Circ Physiol*, **291**, H3058-64 (*Used with Permission*).

4.1A



4.1B



4.1C

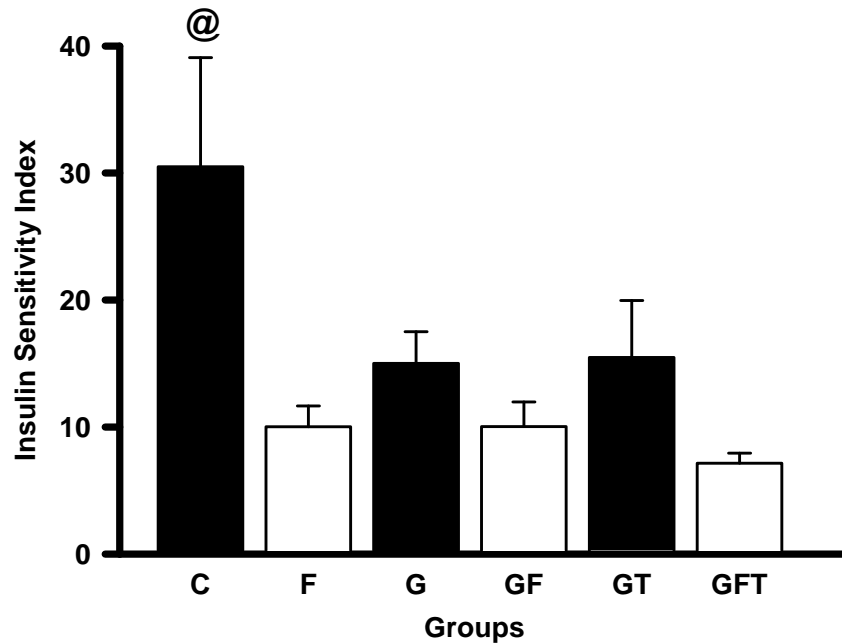


Figure 4.1A-C: Plasma insulin and glucose profiles and insulin sensitivity index (ISI) following an oral glucose challenge. Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats; GT-gonadectomized rats treated with testosterone; GFT-gonadectomized fructose-fed rats treated with testosterone. Testosterone replacement (GFT) did not alter ISI values in gonadectomized fructose-fed rats. (A)-Graph of insulin concentration vs. time. Inset shows area under the curve values (AUC) for each group. (B)-Graph of glucose concentration vs. time. Inset shows area under the curve values (AUC) for each group. (C)-Insulin sensitivity index values for all groups as calculated by the Matsuda and DeFronzo formula (Matsuda *et al.*, 1999). All groups n=4-5. * P<0.05 F, GF and GFT vs. C, G, and GT (1A). @ P<0.05 C vs. F, GF and GFT. All values are presented as mean ± SEM..

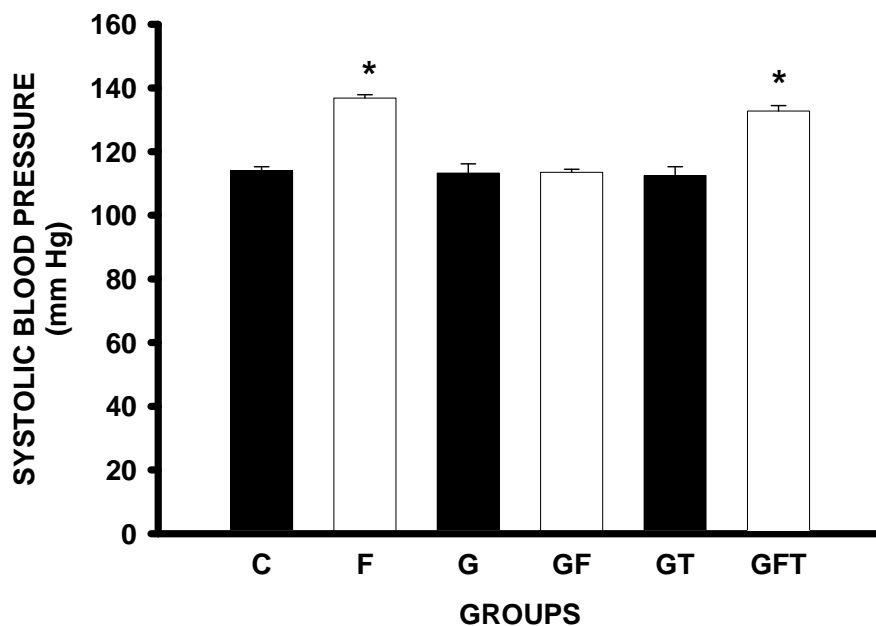


Figure 4.2: Changes in blood pressure after 9 weeks of fructose feeding. Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats; GT-gonadectomized rats treated with testosterone; GFT- gonadectomized fructose-fed rats treated with testosterone. Testosterone treatment was initiated at the end of 5 weeks for a period of 4-5 weeks. Testosterone replacement (GFT) did not alter ISI values in gonadectomized fructose-fed rats. All groups n=4-5. *P<0.05 F and GFT vs. C, G and GT. All values are presented as mean \pm SEM.

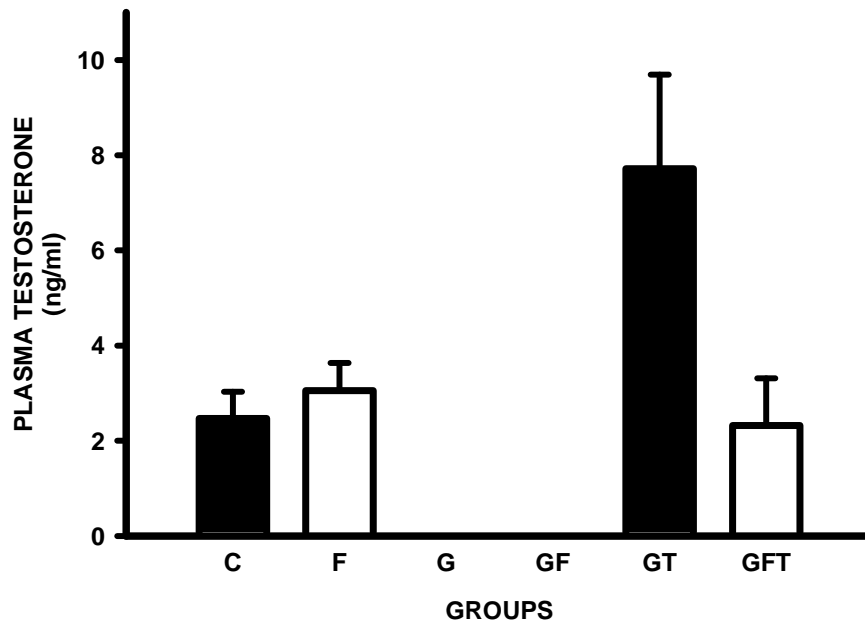


Figure 4.3: Changes in plasma testosterone at termination. Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats; GT-gonadectomized rats treated with testosterone; GFT- gonadectomized fructose-fed rats treated with testosterone. Testosterone treatment was initiated at the end of 5 weeks for a period of 4-5 weeks. Testosterone was measured by a radioimmunoassay kit at termination. Testosterone replacement (GFT) did not alter ISI values in gonadectomized fructose-fed rats. All groups n=4-5. All values are presented as mean \pm SEM

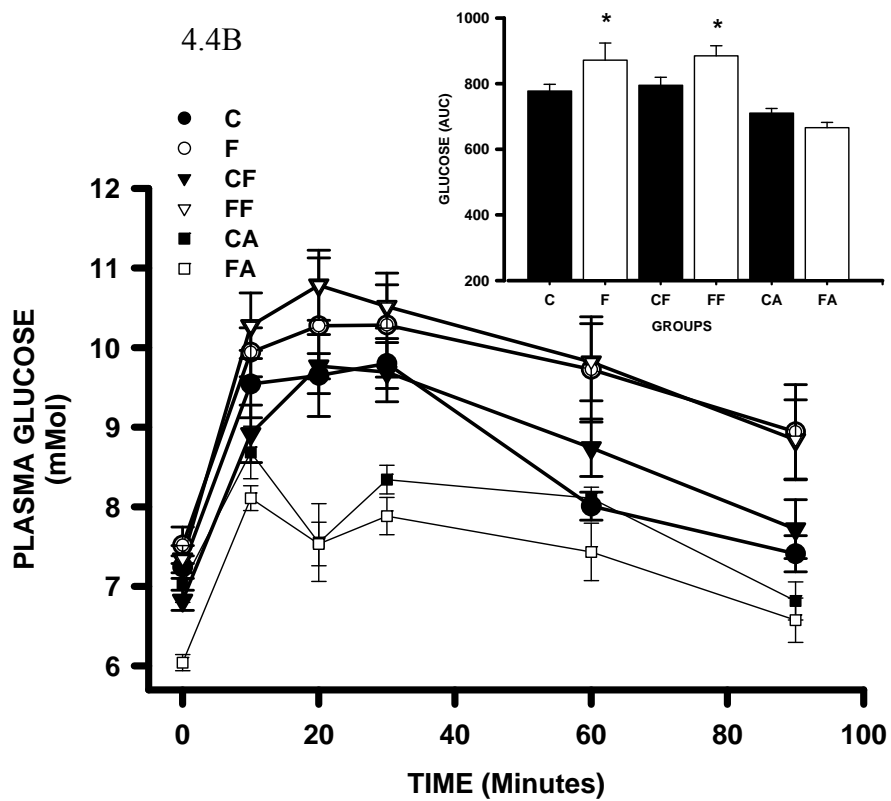
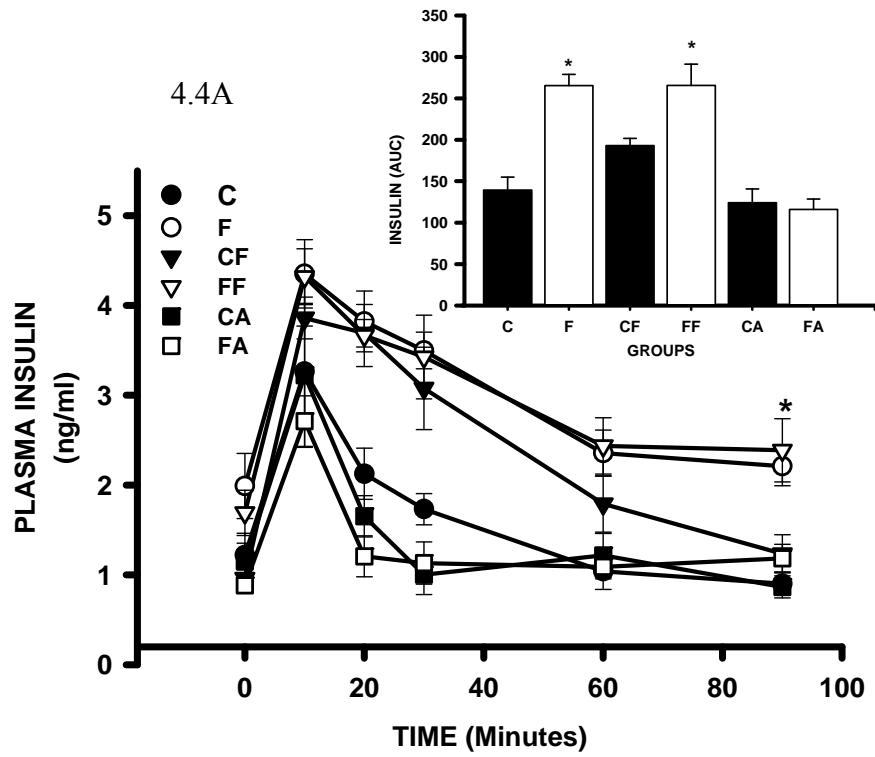
4.2 Role of insulin resistance and testosterone on Cyp4A expression

4.2.1 Insulin sensitivity, blood pressure and testosterone

In this study we attempted to study the role of the androgen receptor and Cyp4A in the regulation of insulin sensitivity and blood pressure. Feeding fructose for 12 weeks induced insulin resistance, which was observed by hyperinsulinemia and delayed glucose clearance. Interestingly although flutamide did not alter the attenuation in insulin sensitivity (FF), treatment with ABT for 3 weeks improved the insulin sensitivity in fructose-fed rats (FA) (Figure 4.4C). This was demonstrated by insulin levels similar to control (C) in FA (Figure 4.4A and inset). Fructose-fed rats were hyperglycemic compared to controls or the ABT-treated rats (Figure 4.4B and inset). Systolic blood pressure was increased in all fructose-fed rats at the end of 9 weeks. Treatment with either flutamide or ABT restored the blood pressure to control values suggesting the involvement of both testosterone and Cyp4A (Figure 4.5). Blocking the androgen receptor increased plasma testosterone levels in both CF and FF groups (Figure 4.6). However control or fructose diets, by themselves, did not affect testosterone levels.

4.2.2 Cyp4A1, 2/3 and COX-2 expression in the SMA

We measured changes in COX-2 expression in the SMA and aorta (Vasudevan and Jiang; unpublished observations). Twelve-week fructose feeding did not affect COX-2 expression in both tissues (Figure 4.7). In the SMA Cyp4A1, 2/3 expression was increased in untreated fructose-fed rats (F). Treatment with flutamide but not ABT decreased Cyp4A expression in these tissues (Figure 4.8).



4.4C

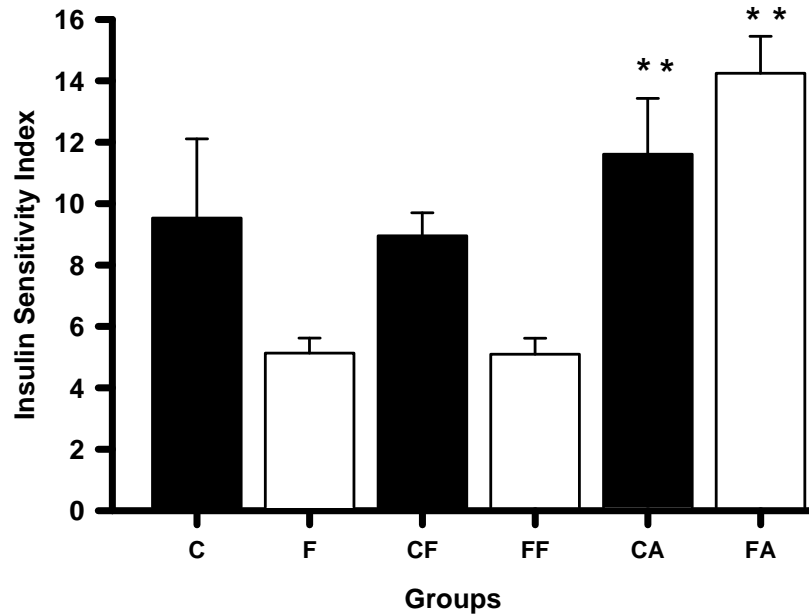


Figure 4.4A-C: Plasma insulin and glucose profiles and insulin sensitivity index (ISI) following an oral glucose challenge. Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; CF-control flutamide-treated rats; FF-fructose-fed flutamide-treated rats; CA-control 1-aminobenzotriazole (ABT)-treated rats; FA-fructose-fed ABT-treated rats. Flutamide (FF) did not alter ISI values in fructose-fed rats. Treatment with ABT improved the insulin sensitivity as demonstrated by high ISI value (4C). A-Graph of insulin concentration vs. time. Inset shows area under the curve values (AUC) for each group. B-Graph of glucose concentration vs. time. Inset shows area under the curve values (AUC) for each group. C-Insulin sensitivity index values for all groups as calculated by the Matsuda and DeFronzo formula (Matsuda *et al.*, 1999). All groups n=6. * P<0.05 F and FF vs. C, CF, CA and FA (10A and B). **P<0.05 F and FF vs. CA and FA. All values are presented as mean \pm SEM.

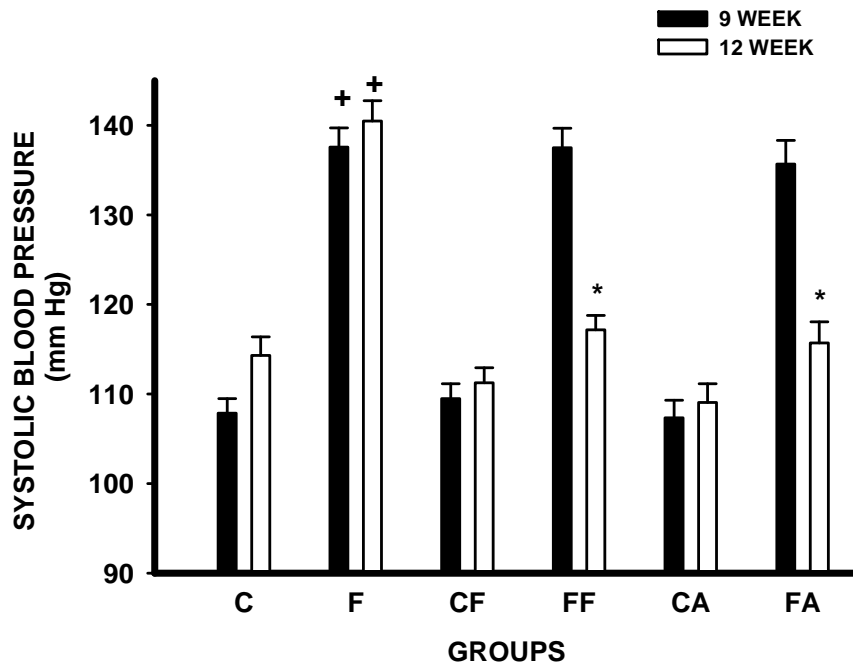


Figure 4.5: Systolic blood pressure values at the end of 9 and 12 weeks of fructose feeding. Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; CF-control flutamide-treated rats; FF-fructose-fed flutamide-treated rats; CA-control 1-aminobenzotriazole (ABT)-treated rats; FA-fructose-fed ABT-treated rats. Treatment with flutamide (8 mg/kg/day s.c.) or ABT (25 mg/kg/day i.p.) was started at the end of 9 weeks. Blood pressure was measured 3 weeks post treatment. All groups n=8. * P<0.05 FF and FA (treated) vs. FF and FA (untreated; black boxes and F (untreated); + P<0.05 F (treated and untreated) vs. C, CF and CA. All values are expressed as mean \pm SEM.

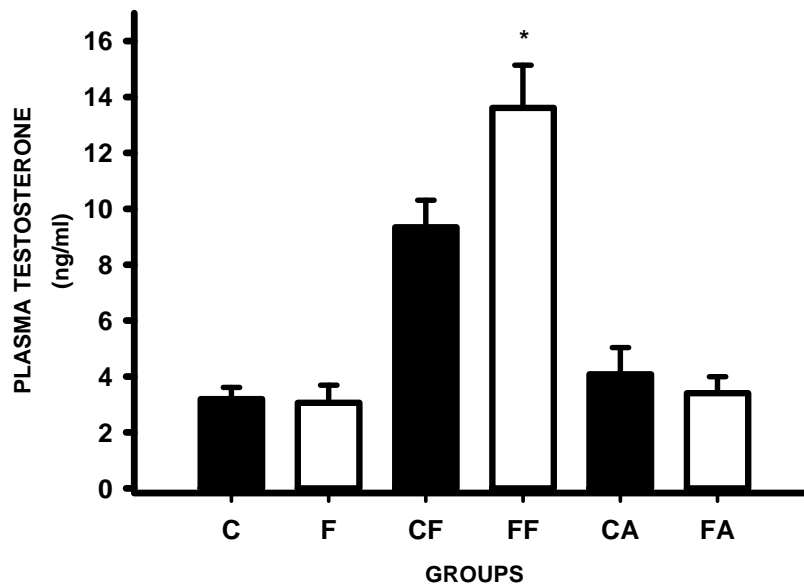


Figure 4.6: Plasma testosterone values at the end of 12 weeks of fructose feeding. Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; CF-control flutamide-treated rats; FF-fructose-fed flutamide-treated rats; CA-control 1-aminobenzotriazole (ABT)-treated rats; FA-fructose-fed ABT-treated rats. Treatment with flutamide (8 mg/kg/day s.c.) or ABT (25 mg/kg/day i.p.) was started at the end of 9 weeks. Testosterone was measured by a radioimmunoassay kit at termination (3 weeks post treatment). All groups n=6* P<0.05 CF and FF vs. C, F, Ca and FA. All values are presented as mean \pm SEM.

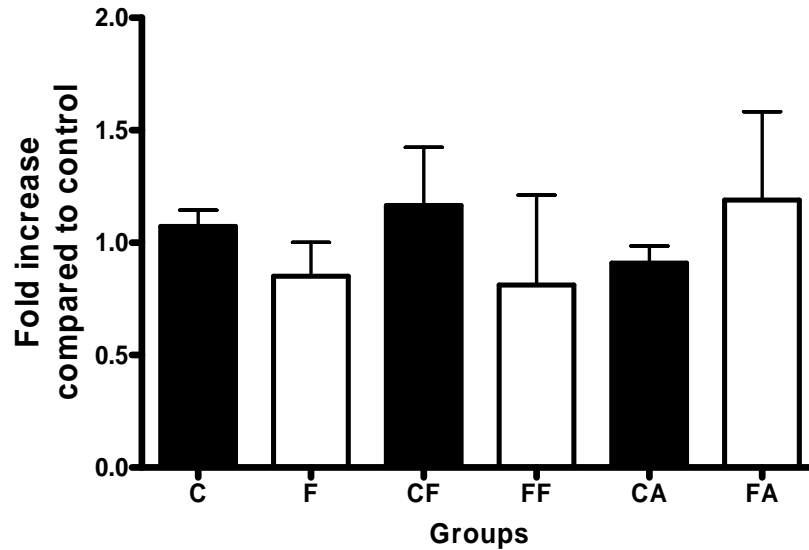


Figure 4.7: Changes in COX-2 in the superior mesenteric arteries bed of control and fructose-fed rats treated with flutamide (8 mg/kg/day s.c.) or ABT (25 mg/kg/day i.p.). Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; CF-control flutamide-treated rats; FF-fructose-fed flutamide-treated rats; CA-intact rats; CA-control 1-aminobenzotriazole (ABT)-treated rats; FA-fructose-fed ABT-treated rats. All groups n=4. Western blotting was performed on samples using COX-2 specific antibody. Controls are GAPDH expression from same samples. All values are presented as mean ± SEM.

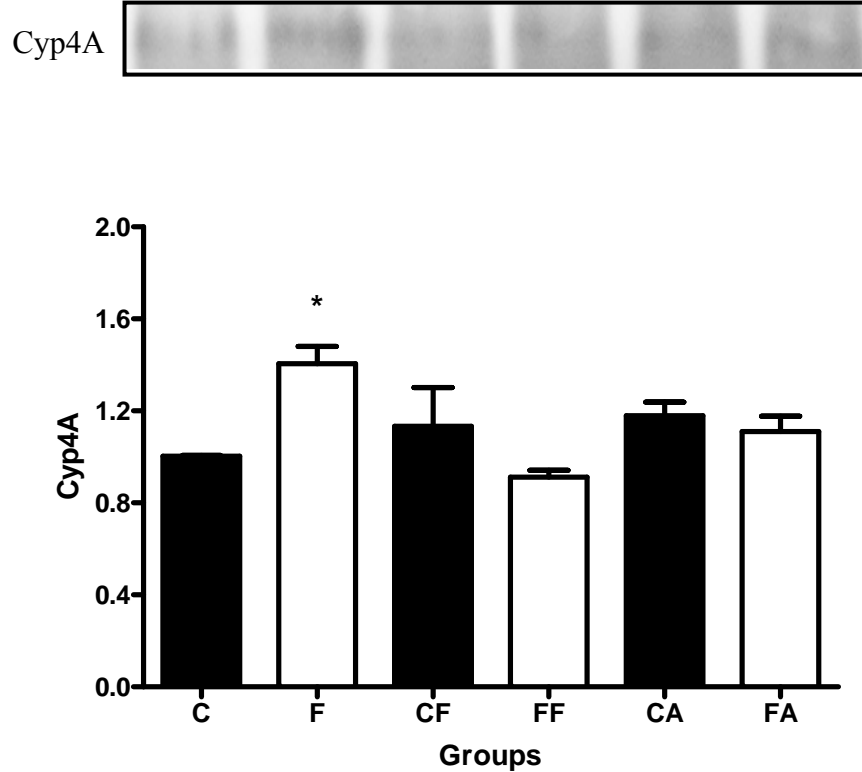


Figure 4.8: Changes in Cyp4A in the superior mesenteric arteries bed of control and fructose-fed rats treated with flutamide (8 mg/kg/day s.c.) or ABT (25 mg/kg/day i.p.). Male Wistar rats were divided into 6 groups, which are: C-intact control rats; F-fructose-fed intact rats; CF-control flutamide-treated rats; FF-fructose-fed flutamide-treated rats; CA-control 1-aminobenzotriazole (ABT)-treated rats; FA-fructose-fed ABT-treated rats. All groups n=3. Western blotting was performed on samples immunoprecipitated with Cyp4A1, 2/3 Ab. * P<0.05 F vs. C & FF. All values are presented as mean ± SEM.

4.3 Effect of a Cyp4A selective inhibitor on insulin sensitivity and blood pressure

4.3.1 Insulin sensitivity and Blood pressure

In rats treated with the selective Cyp4A inhibitor HET0016, both diet and treatment did not affect the body weight (Figure 4.9) Fructose feeding induced insulin resistance as indicated by the reduced insulin sensitivity index (Figure 4.10C). Treatment with HET0016 was inconclusive as the ISI values were not different from both C and F groups. HET0016 treated rats did not vary in their total insulin levels as indicated by the area under curve values (Figure 4.10A inset). Furthermore they were euglycemic, which is a characteristic of this model (Figure 4.10B).

Fructose feeding elevated the blood pressure in F compared to C at the end of 9 weeks. Treatment with HET0016 for 1 week prevented the increase in blood pressure following insulin resistance in FT rats (Figure 4.11).

4.3.2 Cyp4A1, 2/3 expression in the mesenteric bed

Insulin resistance elevated Cyp4A1, 2/3 expression in the mesenteric bed of untreated fructose-fed rats (F). Treatment with HET0016 decreased Cyp4A expression (Figure 4.12).

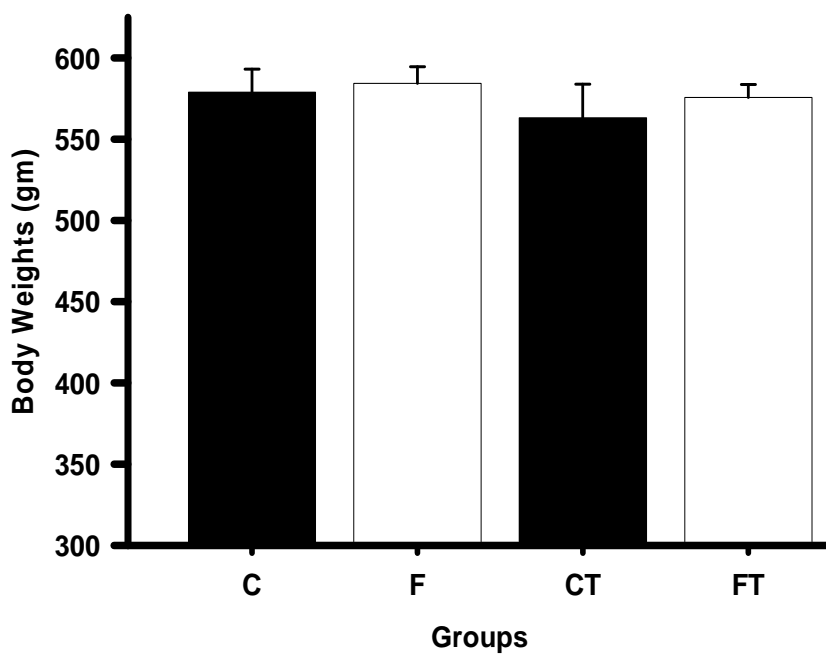
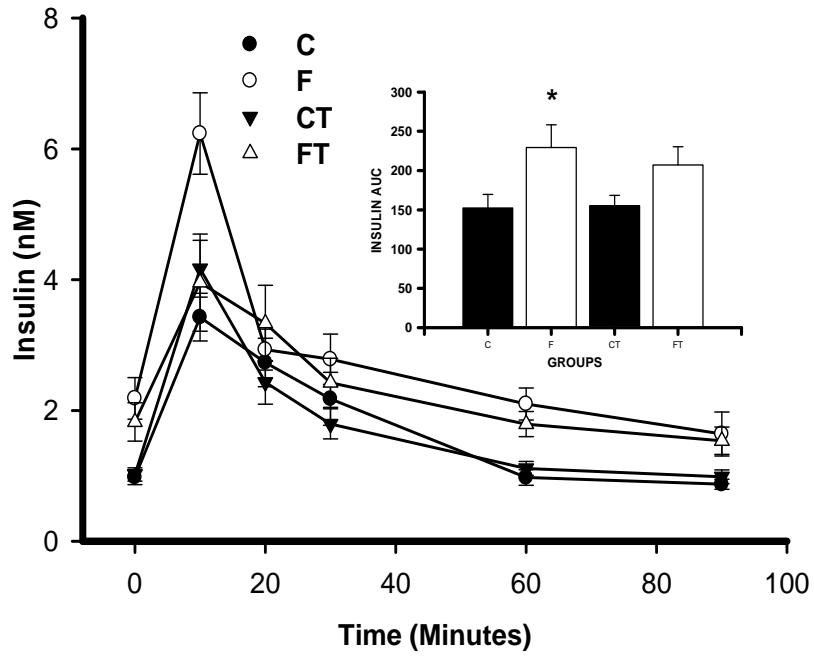
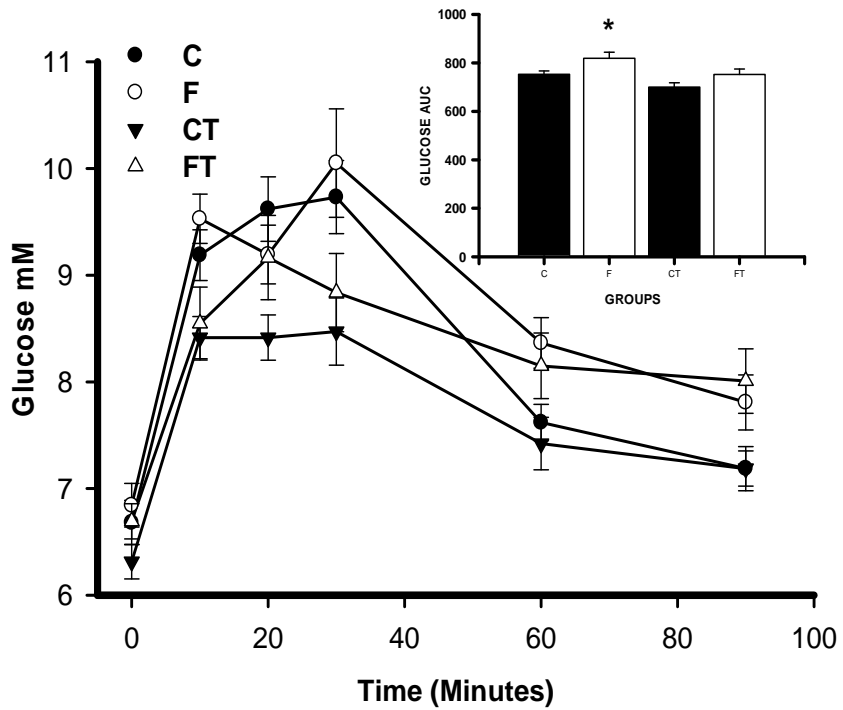


Figure 4.9: Body weights at termination in control and fructose-fed rats treated with HET0016. Intact male Wistar rats were divided into 4 groups, which are C-control; F-fructose-fed; CT-control HET0016-treated; FT-fructose-fed and HET0016 treated. Drug treatment did not produce any change in body weight suggesting that it was safe to use. All groups n=7. All values are presented as mean \pm SEM.

4.10A



4.10B



4.10C

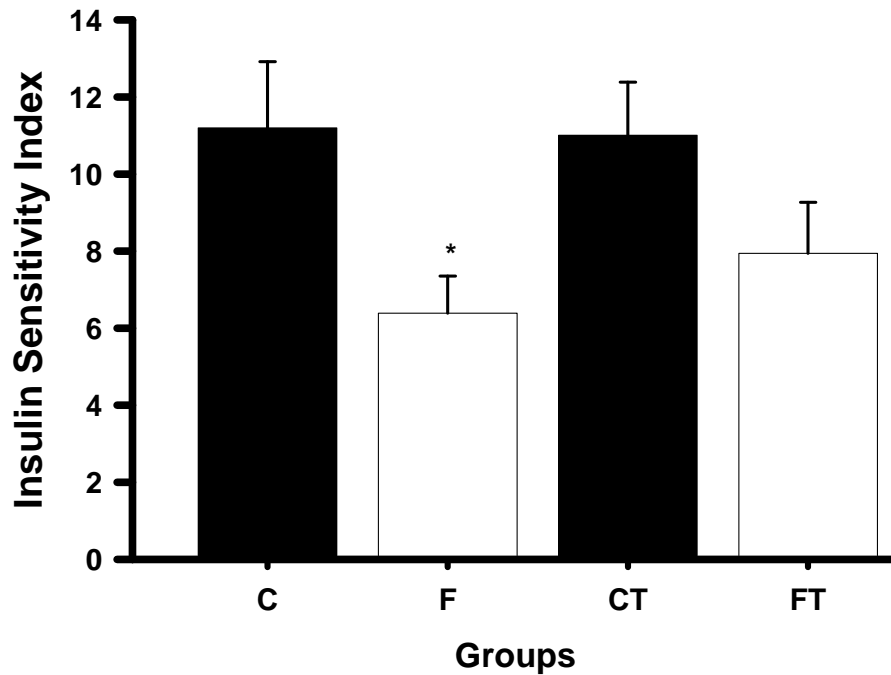


Figure 4.10A-C: Plasma insulin and glucose profiles and insulin sensitivity index (ISI) following an oral glucose challenge. Intact male Wistar rats were divided into 4 groups, which are C-control; F-fructose-fed; CT-control HET0016-treated; FT-fructose-fed and HET0016 treated. HET0016 treated rats did not exhibit statistically different ISI values. (A)-Graph of insulin concentration vs. time. Inset shows area under the curve values (AUC) for each group. (B)-Graph of glucose concentration vs. time. Inset shows area under the curve values (AUC) for each group. (C)-Insulin sensitivity index values for all groups as calculated by the Matsuda and DeFronzo formula(Matsuda *et al.*, 1999). All groups n=7. * P<0.05 F vs. C and CT. All values are presented as mean ± SEM.

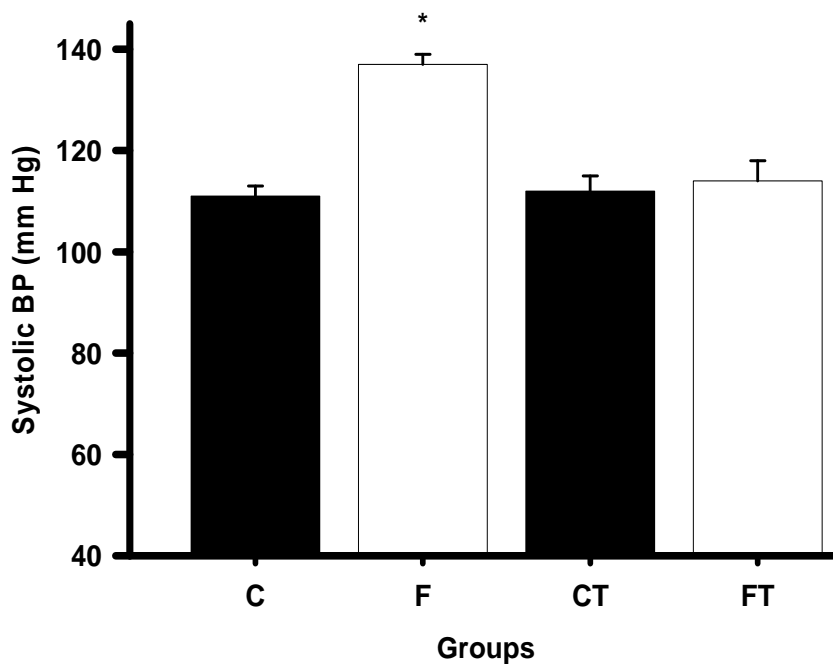


Figure 4.11: Systolic blood pressure values at the end of 9 weeks of fructose feeding.

Intact male Wistar rats were divided into 4 groups, which are C-control; F-fructose-fed; CT-control HET0016-treated; FT-fructose-fed and HET0016 treated. HET0016 (10 mg/kg/day i.p.) was started at the end of 8 weeks. Blood pressure was measured 1 week post treatment. All groups n=7. * P<0.05 F vs. C, CT and FT. All values are presented as mean \pm SEM.

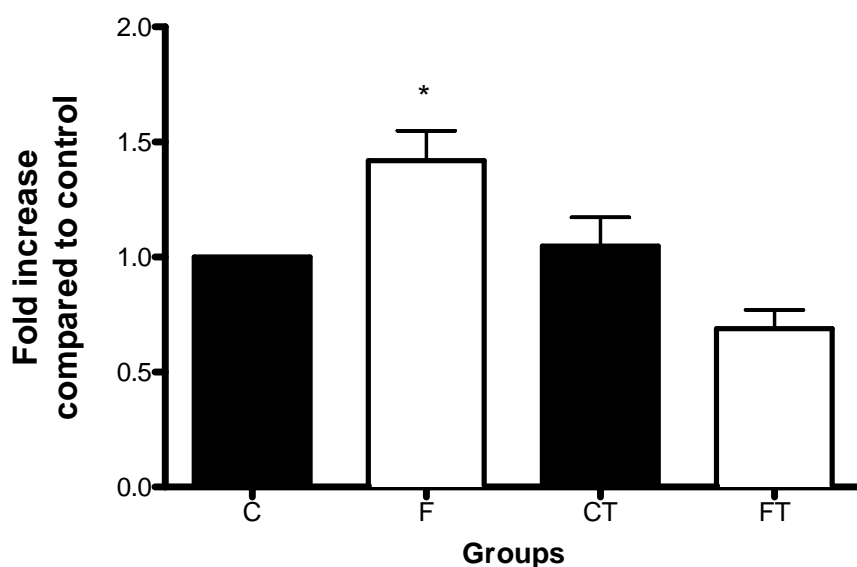
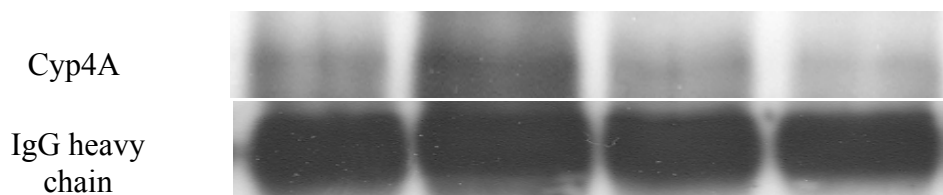


Figure 4.12: Changes in Cyp4A in the mesenteric bed of control and fructose-fed rats treated with HET0016 (10 mg/kg/day i.p.). Intact male Wistar rats were divided into 4 groups, which are C-control; F-fructose-fed; CT-control HET0016-treated; FT-fructose-fed and HET0016 treated. Western blotting was performed on samples immunoprecipitated with Cyp4A1, 2/3 Ab. Controls are bands from antibody IgG used in IP. All groups n=4. *P<0.05 F vs. C, CT and FT. All values are presented as mean \pm SEM.

4.4 Role of insulin resistance and testosterone in vascular arachidonic acid metabolism and vascular reactivity

4.4.1 Insulin Sensitivity and Blood pressure

At the end of 9 weeks, fructose feeding attenuated the insulin sensitivity in both sham-operated and gonadectomized rats (Figure 4.13A-C). Treatment with metformin (FM and GFM) restored normal insulin responses to the glucose challenge.

After 9 weeks of fructose feeding, blood pressure was elevated in intact male rats (F) compared to controls (C). Metformin prevented the elevation in blood pressure in intact rats. Both fructose and metformin did not affect the blood pressure in the gonadectomized groups (Figure 4.14).

4.4.2 Vascular reactivity studies

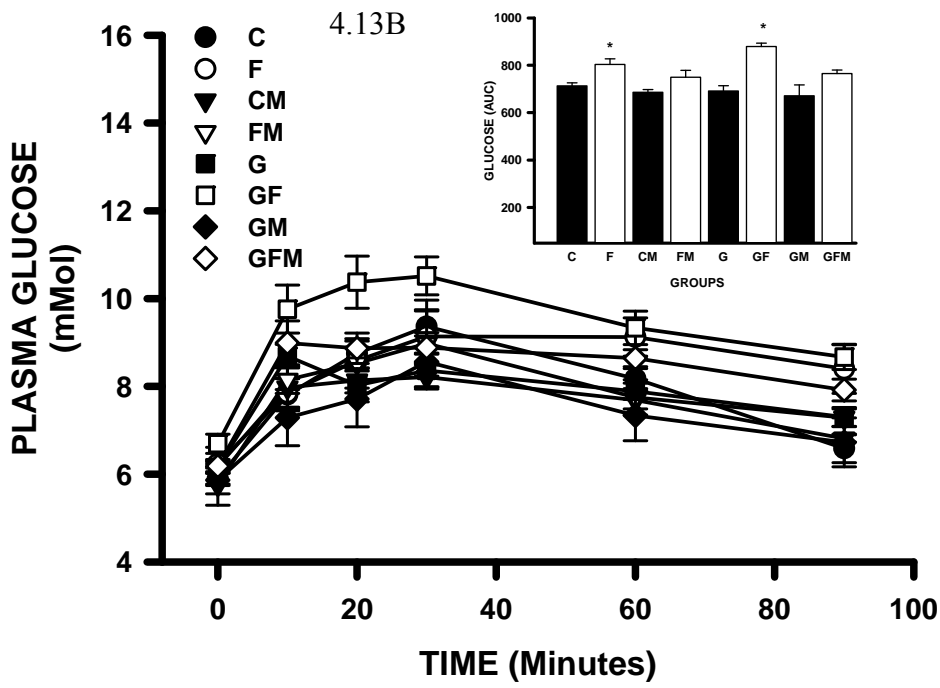
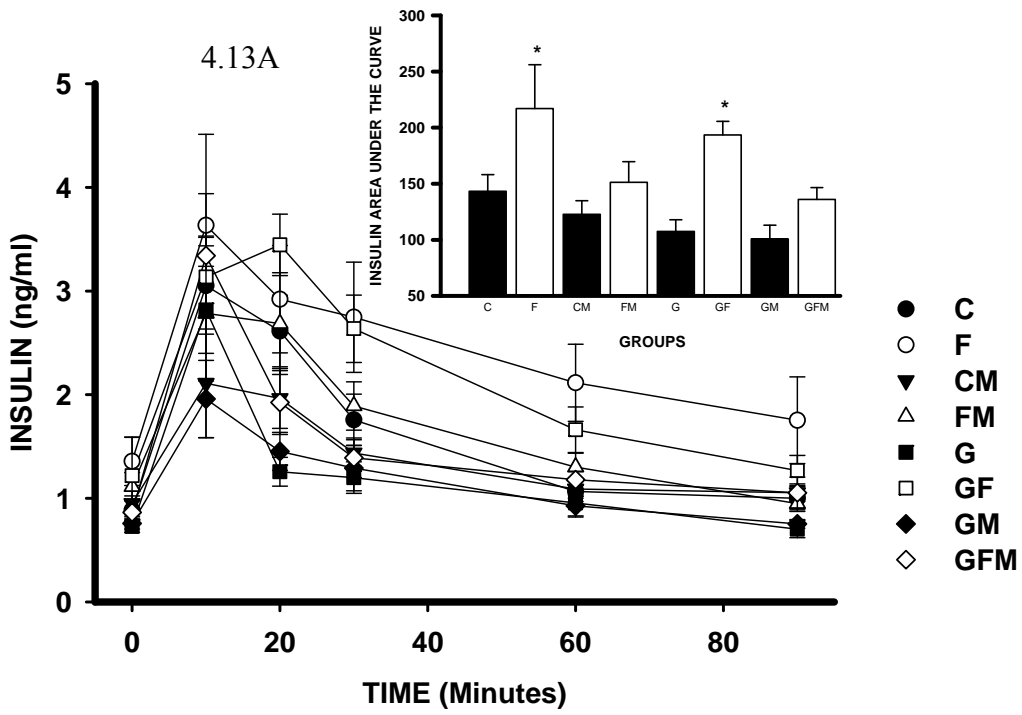
In the isolated superior mesenteric arteries, responses to PE were unchanged by gonadectomy or insulin resistance. As previously reported (Vasudevan et al. 2006), relaxation to acetylcholine was impaired in F but not GF. Metformin treatment restored the endothelium-dependent relaxation in these vessels (Figure 4.15 A and B).

Inhibition of 20-HETE synthesis with DDMS (10^{-5} M) improved the relaxation to ACh in the SM of F but not GF and FM (Figure 4.16). This demonstrates the involvement of androgen-dependent Cyp4A-catalyzed arachidonic acid metabolites in impairing endothelium-dependent vasodilation.

Treatment with indomethacin produced a similar relaxation pattern as observed with DDMS. Endothelium-dependent relaxation was improved in SMA of F. Relaxation was unaffected in FM and GF (Figure 4.17).

Charybdotoxin (CTX) and apamin (APA) block K_{Ca} channels that are opened by EDHF during the process of hyperpolarization. Blocking the EDHF component allows us to study the role of endothelial NO in relaxing the superior mesenteric arteries. In this study, K_{Ca} blockade decreased endothelium-dependent relaxation by about 50% in all groups. However, this decrease was observed to a greater degree in the F but not FM or GF groups suggesting that NO may be the primary endothelial vasodilator in the SMA (Figure 4.18). This may be due to the attenuated NO-dependent relaxation secondary to insulin resistance, which is exacerbated by K_{Ca} blockade.

Treatment with 10^{-6} M L-NAME nearly abolished the relaxation to ACh in all groups (data not shown). This suggests that NO is the major vasodilator in the SMA as there is no compensatory vasorelaxant pathway observed once NO synthesis is inhibited.



4.13C

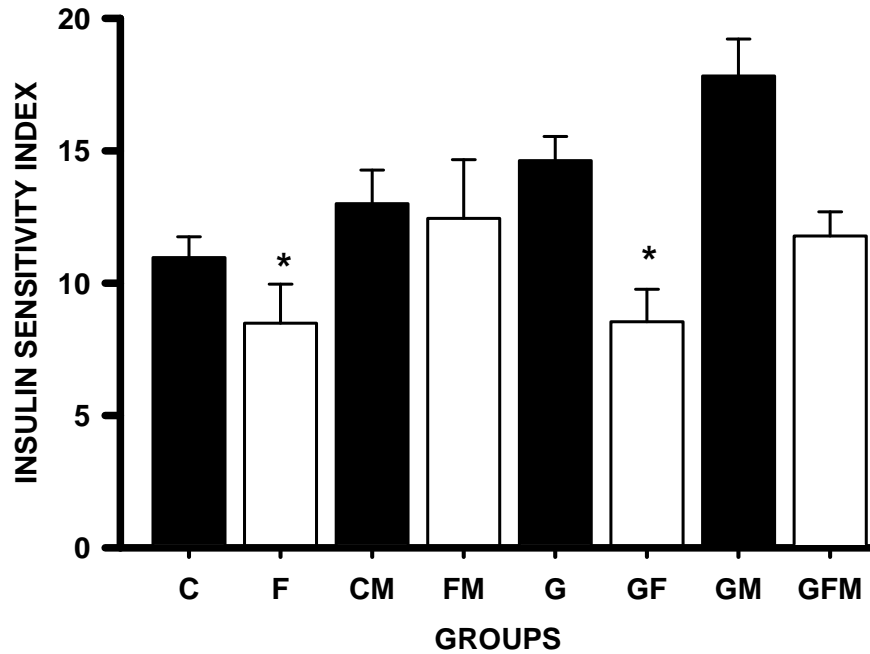


Figure 4.13A-C: Plasma insulin and glucose profiles and insulin sensitivity index (ISI) following an oral glucose challenge. Male Wistar rats were divided into 8 groups, which are: C-intact control rats; F-fructose-fed intact rats; CM-control metformin-treated rats; FM-fructose-fed metformin treated rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats; GM-gonadectomized rats treated with metformin; GFM- gonadectomized fructose-fed rats treated with metformin.

(A)-Graph of insulin concentration vs. time. Inset shows area under the curve values (AUC) for each group. (B)-Graph of glucose concentration vs. time. Inset shows area under the curve values (AUC) for each group. (C)-Insulin sensitivity index values for all groups as calculated by the Matsuda and DeFronzo formula (Matsuda *et al.*, 1999). Metformin treatment prevented the development of hyperinsulinemia in fructose-fed rats. All groups n=8. * P<0.05 F and GF vs. C, CM, FM, G, GM and GFM. All values are presented as mean ± SEM.

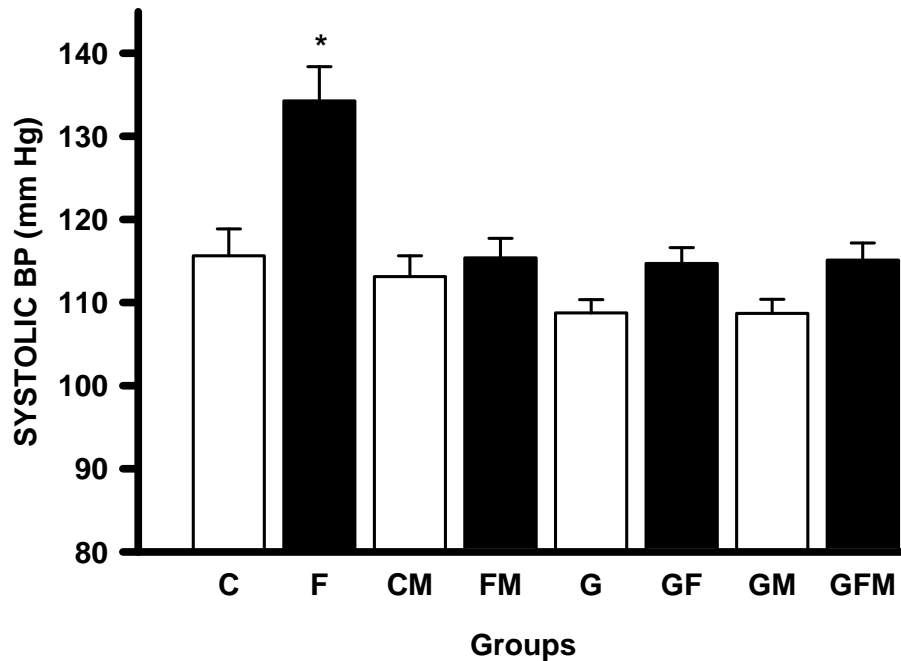


Figure 4.14: Chronic treatment with metformin prevents the increase in blood pressure in fructose fed rats. Male Wistar rats were divided into 8 groups, which are: C-intact control rats; F-fructose-fed intact rats; CM-control metformin-treated rats; FM-fructose-fed metformin treated rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats; GM-gonadectomized rats treated with metformin; GFM- gonadectomized fructose-fed rats treated with metformin. Rats with intact testes had higher blood pressure when fed with fructose (F) as compared to other groups. Treatment with metformin in drinking water during fructose-feeding prevented this increase in BP (FM). All groups n=7-8. * P<0.05 F vs. C, CM, FM, G, GF, GM and GFM. All values are presented as mean \pm SEM.

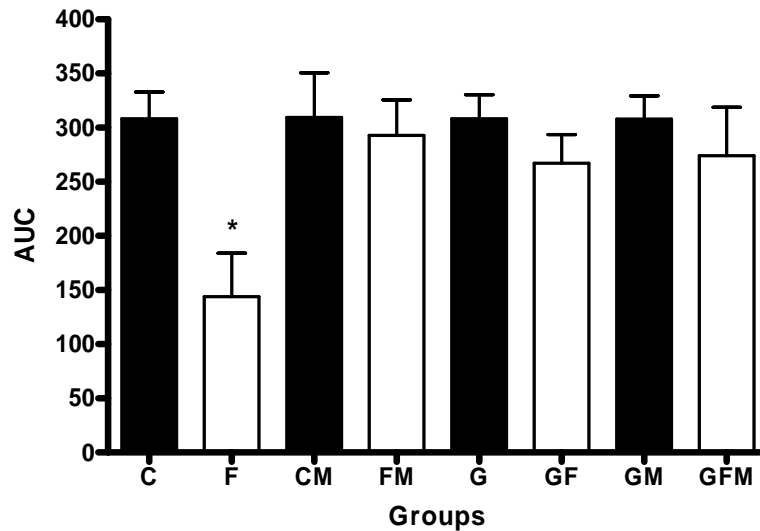
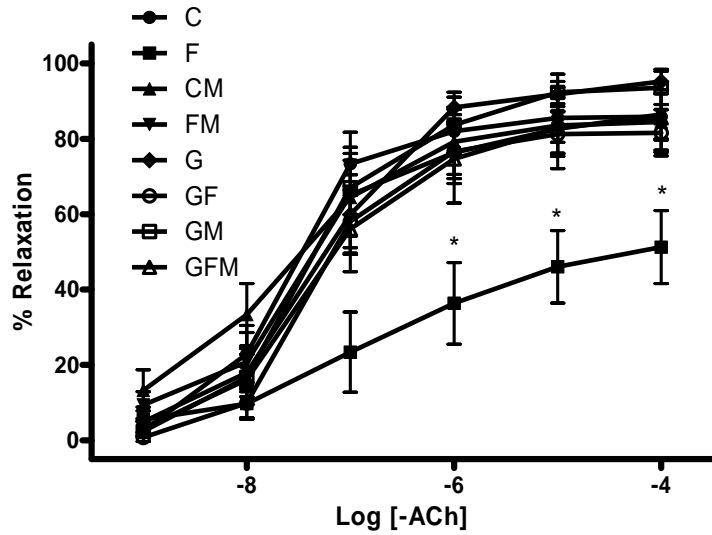


Figure 4.15A-B: Changes in relaxation to acetylcholine in intact and gonadectomized control and fructose-fed rats treated with metformin. Insulin resistance impaired the endothelium dependent relaxation in F (n=6) compared to C (n=6). Metformin prevented endothelial dysfunction in fructose-fed rats (FM: n=6). Gonadectomy prevented endothelial dysfunction in fructose-fed rats (GF: n=6; GFM: n=6). **(B)** AUC values - relaxation to ACh. * P<0.05 F vs. C, CM, FM, G, GF, GM and GFM. All values are presented as mean \pm SEM.

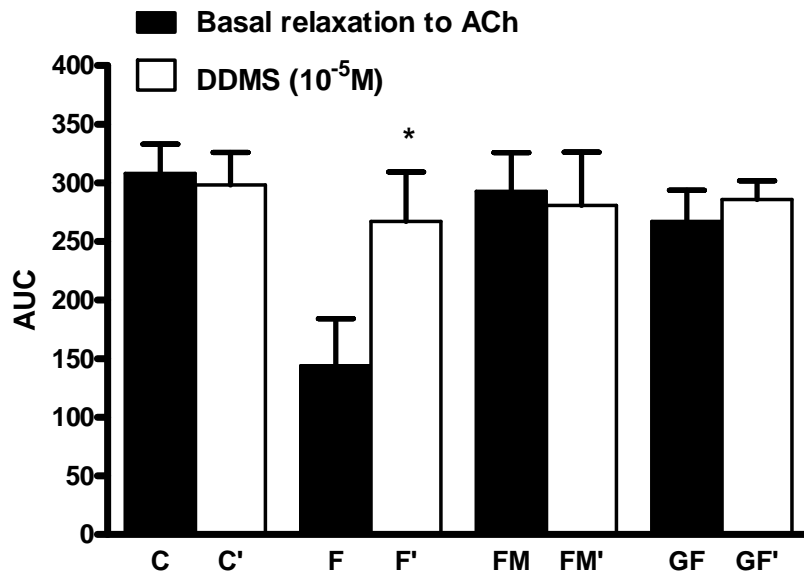


Figure 4.16: Inhibition of 20-HETE synthesis by DDMS improves relaxation to ACh in intact fructose-fed rats. Male Wistar rats were divided into 8 groups, which are: C-intact control rats; F-fructose-fed intact rats; FM-fructose-fed metformin treated rats; GF-gonadectomized fructose-fed rats. Black bars (C, F, FM, GF) are basal responses to acetylcholine in the absence of DDMS; White bars (C', F', FM',GF') are responses to ACh in the presence of DDMS.

Endothelium-dependent relaxation to acetylcholine was unaffected by DDMS in superior mesenteric arteries of of FM and GF rats. Relaxation is shown as AUC values. All groups n=4* P<0.05 F vs. F'. All values are presented as mean ± SEM.

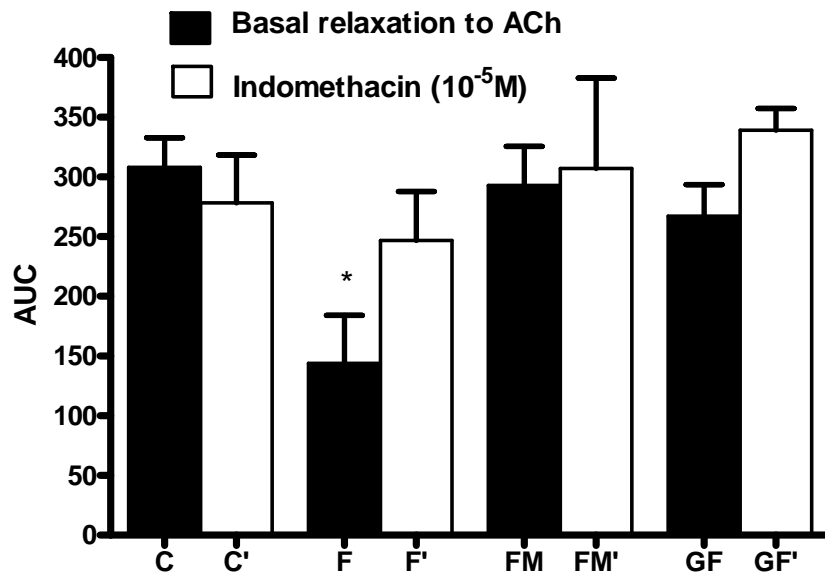


Figure 4.17: Inhibition of COX by indomethacin (INM) improves relaxation to ACh in intact fructose-fed rats. Male Wistar rats were divided into 8 groups, which are: C-intact control rats; F-fructose-fed intact rats; FM-fructose-fed metformin treated rats; GF-gonadectomized fructose-fed rats. Black bars (C, F, FM, GF) are basal responses to acetylcholine in the absence of indomethacin; White bars (C', F', FM', GF') are responses to ACh in the presence of indomethacin.

Endothelium-dependent relaxation to acetylcholine was unaffected by INM in superior mesenteric arteries of FM and GF rats All groups n=4-5.. Relaxation is shown as AUC values.* P<0.05 F vs. F+INM. All values are presented as mean ± SEM.

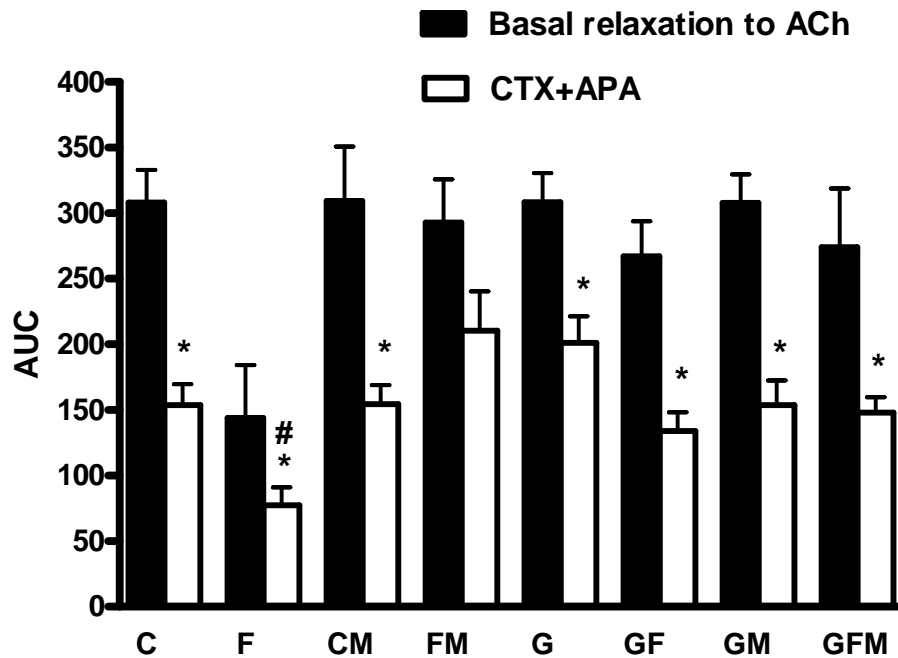


Figure 4.18: Responses to ACh are attenuated to a greater degree in fructose-fed rats in intact rats as compared to gonadectomized or metformin-treated rats following inhibition of EDHF-dependent relaxation. Male Wistar rats were divided into 8 groups, which are: C-intact control rats; F-fructose-fed intact rats; CM-control metformin-treated rats; FM-fructose-fed metformin treated rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats; GM-gonadectomized rats treated with metformin; GFM- gonadectomized fructose-fed rats treated with metformin. AUC values of all groups are compared subsequent to incubation with charybdotoxin (CTX 10^{-8} M) plus apamin (0.25×10^{-6} M). All groups n=4-5. # $P < 0.05$ F vs. C, CM, FM, G, GF, GM and GFM. * $P < 0.05$ relaxation in presence of CTX+APA (white boxes) vs. basal relaxation to ACh (boxes in black). # $P < 0.05$ F (CTX+APA) vs. C, CM, FM, G, GF, GM, GFM (all CTX+APA). All values are presented as mean \pm SEM.

4.5 Role of testosterone and cyclooxygenase 1 and 2 in mediating alpha-adrenoceptor stimulated vasoconstriction

4.5.1 Insulin sensitivity, blood pressure and testosterone

Gonadectomized rats had lower body weights compared to intact rats at the start of the study. This difference was consistent until termination (C; 543±11 gm vs. G; 452±9 gm). Similar to previous reports from our laboratory (Vasudevan, 2005; Vasudevan *et al.*, 2006), fructose attenuated the insulin sensitivity in both intact and gonadectomized rats (F and GF). This was demonstrated by hyperinsulinemia and mild hyperglycemia in response to the glucose challenge (Figure 4.19 A-C).

In both studies blood pressure was elevated in sham-operated fructose-fed rats at the end of 9 weeks (F, 134 ± 2 mm Hg vs. C, 113 ± 2 mm Hg). Fructose did not affect the blood pressure in gonadectomized animals (GF, 113 ± 2 mm Hg) (Figure 4.20).

Testosterone was undetectable in gonadectomized rats. Fructose did not affect testosterone levels (Figure 4.21).

4.5.2 Vascular reactivity studies

Responses to phenylephrine (PE) prior to and post selective/non selective COX inhibition

Superior mesenteric artery (SMA): Endothelium-intact vessels were used for these experiments. In the absence of inhibitors, responses to PE were unchanged in the fructose-fed rats (Figure 4.22). Further these responses were independent of testosterone.

Treatment with NS-398 attenuated the PE-induced vasoconstriction in both control and fructose-fed rats with intact testes (C and F) compared to gonadectomized groups (G and GF; Figure 4.23). When compared with responses in the absence of drug, NS-398 inhibited the responses only in C and F and not in G and GF (Table 4.1).

Interestingly incubation with indomethacin did not decrease PE-evoked responses in C and F compared to the gonadectomized groups (Figure 4.24). However, the responses were significantly attenuated in C and F compared to responses in the absence of the drug (Table 4.1). Similar to NS-398, indomethacin did not affect the responses to PE in G and GF (Table 4.1). This clearly suggests the presence of a testosterone-dependent involvement of COX. In addition, the data point to selective COX-2 involvement in mediating physiological responses to PE.

Treatment with SC-560, the selective COX-1 blocker, did not affect PE-induced vasoconstriction in any of the groups (Figure 4.25). This suggests a lack of involvement for COX-1 in PE-induced constriction.

COX inhibition did not affect the pD_2 values in tissues (Table 4.2). However, the changes in maximum concentration values (R_{max} -Table 4.3) mirrored the drug-dependent changes observed in the concentration response curves to PE discussed earlier (Table 4.1).

Aorta: Similar to the SMA, fructose feeding did not affect the responses to PE in isolated aorta (Figure 4.26). In the presence of NS-398, phenylephrine-evoked contraction was unaffected in all groups except F (Figure 4.27). NS-398 decreased the responses in F compared to basal values but not in C (Table 4.4), thus suggesting insulin resistance to be responsible for the augmented dependence on COX-2 in aorta. COX-2 dependent modulation

of constriction was absent in the gonadectomized rats where the degree of attenuation of PE-induced vasoconstriction was greater in F compared to GF (Figure 4.27).

Indomethacin produced a contraction pattern similar to that observed in presence of NS-398. Thus while indomethacin did not affect the responses in C, contraction to PE was attenuated in intact fructose-fed rats. Tissues from G and GF were unaffected by the presence of indomethacin (Figure 4.28). Similar to NS-398, PE-evoked contraction was significantly lower only in F following indomethacin treatment.

Responses to PE were unaffected in all groups following selective COX-1 blockade (Figure 4.29). Further SC-560 did not affect the responses compared to basal values in any of the groups (Table 2A). This is similar to the results observed in SMA.

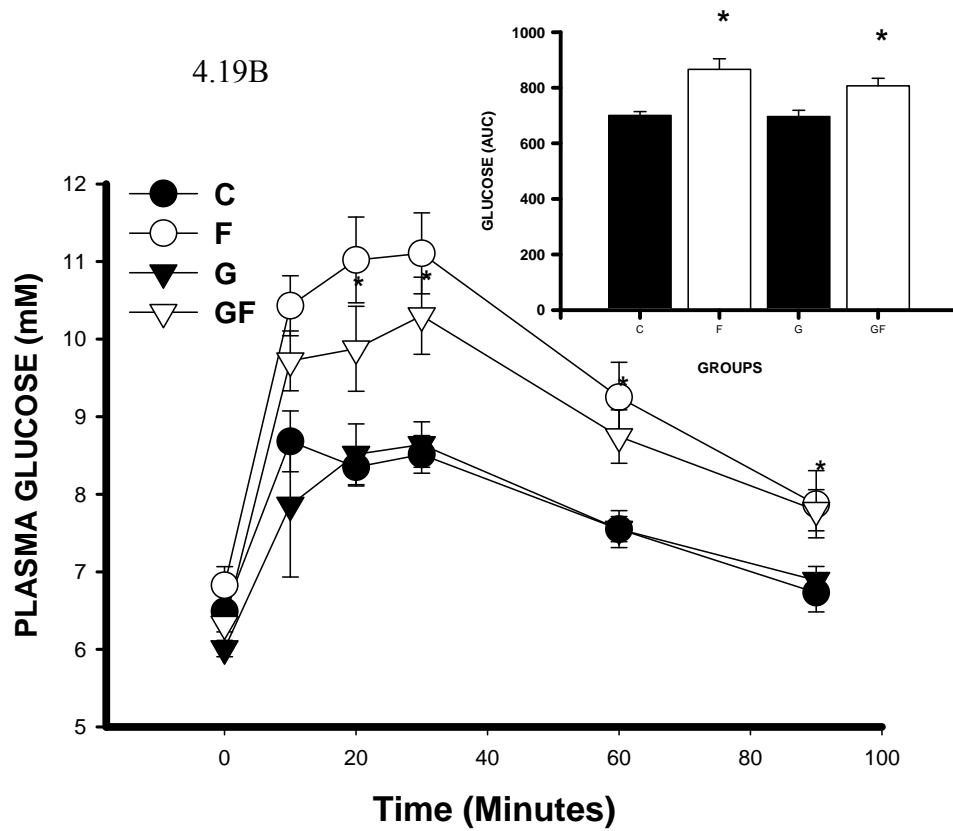
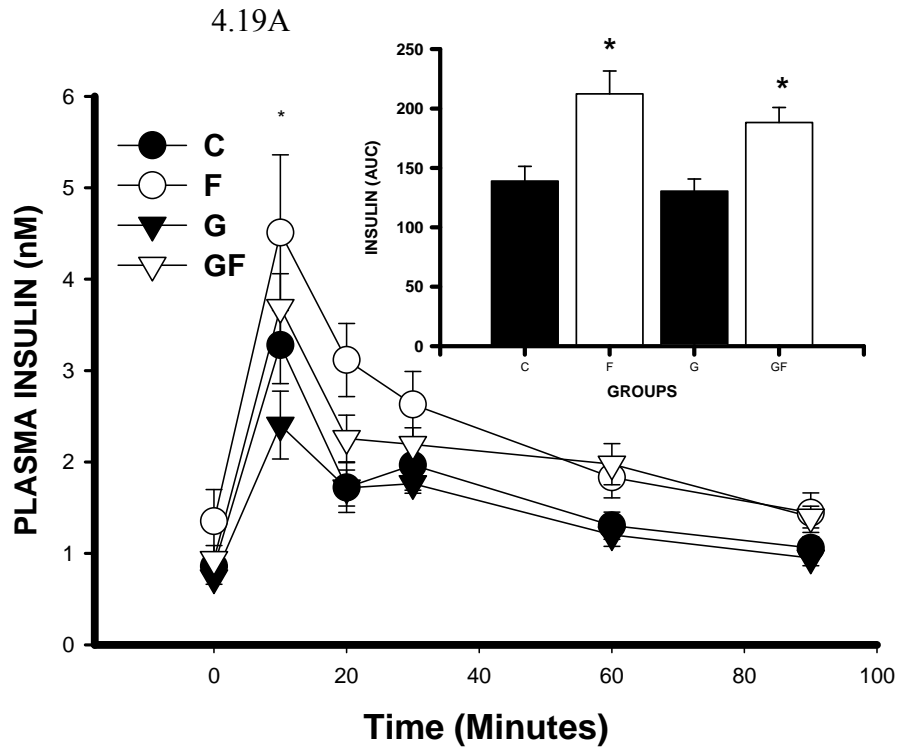
Similar to the SMA, pD₂ values were unaffected in all groups (Table 4.5). However inhibition of cyclooxygenase attenuated the R_{max} values in F but not C, G and GF (Table 4.6).

4.5.3 Cyp4A1, 2/3 and COX-2 expression in the aorta and SMA

In the SMA or aorta, both fructose and gonadectomy did not affect COX-2 expression. (Figures 4.30 and 4.31 respectively). Results from immunoprecipitation are not shown as the data was inconsistent. On the other hand, Cyp4A expression was increased in F compared to C in the SMA, which was prevented by gonadectomy (Figure 4.33). Cyp4A expression was unaffected by diet or testosterone in the aorta (Figure 4.32).

4.5.4 Plasma Thromboxane A2

Plasma thromboxane levels were increased in both the fructose-fed groups (F and GF). Interestingly, the TXA2 levels were the highest in GF compared to other groups, suggesting that the presence of testosterone may be essential to its pro-hypertensive function (Figure 4.34).



4.19C

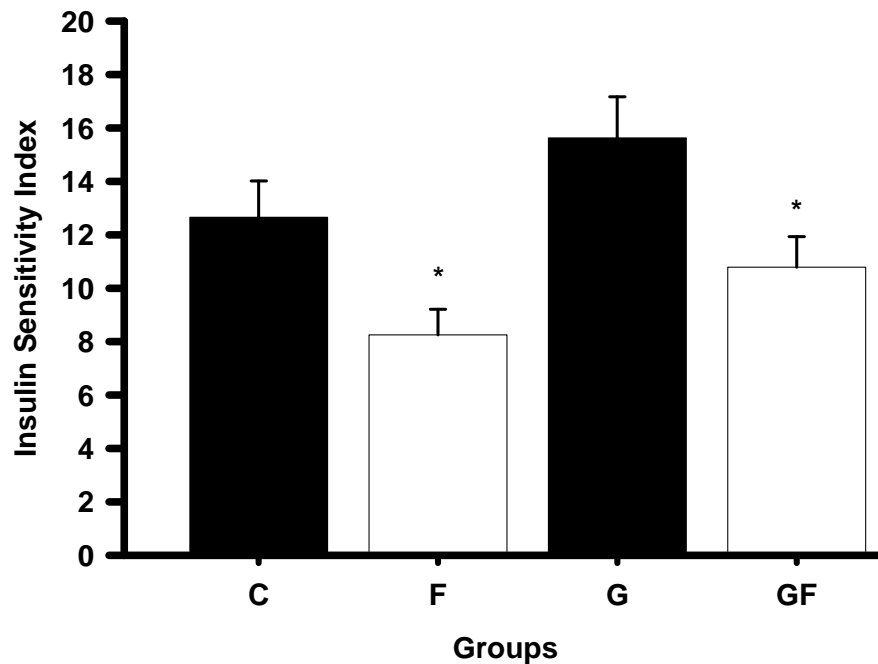


Figure 4.19A-C: Plasma insulin and glucose profiles and insulin sensitivity index (ISI) following an oral glucose challenge. Groups are C-control and intact rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats. (A)-Graph of insulin concentration vs. time. Inset shows area under the curve values (AUC) for each group. (B)-Graph of glucose concentration vs. time. Inset shows area under the curve values (AUC) for each group. (C)-Insulin sensitivity index values for all groups as calculated by the Matsuda and DeFronzo formula (Matsuda *et al.*, 1999). Plasma insulin levels were the highest in F and GF compared to rats fed on normal chow. Gonadectomy did not affect hyperinsulinemia subsequent to fructose feeding. * $P < 0.05$ sham-operated fructose-fed male rats (F, $n=8$) and gonadectomized high fructose-fed (GF, $n=8$) vs. normal chow-fed controls (C, $n=8$) and gonadectomized normal chow-fed (G, $n=10$). All values are presented as mean \pm SEM.

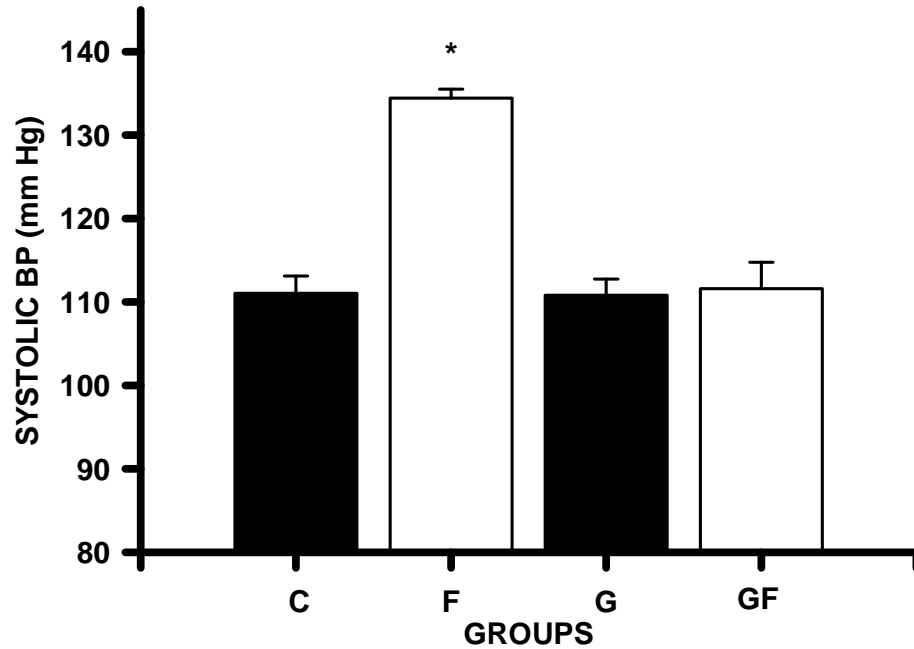


Figure 4.20: Gonadectomy prevented the development of hypertension in fructose-fed rats (GF). Groups are C-control and intact rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats. Systolic blood pressure was measured in sham-operated (F, n=8) and gonadectomized (GF, n=9) male fructose-fed rats. Measurements were taken at 9 weeks (prior to termination). * $P < 0.05$ F vs. C, G and GF. All values are presented as mean \pm SEM.

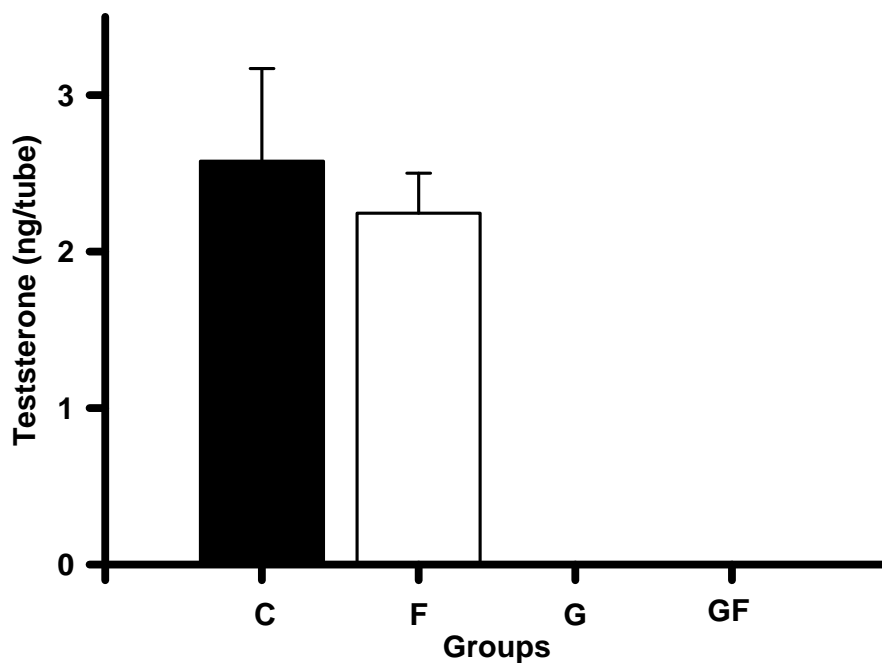


Figure 4.21: Plasma testosterone levels in intact and gonadectomized rats after feeding with fructose. Fructose feeding did not alter the testosterone levels in intact male rats (F) as compared to normal chow-fed controls (C). Testosterone levels were undetectable in the gonadectomized normal chow-fed (G) and gonadectomized high fructose-fed (GF) rats. Testosterone was measured using a commercially available RIA kit (MP Biomedicals). All groups n=8. All values are presented as mean \pm SEM.

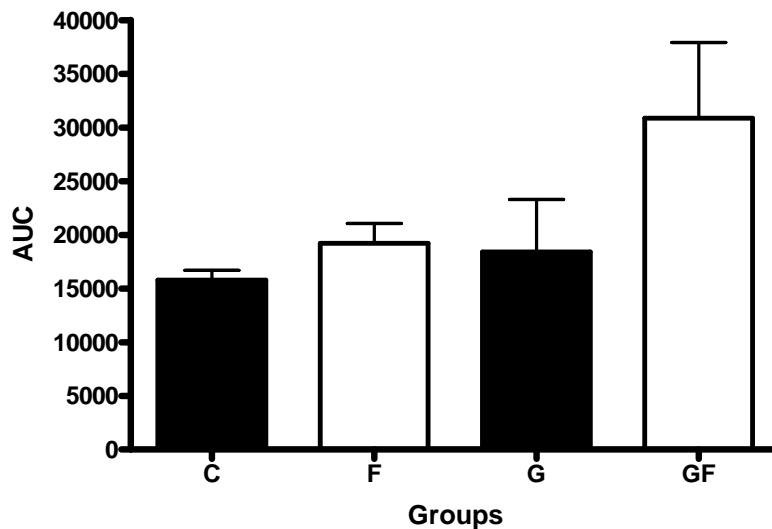
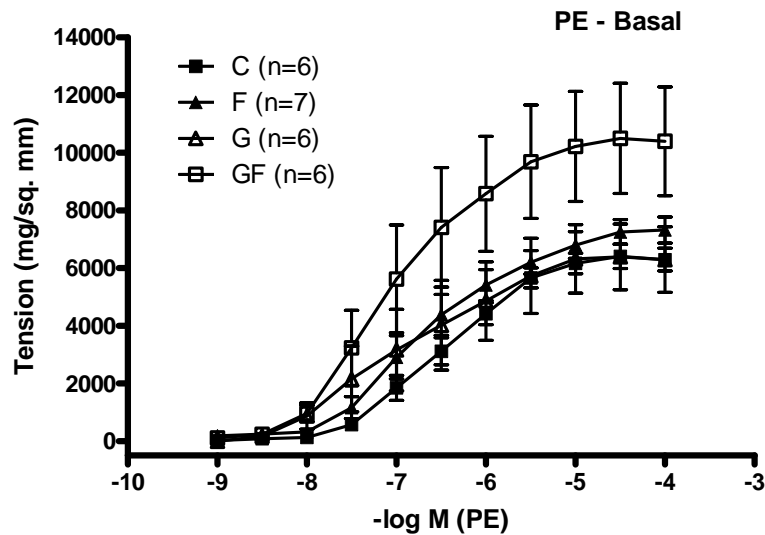


Figure 4.22: Fructose feeding (F) and gonadectomy do not affect responses to phenylephrine (PE) in the superior mesenteric arteries (SMA). Responses were obtained to PE (10^{-9} to 10^{-4} mol/L) in the absence of inhibitors in the four experimental groups; control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of mg/square mm. The lower graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. All values are presented as mean \pm SEM.

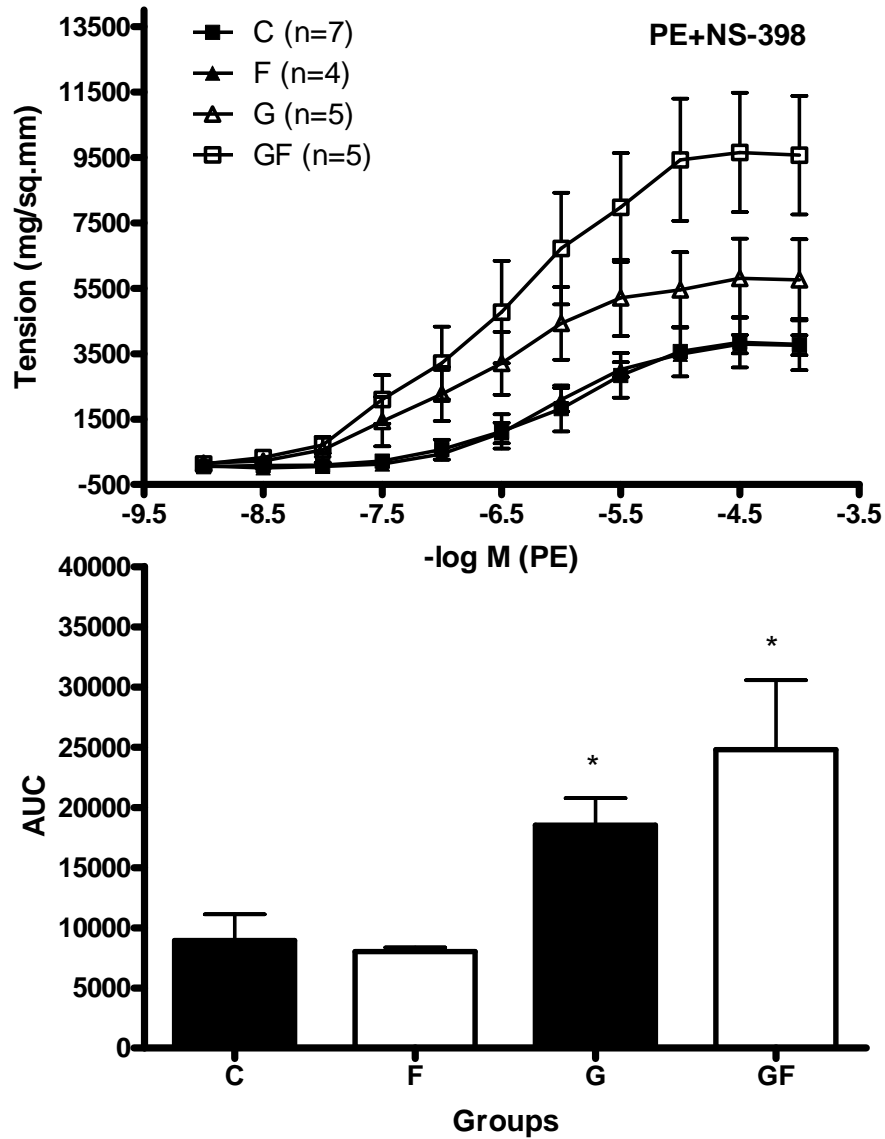


Figure 4.23: Selective COX-2 blockade attenuates PE-evoked responses in the SMA of intact (C and F) but not in gonadectomized (G and GF) rats. Responses to PE (10^{-9} to 10^{-4} mol/L) were obtained in the presence of the COX-2 selective inhibitor NS-398 (10^{-6} M) in control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of mg/square mm. The adjacent graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. * $P < 0.05$ G and GF vs. C and F. All values are presented as mean \pm SEM.

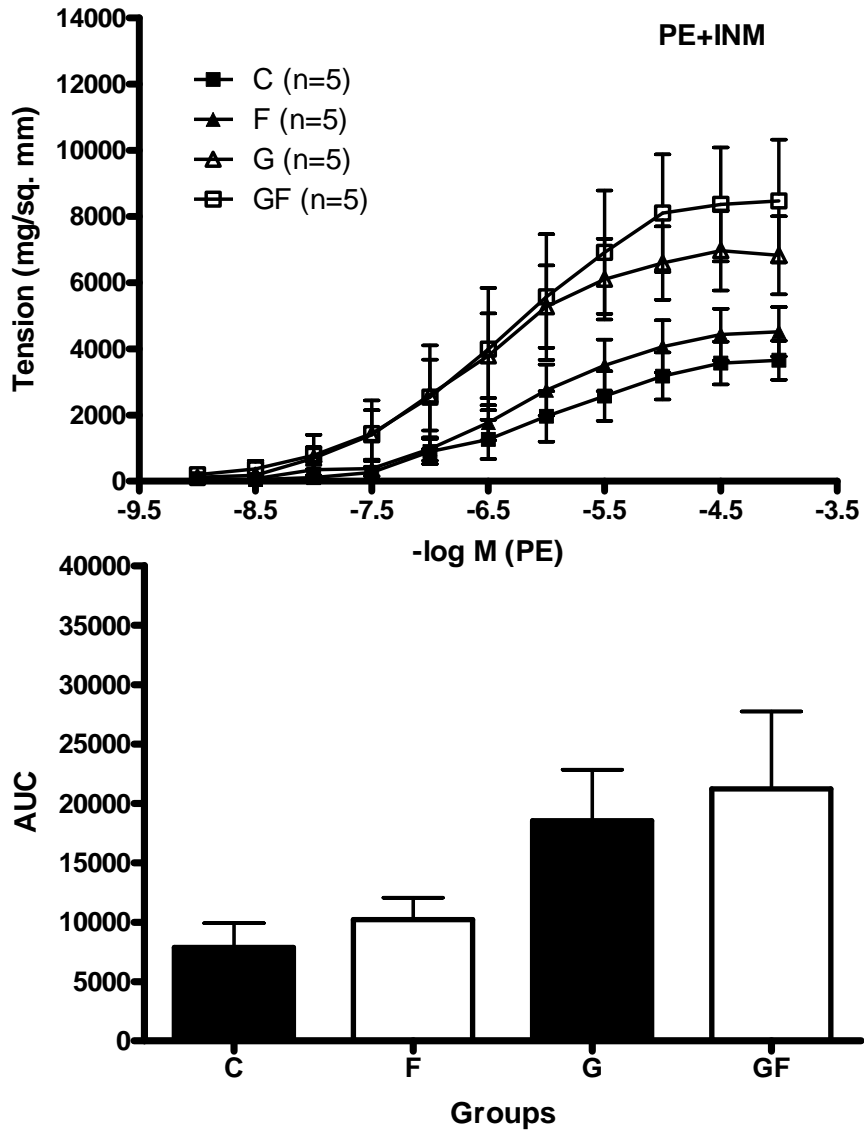


Figure 4.24: Fructose feeding and gonadectomy did not affect responses to PE subsequent to non-selective COX isoform inhibition using indomethacin (10^{-5} M). Responses were obtained to PE (10^{-9} to 10^{-4} mol/L) in control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of mg/square mm. The lower graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. All values are presented as mean \pm SEM.

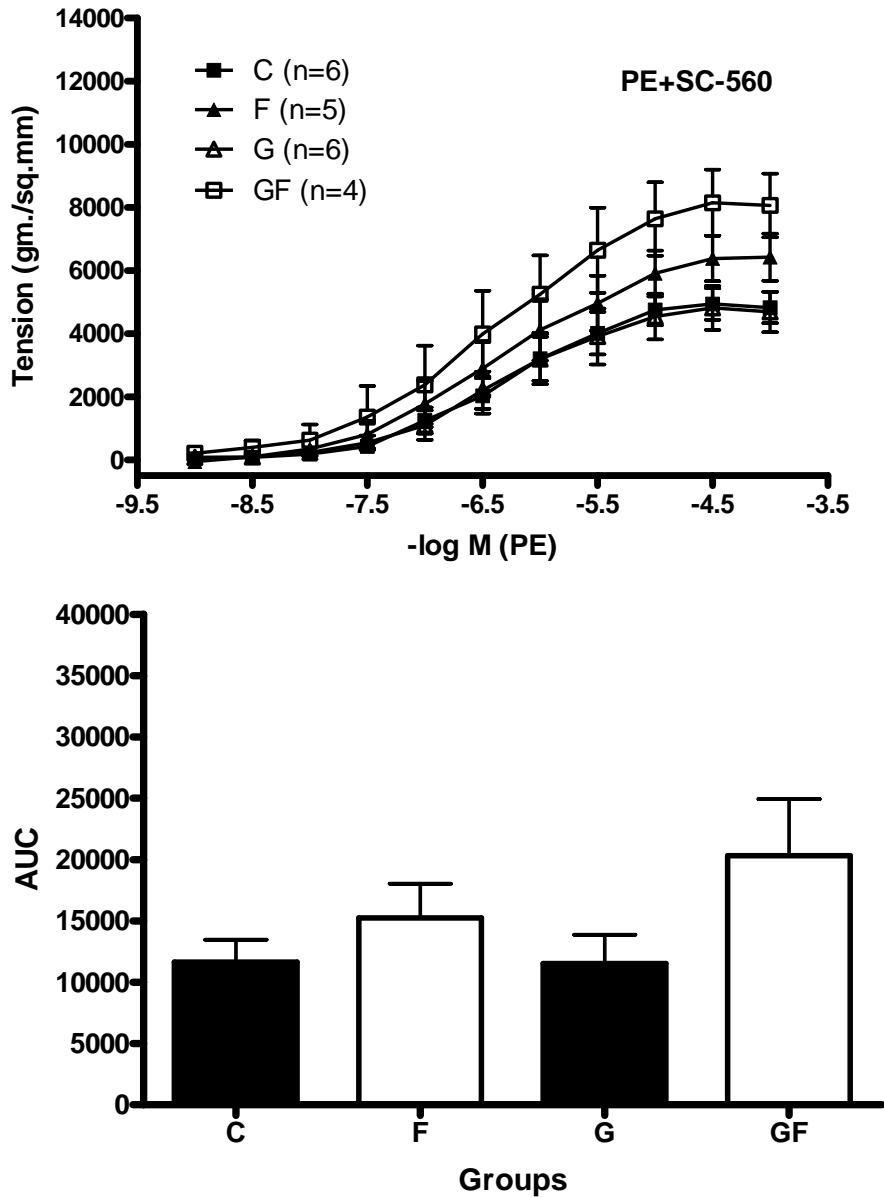


Figure 4.25: Fructose feeding and gonadectomy did not affect responses to PE subsequent to selective COX-1 inhibition using SC-560 (10^{-5}M). Responses were obtained to PE (10^{-9} to 10^{-4} mol/L) in control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of mg/square mm. The lower graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. All values are presented as mean \pm SEM.

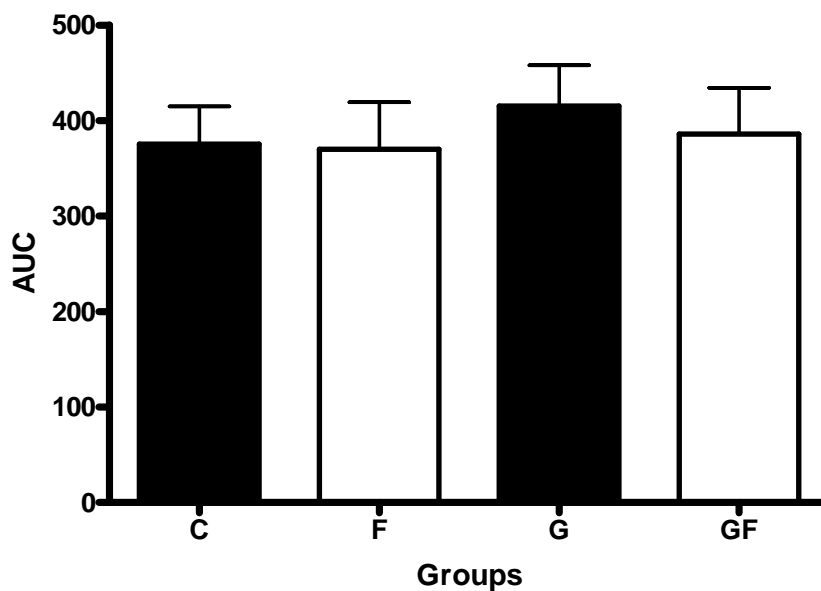
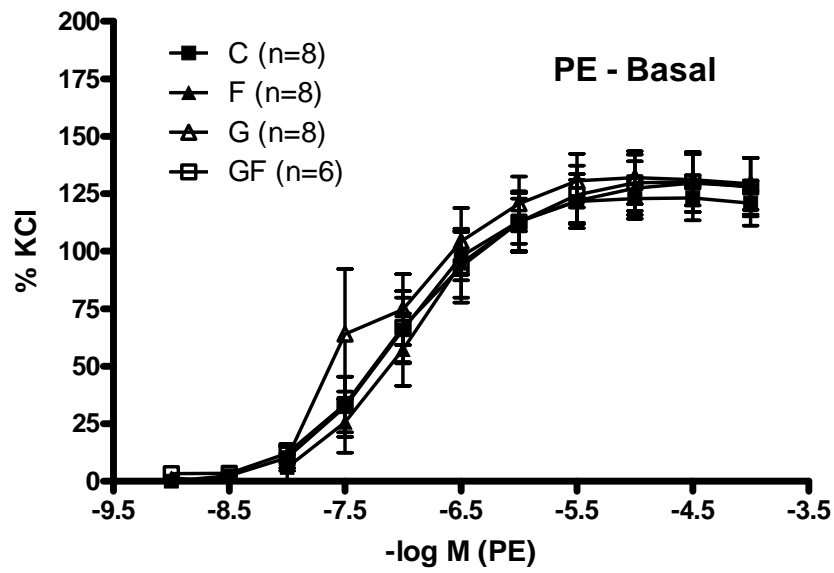


Figure 4.26: Fructose feeding (F) and gonadectomy do not affect responses to phenylephrine (PE) in the aorta. Responses were obtained to PE (10^{-9} to 10^{-4} mol/L) in the absence of inhibitors in control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of % maximum response to KCl. The lower graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. All values are presented as mean \pm SEM.

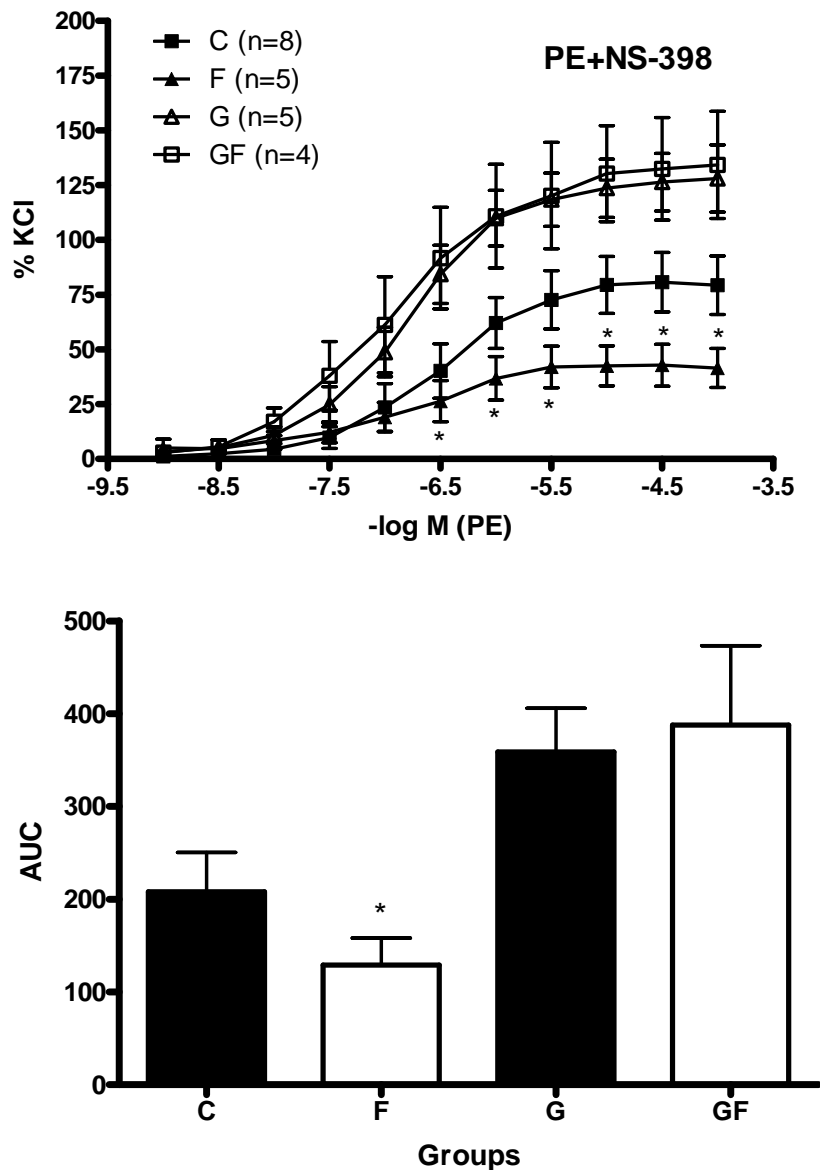


Figure 4.27: Selective COX-2 blockade attenuates PE-evoked responses in the aorta of intact (C and F) but not in gonadectomized (G and GF) rats. Responses to PE (10^{-9} to 10^{-4} mol/L) were obtained in the presence of the COX-2 selective inhibitor NS-398 (10^{-6} M) in control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of % maximum response to KCl. The lower graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. * $P < 0.05$ G and GF vs. C and F. All values are presented as mean \pm SEM.

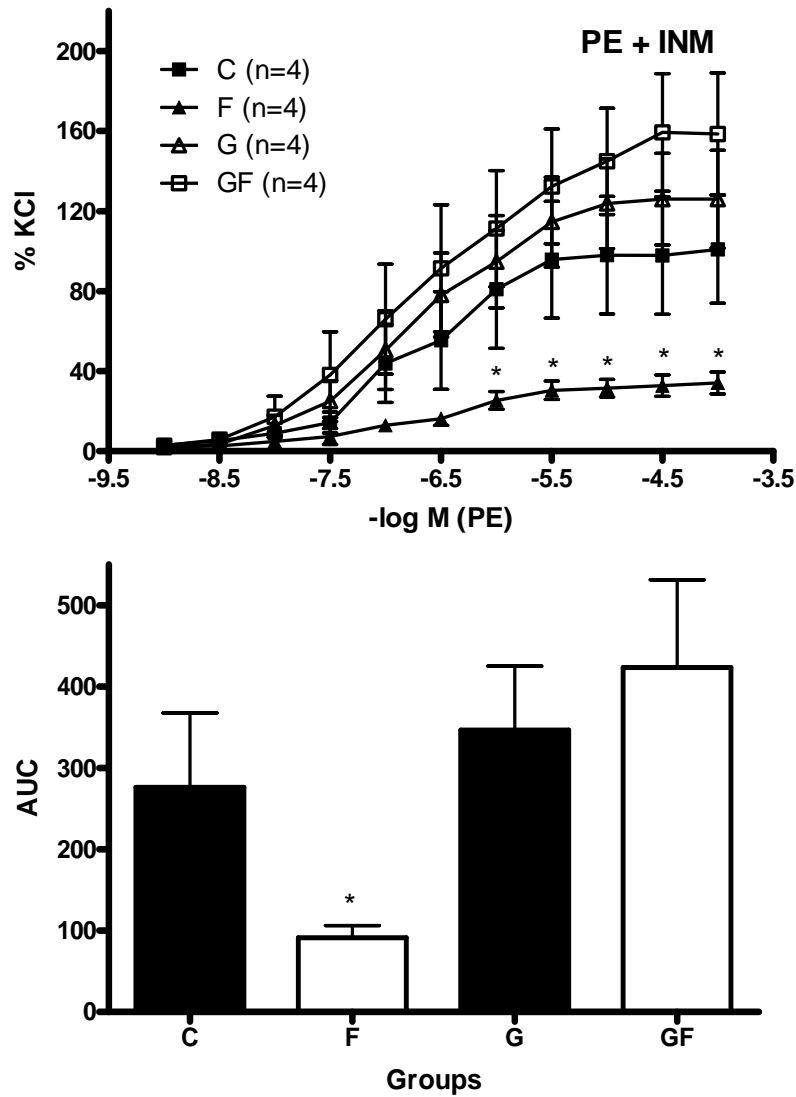


Figure 4.28: Non-selective COX-2 blockade attenuates PE-evoked responses in the aorta of intact (C and F) but not in gonadectomized (G and GF) rats. Responses to PE (10^{-9} to 10^{-4} mol/L) were obtained in the presence of indomethacin (10^{-5} M) in control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of % maximum response to KCl. The lower graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. * $P < 0.05$ G and GF vs. C and F. All values are presented as mean \pm SEM.

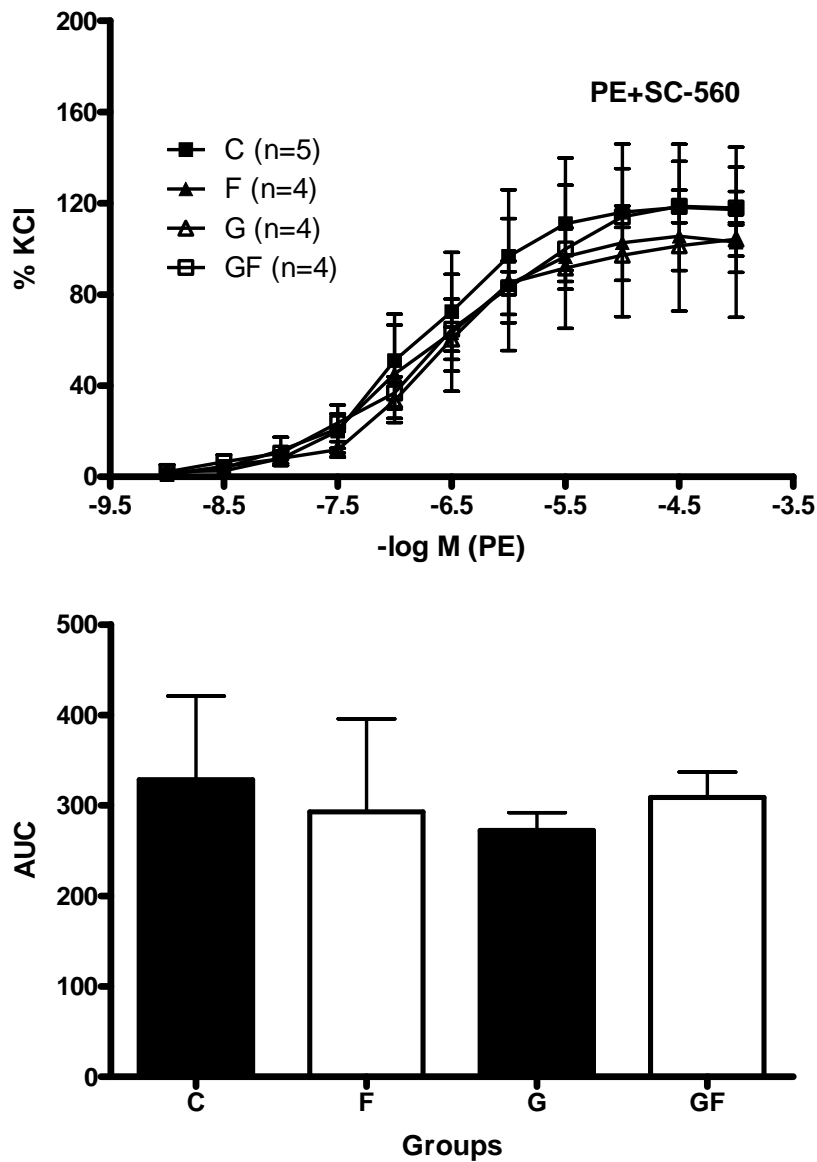


Figure 4.29: Fructose feeding and gonadectomy did not affect responses to PE subsequent to selective COX-1 inhibition using SC-560 (10^{-5} M). Responses were obtained to PE (10^{-9} to 10^{-4} mol/L) in control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose-fed (GF). Values are in terms of % maximum response to KCl. The lower graph shows corresponding area under the curve (AUC) values for the curve. “n” values are indicated on the graph. All values are presented as mean \pm SEM.

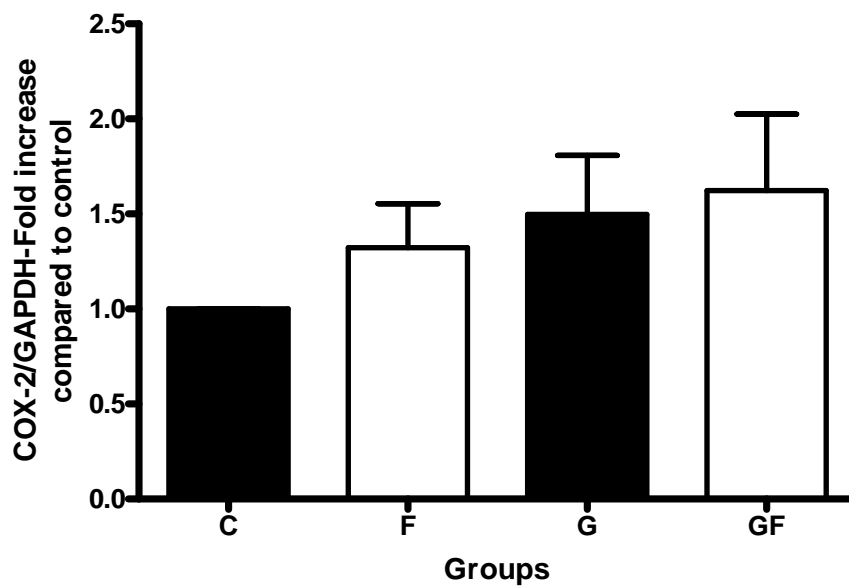
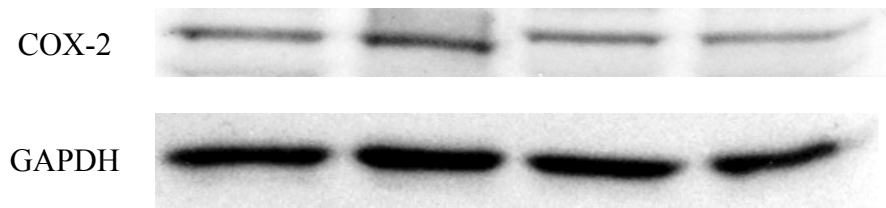


Figure 4.30: Changes in COX-2 expression in the aorta of intact and gonadectomized rats. Groups are C-control and intact rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats. Western blotting was performed on protein lysates. Controls are bands from GAPDH, which was used as the housekeeping protein. All groups n=6. All values are presented as mean \pm SEM.

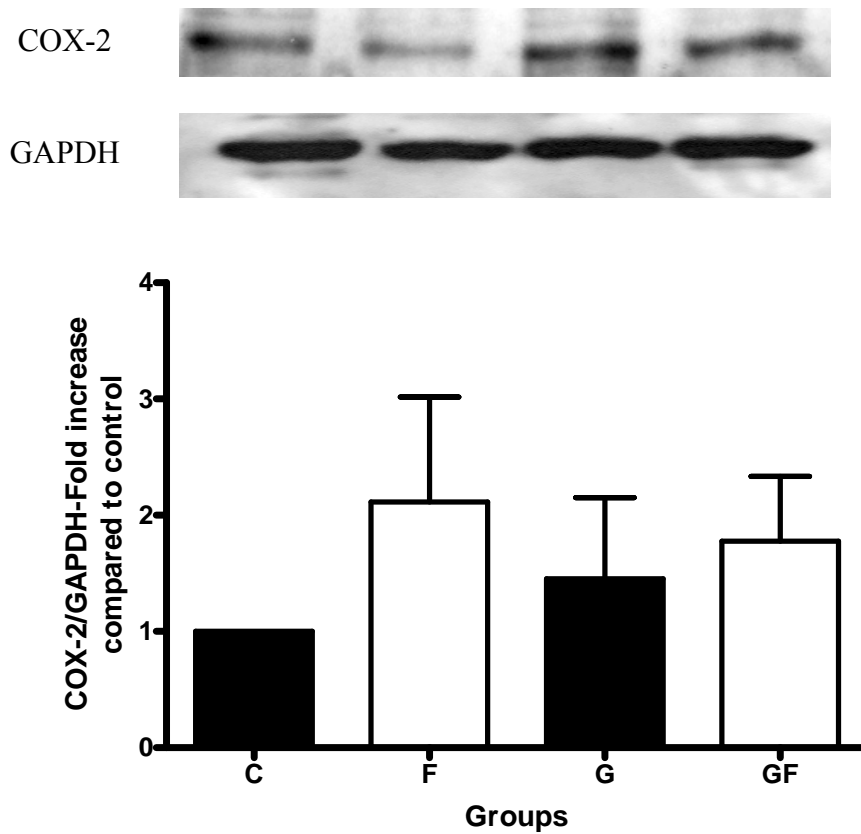


Figure 4.31: Changes in COX-2 expression in the superior mesenteric artery of intact and gonadectomized rats. Groups are C-control and intact rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats. Western blotting was performed on protein lysates. Controls are bands from GAPDH, which was used as the housekeeping protein. All groups n=6. All values are presented as mean \pm SEM.

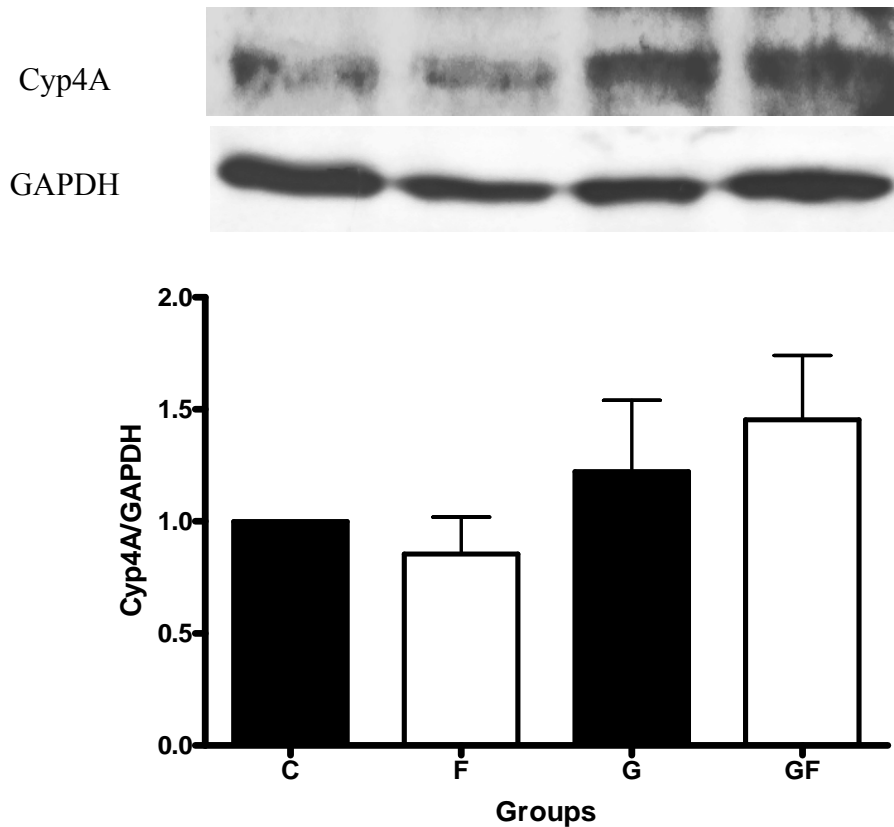


Figure 4.32: Changes in Cyp4A1, 2/3 in the aorta of intact and gonadectomized rats. Groups are C-control and intact rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats. Western blotting was performed on protein lysates. Controls are bands from GAPDH, which as used as the housekeeping protein. All groups n=4. All values are presented as mean \pm SEM.

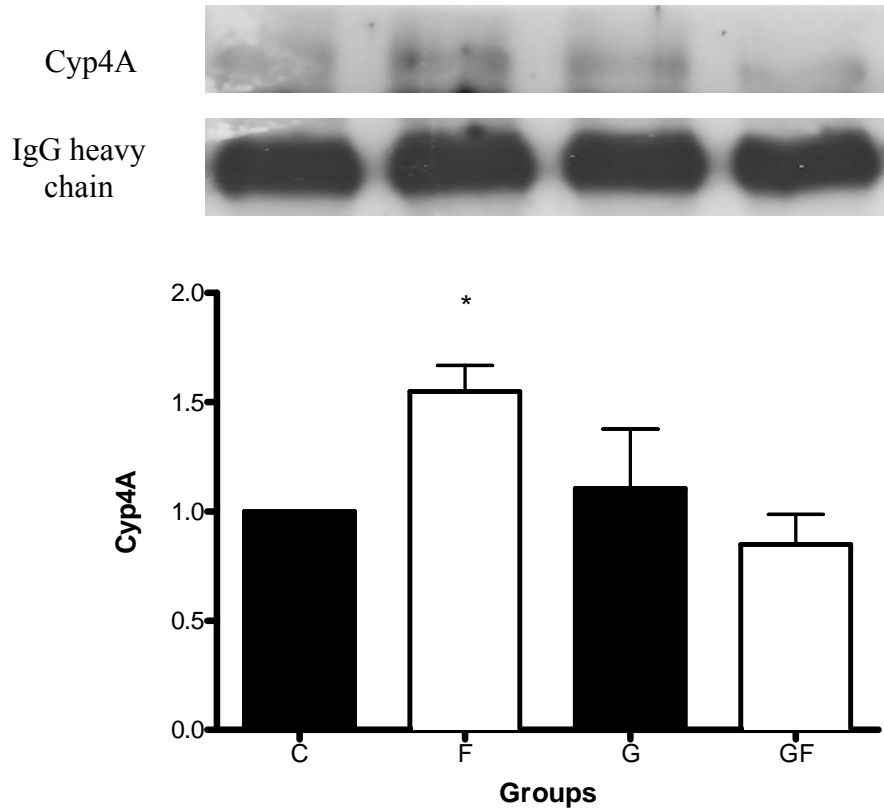


Figure 4.33: Changes in Cyp4A1, 2/3 expression in the superior mesenteric artery of intact and gonadectomized rats. Groups are C-control and intact rats; F-fructose-fed intact rats; G-gonadectomized rats; GF-gonadectomized fructose-fed rats. Western blotting was performed on protein lysates. Controls are bands from, which was used as the housekeeping protein. All groups n=4. *P<0.05 F vs. C, and GF. All values are presented as mean ± SEM.

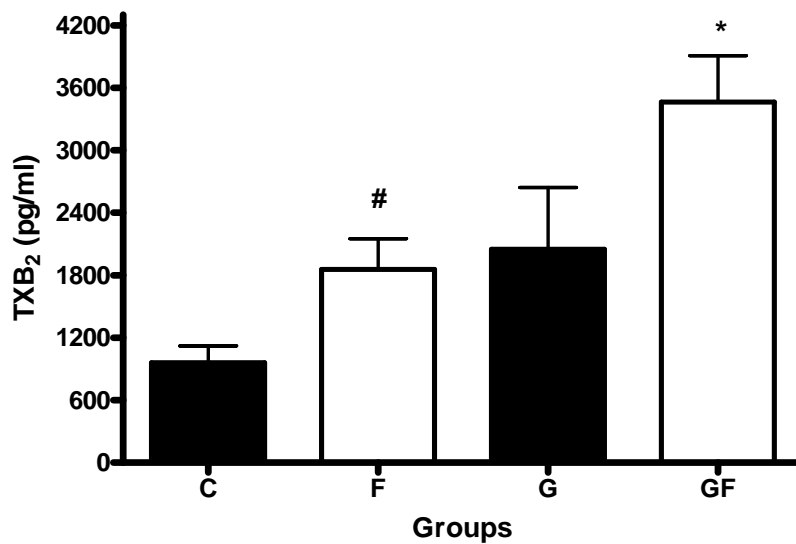


Figure 4.34: Changes in plasma thromboxane B2 (TXB2) levels in control (C; n=5), fructose (F; n=7), gonadectomized (g; n=6) and gonadectomized fructose-fed (GF; n=5). Thromboxane A2 was measured in terms of its stable metabolite TXB2 using an EIA kit. All values are mean \pm sem. *P<0.05 GF vs. C, F and G; # P<0.05 F vs. C.

Table 4.1: Area under the curve values in the superior mesenteric artery. Pressor responses to phenylephrine (PE) were evaluated in control (C, n=5-7), fructose-fed (F, n=4-7), gonadectomized (G, n=5-6) and gonadectomized fructose-fed (GF, n=4-6) subsequent to selective (NS-398 and SC-560) and non-selective (indomethacin (INM)) inhibition of COX 1 and 2. All values are mean \pm SEM. * P<0.05 PE+NS-398 and PE+INM vs. PE (Basal).

Groups	PE (Basal) (AUC)	PE+NS-398 (AUC)	PE+SC-560 (AUC)	PE+INM (AUC)
C	15784 \pm 897	8928 \pm 2193*	10427 \pm 1958	7865 \pm 2043*
F	19223 \pm 1832	8006 \pm 360*	13554 \pm 2848	10224 \pm 1827*
G	11345 \pm 4936	18509 \pm 2264	11528 \pm 2345	18547 \pm 4282
GF	30856 \pm 7048	24871 \pm 5733	20300 \pm 4624	21210 \pm 6537

Table 4.2: pD₂ values to phenylephrine (PE) in the superior mesenteric artery (SMA). Pressor responses to phenylephrine (PE) were evaluated in control (C, n=5-7), fructose-fed (F, n=4-7), gonadectomized (G, n=5-6) and gonadectomized fructose-fed (GF, n=4-6) subsequent to selective (NS-398 and SC-560) and non-selective (indomethacin (INM)) inhibition of COX 1 and 2. All values are mean ± SEM. * P<0.05 PE+NS-398 and PE+INM vs. PE; † P<0.05 GF vs. C, F and G (all PE+NS-398).

Groups	pD ₂			
	PE	PE + NS-398	PE + SC-560	PE + INM
C	6.5 ± 0.2	6.0 ± 0.2	6.1 ± 0.2	6.0 ± 0.4
F	6.7 ± 0.2	6.1 ± 0.2	6.3 ± 0.3	6.5 ± 0.3
G	6.6 ± 0.4	6.7 ± 0.2	6.2 ± 0.2	6.4 ± 0.4
GF	6.9 ± 0.3	6.4 ± 0.2	6.4 ± 0.3	6.3 ± 0.3

Table 4.3: R_{max} values to PE in the SMA. Pressor responses to phenylephrine (PE) were evaluated in control (C, n=5-7), fructose-fed (F, n=4-7), gonadectomized (G, n=5-6) and gonadectomized fructose-fed (GF, n=4-6) subsequent to selective (NS-398 and SC-560) and non-selective (indomethacin (INM)) inhibition of COX 1 and 2. All values are mean \pm SEM. * $P < 0.05$ PE+NS-398 and PE+INM vs. PE; $\dagger P < 0.05$ GF vs. C, F and G (all PE+NS-398).

Groups	R_{max}			
	PE	PE + NS-398	PE + SC-560	PE + INM
C	6463 \pm 397	3916 \pm 772 *	4589 \pm 561	3850 \pm 546*
F	7148 \pm 417	3879 \pm 313 *	5825 \pm 920	4897 \pm 817*
G	4876 \pm 1673	4311 \pm 1404	3991 \pm 712	6722 \pm 1863
GF	10325 \pm 1844	9839 \pm 2031 \dagger	8321 \pm 1037	8527 \pm 1819

Table 4.4: Area under the curve values in the aorta. Pressor responses to phenylephrine (PE) were evaluated in control (C, n=4-8), fructose-fed (F, n=4-8), gonadectomized (G, n=4-8) and gonadectomized fructose-fed (GF, n=4-6) subsequent to selective (NS-398 and SC-560) and non-selective (indomethacin (INM)) inhibition of COX 1 and 2. All values are mean \pm SEM. * P<0.05 PE+NS-398 and PE+INM vs. PE (Basal) and PE+SC-560.

Groups	PE (Basal) (AUC)	PE+NS-398 (AUC)	PE+SC-560 (AUC)	PE+INM (AUC)
C	375 \pm 40	208 \pm 43	329 \pm 92	276 \pm 92
F	370 \pm 49	129 \pm 29*	293 \pm 103	91 \pm 15*
G	415 \pm 43	359 \pm 47	272 \pm 20	346 \pm 79
GF	386 \pm 48	388 \pm 85	309 \pm 28	424 \pm 108

Table 4.5: pD2 values to phenylephrine (PE) in the aorta. Pressor responses to phenylephrine (PE) were evaluated in control (C, n=4-8), fructose-fed (F, n=4-8), gonadectomized (G, n=4-8) and gonadectomized fructose-fed (GF, n=4-6) subsequent to selective (NS-398 and SC-560) and non-selective (indomethacin (INM)) inhibition of COX 1 and 2. All values are mean \pm SEM.

Groups	pD2			
	PE	PE + NS-398	PE + SC-560	PE + INM
C	7.1 \pm 0.14	6.5 \pm 0.14	6.6 \pm 0.21	6.7 \pm 0.21
F	6.9 \pm 0.14	6.9 \pm 0.40	6.6 \pm 0.2	6.3 \pm 0.41
G	7.3 \pm 0.14	6.7 \pm 0.13	6.7 \pm 0.07	6.5 \pm 0.023
GF	6.9 \pm 0.20	6.8 \pm 0.27	6.4 \pm 0.24	6.5 \pm 0.39

Table 4.6: R_{max} values to PE in the aorta. Pressor responses to phenylephrine (PE) were evaluated in control (C, n=4-8), fructose-fed (F, n=4-8), gonadectomized (G, n=4-8) and gonadectomized fructose-fed (GF, n=4-6) subsequent to selective (NS-398 and SC-560) and non-selective (indomethacin (INM)) inhibition of COX 1 and 2. All values are mean \pm SEM.
 * $P < 0.05$ PE+NS-398 and PE+INM vs. PE and PE+SC-560; $\dagger P < 0.05$ F vs. G and GF.

Groups	R_{max}			
	PE	PE + NS-398	PE + SC-560	PE + INM
C	122 \pm 9	82 \pm 13	107 \pm 26	101 \pm 29
F	127 \pm 12	44 \pm 10 * \dagger	106 \pm 34	40 \pm 10 * \dagger
G	130 \pm 11	111 \pm 12	102 \pm 5	130 \pm 24
GF	129 \pm 12	131 \pm 22	125 \pm 10	175 \pm 40

5. DISCUSSION

5.1 General overview

The metabolic syndrome is a cluster of phenotypes which includes insulin resistance and hypertension. A combination of genetic and environmental factors causes insulin resistance. Insulin resistance has been extensively studied in the skeletal muscle, adipose tissue and liver. In the past decade insulin resistance has also been characterized and studied in the cardiovascular system (Eckel *et al.*, 2000). Some of the early complications associated with insulin resistance are hyperinsulinemia, increased triglycerides (Galipeau *et al.*, 2001; Jiang *et al.*, 2007) and free fatty acids (Alzamendi *et al.*, 2009) and vascular endothelial dysfunction (Vasudevan *et al.*, 2006), which abets the increase in blood pressure. All of these features except obesity have been characterized in diet-induced models of the metabolic syndrome such as the fructose fed rat. However, fructose has been shown to increase adipocyte mass and size in the abdomen (Alzamendi *et al.*, 2009). Eating or drinking fructose has been shown to induce insulin resistance as early as 2-3 weeks accompanied by endothelial dysfunction (Katakam *et al.*, 1998). Similar symptoms are observed in sucrose-fed rats (El Hafidi *et al.*, 2006). However these findings have been recorded anywhere between 2-24 weeks of feeding the high carbohydrate containing diet.

The induction of endothelial dysfunction as demonstrated by attenuated relaxation to acetylcholine may be due to reduced synthesis of or response to endothelial vasorelaxants and/or increased synthesis or response to vasoconstrictors. We and others have previously demonstrated the loss of endothelial NO-dependent relaxation in the mesenteric arteries (Katakam *et al.*, 1998; Vasudevan *et al.*, 2006). Preventing insulin resistance by metformin

prevented endothelial dysfunction (Figure 4.15A-B). In addition, we have also previously demonstrated attenuated EDHF-dependent relaxation in insulin resistance (Vasudevan *et al.*, 2006). One of the key factors in regulating hemodynamics in the metabolic syndrome are the sex hormones estrogen and testosterone. Our laboratory has previously demonstrated that estrogen (Galipeau *et al.*, 2002a; Galipeau, 2001) and testosterone (Song *et al.*, 2004) have been separately shown to prevent and permit, respectively, the increase in blood pressure and endothelial dysfunction (Vasudevan, 2005; Vasudevan *et al.*, 2006) following insulin resistance. Furthermore we have also reported that, more than the absence of estrogen, the presence of testosterone is essential in impairing relaxation and increasing blood pressure. Gonadectomy prevented the loss of NO-dependent relaxation in the SMA (Vasudevan *et al.*, 2006). However as testosterone did not affect insulin sensitivity, we asked if insulin resistance triggered vasoconstrictor pathway(s) which were dependent on testosterone.

The current work extended the previous findings by investigating the individual contributions of testosterone-dependent vasoactive pathways. The objectives were:

1. To confirm the role of testosterone in increasing the blood pressure secondary to insulin resistance. In addition to replacing testosterone, we studied if testosterone-dependent hemodynamic changes were mediated through the androgen receptor.
2. To determine if and to what extent does testosterone affect arachidonic acid metabolism in insulin resistance. Both cyclooxygenase-2 and Cyp4A have been shown to be regulated by testosterone.
3. To determine if insulin resistance by itself alters the equilibrium in the production of various prostanoids and eicosanoids.

5.2 Fructose-fed rat- a model for insulin resistance and blood pressure

Feeding fructose has been shown by various groups to induce insulin resistance, elevated plasma insulin and triglycerides and hypertension without affecting glucose levels (Galipeau *et al.*, 2001; Hwang *et al.*, 1987; Katakam *et al.*, 1998; Vasudevan, 2005; Verma *et al.*, 2000). In these rats, insulin resistance precedes vascular complications and high blood pressure (Katakam *et al.*, 1998). Our laboratory has shown that both Wistar and SD rats develop the above mentioned symptoms upon feeding with fructose for 6-9 weeks without any change in body weight or weight gain (Vasudevan *et al.*, 2006). Treatment with metformin prevents insulin resistance and associated endothelial dysfunction (Figures 4.13A-C and 4.15A-B respectively), which is in agreement with previously published reports (Verma *et al.*, 1994a). However fructose feeding for 9 weeks mirrors only the initial stages of the metabolic syndrome and does not cause obesity or Type 2 diabetes. Only one report has suggested the development of diabetes in rats fed with sucrose for up to 15 months (Pierce *et al.*, 1989). Fructose diet/syrup has been implicated in the development of obesity in humans (Elliott *et al.*, 2002; Melanson *et al.*, 2008). However, additional evidence is required to confirm if these findings are reflected in sugar-fed rats. Several reports have shown increased adipose size and mass in the abdomen of fructose-fed rats (Alzamendi *et al.*, 2009; El Hafidi *et al.*, 2006), but no direct evidence for obesity. Unpublished data from our laboratory agree with the clinical findings as feeding fructose for a year may induce obesity in Wistar rats. However, fructose-fed SD rats did not become obese (Yuen *et al.* 2009), which suggests the involvement of strain difference as a possible factor.

One of the main areas of debate related to this model is the increase in blood pressure. Various groups have reported increased blood pressure in SD/Wistar fructose-fed rats over 4-9 weeks (Lin *et al.*, 2007; Vasudevan *et al.*, 2006; Verma *et al.*, 1997a). Using the non-invasive tail cuff method, our laboratory has observed a significant increase in systolic blood pressure from 6-9 weeks in Wistar rats (Galipeau *et al.*, 2001; Vasudevan *et al.*, 2006). This method enabled us to record changes in blood pressure over a long period and is similar to using a sphygmomanometer in the clinic. Similar results have been obtained by directly measuring blood pressure in conscious rats whose femoral arteries were cannulated and subsequently allowed adequate rest for normalization of baroreflexes (Dai *et al.*, 1994; Hsieh *et al.*, 2005; Song *et al.*, 2005). However there is one conflicting report that suggests the increases in BP in fructose-fed rats is a stress-induced artifact when measured by the tail cuff method. Measurement of blood pressure in conscious fructose-fed rats by radiotelemetry did not reveal any difference between basal and 8-week blood pressure, although insulin resistance and endothelial dysfunction were observed (D'Angelo *et al.*, 2005). In our opinion, this is an isolated case as several studies in various rodent models of hypertension have compared and validated the tail cuff method against the direct and/or telemetric methods and reported similar values for blood pressure (Fraser *et al.*, 2001; Ibrahim *et al.*, 2006; Van Vliet *et al.*, 2000). Ibrahim *et al.* (2006) argue that both direct and tail cuff methods, despite their potential limitations, were accurate compared to telemetric measurements, which showed high variability between data points and may not be a true snapshot of changes in blood pressure. In addition, telemetric experiments are expensive and resource-intensive which limit their use. Tail cuff measurements have been shown to be accurate and suitable for high throughput experimental protocols in mice (Feng *et al.*, 2008; Kurtz *et al.*, 2005). In our

studies, as mentioned in Section 2, rats were conditioned to being restrained prior to and during the course of the study. The temperature inside the blood pressure chamber was maintained between 24°C and 27°C. Once inside the restrainer, rats were allowed enough time to rest and acclimatize to the chamber, following which at least 5 readings were taken in conscious and still rats. Thus, our results indicate the true changes in blood pressure over time.

5.3 Role of testosterone and androgen receptor in insulin resistance

The male sex hormone, testosterone, has been the subject of extensive research. In addition to its role as a steroidal sex hormone, testosterone is also involved in other physiological processes. Of these, its role in the regulation of insulin sensitivity has been controversial. Women with polycystic ovary syndrome (PCOS) show high levels of testosterone, which has been suggested to promote insulin resistance (Hart *et al.*, 2004). Improving insulin sensitivity with metformin reduced both the blood pressure and androgen levels in these patients (Glueck *et al.*, 2004; Kriplani *et al.*, 2004). Further, androgen has been recommended as a potential indicator of cardiovascular risk in post menopausal women as there is a relative increase in testosterone following menopause (Mesch *et al.*, 2008). We have previously demonstrated that testosterone is essential for the development of endothelial dysfunction and hypertension in FFR as gonadectomy prevents these symptoms (Song *et al.*, 2004; Vasudevan *et al.*, 2006). However, we needed to confirm that testosterone is indeed involved in regulating this phenomenon. In our experiments, we studied the effects of testosterone replacement on insulin levels and insulin sensitivity in fructose-fed rats in study A. In

fructose-fed rats (F, GF and GFT), we found sustained hyperinsulinemia (Figure 4.1A) and attenuated insulin sensitivity (Figure 4.1C) as compared to normal chow-fed rats (C, G and GT). However, all rats were normoglycemic (Figure 4.1B). Thus fructose feeding induced insulin resistance, which is a characteristic of this model. Insulin resistance was induced in both sham-operated and gonadectomized fructose-fed rats, regardless of the presence or absence of testosterone. (Figure 4.1C). Although the present and previous studies (Vasudevan, 2005) show high levels of plasma testosterone, its effects on other systems are unclear. Supraphysiological levels of testosterone in rats were shown to depress insulin sensitivity (Holmang *et al.*, 1992; Rizza, 2000). However in humans low-dose testosterone (replacement dosages) may increase insulin action, whereas high-dose testosterone (pharmacological dosages) appears to decrease insulin action (Rizza, 2000). Interestingly in our studies, the high concentrations of testosterone did not affect the depressed insulin sensitivity induced by fructose. Conversely, insulin resistance did not affect circulating testosterone levels in the sham-operated groups (Figure 4.3). Our findings are in agreement with previous results from our laboratory and other groups, which have negated any role for testosterone in influencing insulin sensitivity (Song *et al.*, 2004; Tamaya-Mori *et al.*, 2002).

5.4 Role of testosterone and androgen receptor on insulin resistance-induced hypertension

The effects of gonadectomy and testosterone replacement have been previously discussed in detail (Vasudevan, 2005). Our results (Figures 4.1, 4.19-4.21) are in agreement with these reports. Treatment with testosterone increased the blood pressure in gonadectomized FFR. Blood pressure values were similar to those observed in intact FFR (Figure 2). As previously

reported, insulin resistance was unaffected by the presence or absence of testosterone (Figure 4.1A-C). Thus we have reproducibly confirmed the need for testosterone in the induction of hypertension. Testosterone levels did not vary in the treated groups compared to C or F (Figure 4.3). Based on previous data, we had speculated that testosterone may mediate its prohypertensive effects following insulin resistance, by acting on its receptor, which in turn activates downstream effectors. This is supported by reports in which blocking the androgen receptor in SHR by the anti-androgen flutamide resulted in a fall in blood pressure (Reckelhoff *et al.*, 1999). We tested this hypothesis in rats fed with fructose for 12 weeks. The rats were treated with flutamide for 3 weeks (CF and FF; Weeks 10-12). Blocking the androgen receptor resulted in increased plasma testosterone (Figure 4.6). However insulin sensitivity was unaffected which confirms our finding that insulin resistance is independent of testosterone (Figure 4.4A-C). Systolic blood pressure was elevated at the end of 9 weeks prior to initiating treatment. Flutamide reduced the blood pressure (Figure 4.5) and restored it to control levels thereby implicating the androgen receptor in mediating the hemodynamic actions of testosterone. We also investigated the effects of insulin resistance and androgen receptor blockade on vasoconstrictor pathways such as COX-2 and Cyp4A. These will be discussed in the subsequent sections.

The involvement of 5-dihydrotestosterone (DHT), the active metabolite of testosterone, in the induction of blood pressure is unclear as conflicting reports exist with regard to its actions. In SHR, inhibiting 5 α -reductase (which converts testosterone to 5-dihydrotestosterone) did not reduce hypertension (Reckelhoff *et al.*, 1999). However, blood pressure is elevated in rats treated with DHT for up to 14 days (Nakagawa *et al.*, 2003; Singh *et al.*, 2007) suggesting the need for additional studies to clarify this effect.

5.5 Testosterone and Cyp4A in vascular reactivity

Although *in vitro* testosterone relaxes both the aorta (Tep-areenan *et al.*, 2003) and mesenteric arteries (Tep-areenan *et al.*, 2002), testosterone promotes vasoconstriction and hypertension when present *in vivo*. We have reported this effect in our previous studies where gonadectomy prevented the loss in endothelial relaxation in FFR (Vasudevan *et al.*, 2006; Vasudevan *et al.*, 2005). Additional experiments suggested that in the presence of testosterone, endothelial NO-dependent relaxation is attenuated in the superior mesenteric arteries (SMA). However, testosterone did not affect the EDHF component in SMA (Vasudevan *et al.*, 2006). Testosterone is essential for the synthesis and function of several endogenous vasoconstrictors, which contribute to endothelial dysfunction. Preliminary investigations in fructose-fed rats revealed the involvement of 20-HETE in attenuating endothelial relaxation. Inhibiting 20-HETE synthesis with 1-aminobenzotriazole (ABT) ameliorated the relaxation to ACh in the SMA of intact but not in gonadectomized fructose-fed rats (Vasudevan, 2005). ABT was used as it was the drug of choice for inhibiting Cyp4A. Following the development of selective 20-HETE synthesis inhibitors, we used the Cyp4A selective inhibitor DDMS (N-methylsulfonyl-12, 12-dibromododec-11-enamide) *in vitro* to confirm our earlier findings. DDMS produced the same effect as ABT on ACh-induced relaxation in the SMA of intact FFR (Figure 4.16) without altering the relaxation in gonadectomized rats. This suggested that in the presence of testosterone, 20-HETE might indeed be involved in mediating endothelial dysfunction. Protein expression of Cyp4A1, 2/3 was upregulated in the SMA of untreated fructose-fed rats which was reduced by flutamide (Figure 4.8), which additionally implicates the involvement of the androgen receptor in

mediating these effects. Similarly gonadectomy prevented the increase in Cyp4A1, 2/3 expression in the FFR SMA but not in the aorta (Figures 4.32 and 4.33). Thus preventing the gonadal production or receptor-mediated functions of testosterone attenuates Cyp4A expression and therefore 20-HETE synthesis. Our findings are in agreement with the existing evidence to support the role of testosterone in regulating Cyp4A/20-HETE synthesis. Cyp4A expression and 20-HETE synthesis were attenuated by gonadectomy and restored subsequent to testosterone replacement in Cyp4A14 knock-out mice (Holla *et al.*, 2001). In another study, 14-day infusion of dihydrotestosterone (DHT) increased Cyp4A1 protein expression in the renal interlobar arteries, which was accompanied by increased 20-HETE levels (Singh *et al.*, 2007). Treatment with DHT also induced endothelial dysfunction in these vessels as measured by the loss in relaxation to ACh. Although in both our results and those of Singh *et al.* (2007), 20-HETE synthesis is androgen-dependent, it has been demonstrated that supraphysiological levels of androgens can increase 20-HETE synthesis (Nakagawa *et al.*, 2003). In our studies, both control and fructose-fed rats had similar testosterone levels (Figures 3, 6 and 21). While testosterone was undetectable in gonadectomized rats, flutamide elevated testosterone in the plasma (Figure 4.6), which confirmed its anti-androgen effects. However, flutamide also produces endothelium-dependent relaxation *in vitro* (Ba *et al.*, 2002), which is not regulated by the androgen receptor (Iliescu *et al.*, 2003). Currently there are no reports suggesting an interaction between flutamide and Cyp4A. Taken together, a functional testosterone-androgen receptor signaling system is essential for Cyp4A/20-HETE to influence vascular function and blood pressure. Secondly, the data support our hypothesis that in insulin resistance, testosterone upregulates prohypertensive agents such as 20-HETE, which in part contributes to the secondary complications.

5.6 Testosterone and Cyclooxygenase in vascular reactivity

Testosterone affects COX-mediated pathways in the development of hypertension. Song *et al.* (2004) reported a testosterone-dependent elevation in aortic COX-2 mRNA expression in fructose-fed rats, which may play a key role in altering vascular reactivity. However, no pharmacological data are available regarding the role of testosterone in the effects of individual COX isoforms on vascular tone. In this study, we had 3 main objectives: (1) To examine the effect of COX-inhibition on endothelium-dependent relaxation. (2) To identify the individual contributions of COX-1 and COX-2 to PE-induced vasoconstriction in the SMA and aorta of normal and fructose-fed rats and (3) To determine whether testosterone affects the effects of COX in regulating vascular reactivity. We have identified that COX-2 is the specific cyclooxygenase (COX) isoform that mediates PE-induced vasoconstriction in the aorta secondary to insulin resistance. In addition, we have also demonstrated this vessel-specific involvement of COX-2 to be dependent on testosterone. Although previous studies from our laboratory have shown changes in vascular COX-2 protein (Jiang *et al.*, 2007) and mRNA levels (Song *et al.*, 2004), its functional contributions were unknown. The availability of isoform selective inhibitors of COX enabled us to look at the influence of testosterone on the individual contributions of COX-1 and COX-2 to the vasculature. Thus insulin resistance promotes an increased participation of COX-2 in mediating vasoconstriction to PE in the aorta. This is in agreement with previously published data in SHR which demonstrates increased involvement of COX-2 in mediating responses to PE (Alvarez *et al.*, 2005).

Interestingly, fructose failed to alter the degree of COX-2-dependent responses to PE in the SMA suggesting a diet-independent effect of COX (Figures 4.23-4.25). Gonadectomy

prevented the involvement of COX-2 in vasoconstriction as demonstrated by the decrease in vasoconstrictor activity (Figures 4.23-4.25) and corresponding improvement in endothelium-dependent relaxation (Figure 4.17) subsequent to insulin resistance. This suggests a pro-vasoconstrictor role for COX, which is confirmed by the improvement in endothelium-dependent relaxation following treatment with indomethacin (Figure 4.17). Furthermore this phenomenon was observed only in rats with intact testes suggesting the involvement of testosterone in mediating COX-dependent vasoconstriction. Thus in fructose-fed rats, COX plays an important role in attenuating vasodilation and promoting vasoconstriction.

Insulin resistance and testosterone did not alter COX-2 protein expression in both aorta and SMA (Figures 4.30 and 4.31). Similarly treatment with flutamide failed to alter COX-2 expression in the SMA (Figure 7). This contradicts previous reports from our laboratory, which showed increased COX-2 expression in the aorta of 9-week fructose-fed rats. While we are unable to explain this variation, one possibility is to examine COX-2 expression in aorta that has been incubated with PE. This would mimick the milieu present when we investigated the changes in vascular reactivity and help us confirm the effects of insulin resistance on the contributions in mediating α -adrenoceptor-stimulated vasoconstriction.

Thromboxane A2 levels were elevated by fructose feeding in the plasma (Figure 4.34). According to our initial hypothesis, TXA2 levels should be mirroring COX-2 function as Jiang et al. (2007) showed no change in thromboxane receptor (TPR) expression in FFR aorta. Interestingly, TXA2 levels were the highest in the GF group, which were normotensive. This suggests that the presence of testosterone may be essential to its pro-hypertensive function. We could also speculate that the TPR may be inactivated subsequent

to gonadectomy. Our findings are in agreement with reports from Balfagon's group where both basal and agonist-stimulated TXA2 levels were increased in the SMA of gonadectomized rats as compared to intact rats. This increase in TXA2 was suggested to mediate responses to α -adrenergic stimulation (Blanco-Rivero *et al.*, 2006). Further investigations are needed in to resolve these issues.

Although the degree of COX involvement is altered following insulin resistance, both insulin resistance and testosterone do not affect basal PE-evoked contraction in both the aorta and SMA of F when compared to controls (C) (Figures 4.26 and 4.22 respectively). While separate studies have shown upregulation in the endothelin-1 (Lee *et al.*, 2001; Miller *et al.*, 2002), renin-angiotensin (Hsieh *et al.*, 2005; Tran *et al.*, 2009) and sympathetic nervous systems (Esler *et al.*, 2006; Straznicky *et al.*, 2008; Verma *et al.*, 1999) along with parallel attenuation in NO and EDHF-dependent relaxation (Katakam *et al.*, 1999; Vasudevan *et al.*, 2006), the responses to a vasoconstrictor such as PE are unchanged in intact fructose-fed rats. In control intact and gonadectomized rat vessels, achievement of homeostasis can be attributed to a robust endothelial NO-dependent relaxation, which is impaired in F. Therefore we hypothesize the involvement of endothelium-independent mechanisms in preventing exaggerated vasoconstrictor responses to PE. These pathways however may not be salutary to the overall hemodynamics of the rats. Nitric oxide produced by inducible NOS (iNOS) is one such endothelium-independent pathway present in the vascular smooth muscle which could mediate this phenomenon. Increased oxidative stress is involved in diet-induced insulin resistance and elevation in blood pressure (Song *et al.*, 2005). iNOS-dependent NO synthesis is increased following elevated oxidative stress due to insulin resistance (Wang *et al.*, 2006). Interestingly, androgen also increases the susceptibility to oxidative stress (Singh *et al.*,

2007), which if demonstrated in fructose-fed rats, could account for the testosterone-dependent differences in vascular reactivity. In principle, the potential increase in vasoconstriction could be neutralized by NO thus achieving homeostasis.

It would also be interesting to evaluate the position of COX-2 in the signaling pathways involved in PE-evoked vasoconstriction. PE constricts blood vessels through various mediators such as the renin-angiotensin system (RAS) (Alvarez *et al.*, 2007) and COX-2 (Alvarez *et al.*, 2005). Interestingly, both mediators are regulated by testosterone (Sartori-Valinotti *et al.*, 2008; Song *et al.*, 2004) and have been implicated in elevating blood pressure secondary to insulin resistance (Nyby *et al.*, 2007; Song *et al.*, 2004; Vasudevan *et al.*, 2006). However there is no direct evidence linking angiotensin-2 and COX-2 in insulin resistant conditions. Oxidative stress associated with the metabolic syndrome has been shown to increase the synthesis of vasoactive prostanoids and eicosanoids (Tsai *et al.*, 2009), which suggests oxidative stress to play a key role in impairing vasoactive equilibrium associated with insulin resistance. Another interesting study would be to examine the association of 20-HETE (20-hydroxyeicosatetraenoic acid) with RAS and COX-2 in fructose-fed rats. Similar to COX-2 and RAS, 20-HETE synthesis is also testosterone dependent as shown by us (Figures 4.8, 4.16 and 4.33 respectively) and others (Holla *et al.*, 2001). Thus although the effects of these systems have been investigated individually, sparse information exists with regard to any interplay among these systems. We believe that the testosterone-dependent COX-2, RAS and Cyp4A pathways could be interrelated and together contribute to endothelial dysfunction and hypertension.

5.7 Cyp4A/20-HETE and insulin resistance

We have demonstrated for the first time that Cyp4A-mediated synthesis of 20-HETE plays an important role in the development of high blood pressure following insulin resistance. Previous reports have studied the changes in 20-HETE synthesis in high fat fed rats (Laffer *et al.*, 2004). Although 20-HETE levels decreased, there was no correlation made between insulin resistance and 20-HETE synthesis in the studies. As the nine-week fructose-fed rat exhibits both insulin resistance and elevated blood pressure without developing obesity, we studied the effects of insulin resistance on 20-HETE synthesis and its role in regulating blood pressure. Our hypothesis was additionally supported by reports implicating 20-HETE as a downstream target for several endogenous vasoconstrictors such as angiotensin-2 (Croft *et al.*, 2000; Miyata *et al.*, 2005) and norepinephrine (Berezan *et al.*, 2008), which have been previously implicated in the development of high blood pressure secondary to insulin resistance. In addition to increasing blood pressure, we have also shown that preventing 20-HETE synthesis by incubating with ABT (Vasudevan, 2005) or DDMS (Figure 4.16) *in vitro* improved the relaxation to acetylcholine in the SMA of fructose-fed rats.

As ABT is a suicide substrate inhibitor for Cyp4A, we did not anticipate any change in Cyp4A expression. Our data show no change in the SMA Cyp4A expression, which confirms our hypothesis (CA and FA; Figure 4.8). However, ABT reduced plasma insulin levels and ameliorated glucose clearance similar to controls (Figures 4.4A-C). We initially used ABT as it was the agent of choice to study the role of 20-HETE; but its inability to selectively inhibit Cyp4A (Linder *et al.*, 2009) and the advent of more potent and Cyp4A

selective drugs such as HET0016 (Miyata *et al.*, 2001; Seki *et al.*, 2005) made us revisit our findings. We wanted to confirm our preliminary findings before arriving at any conclusions. We were fortunate to receive a timely gift sample of HET0016 from Taisho Pharma. As further evidence we directly inhibited Cyp4A *in vivo* with HET0016. Our results show that HET0016 inhibits Cyp4A expression (Figure 4.12). We did not find any similar results in our literature search, which makes this finding novel. The work published to date only measured changes in 20-HETE levels and not Cyp4A expression in HET0016 treated rats. Although previous work published by Seki *et al.* shows an irreversible inhibition of Cyp4A by HET0016 (Seki *et al.*, 2005), we do not know of any reported mechanism for our observations. The limited availability of HET0016 prevented long term or multiple dosing. However, it also presented additional problems. One such problem was solubility for injection. We prepared the suspension as per the manufacturer's instructions (Section 3.2). Secondly, HET0016 has a short half-life in the body, which had to be accounted for. It was well tolerated by the rats as there was no weight loss observed during the treatment period. The other challenge we faced was the unavailability of a Cyp4A isoform selective antibody. Although separate reports suggest that Cyp4A1, 2/3 and 8 are involved in regulating blood pressure, isoform selective antibodies are commercially unavailable owing to the high sequence homology between the isoforms. Our initial western blots had high background non-specific binding, which made it difficult to detect the Cyp4A1, 2/3 band at ~ 51kD. Hence we immunoprecipitated the protein to amplify the signal: noise ratio.

Compared to untreated fructose-fed rats, HET0016 treatment did not increase the ISI value in FT. However it was also not different from C suggesting high variability in its effects on glucose disposal. Interestingly ABT normalized the glucose disposal profile in

fructose-fed rats (4.13A-C). We have no mechanism to support this finding but it would be interesting to know if ABT reduces blood pressure by improving insulin sensitivity and the pathways recruited in the process. Studies are required at the molecular level to investigate the effects of ABT on insulin sensitivity and the effects of HET0016 on individual Cyp4A isoforms at the protein and message levels.

5.8 PERSPECTIVES

The relationship between insulin resistance and blood pressure in the context of the metabolic syndrome is very complex involving several pathways. Testosterone plays an important role in the development of vascular complications secondary to insulin resistance. However, several other processes and pathways associated with both insulin resistance and testosterone may provide vital clues on the molecular mechanisms involved in the development of vascular complications. Although it is unclear with regard to which factor(s) trigger the cascade, several reports have implicated increased oxidative stress as one of the key players in the development of insulin resistance and its secondary complications. Indeed Song *et al.* (2005) demonstrated that treating fructose-fed rats with the antioxidant, N-acetylcysteine, prevented insulin resistance and hypertension (Song *et al.*, 2005). Oxidative stress has been linked to a number of factors directly or indirectly associated with the metabolic syndrome. Increased oxidative stress accompanied by decreased antioxidant capacity induces inflammation in various tissues such as the liver, skeletal muscle and adipose tissue, which is suggested to cause insulin resistance (Shoelson *et al.*, 2006; Tilg *et al.*, 2008). Markers of inflammation such as tumor necrosis factor alpha (TNF- α) and IKK beta (Shoelson *et al.*, 2003; Yuan *et al.*, 2001) have been shown to be upregulated in insulin resistance. Although interleukin-6 (IL-6), whose synthesis is upregulated by TNF- α , may be involved further evidence is needed in this regard (Tilg *et al.*, 2008).

Changes in oxidative stress are also regulated by sex hormones. Thus androgens increase oxidative stress, which upregulates the synthesis of vasoconstrictor prostanoids such as F₂-isoprostanes (Fortepiani *et al.*, 2003) and eicosanoids such as 20-HETE (Singh *et al.*, 2007).

Interestingly, Fortepiani et al. (2003) showed increased F2-isoprostane levels in postmenopausal rats, in which testosterone was relatively higher, to be similar as the levels in male rats; which suggests that the presence of testosterone predisposes rats to oxidative insult. In addition, the renin-angiotensin system (RAS) is also a pro-oxidant and pro-inflammatory pathway (Sowers, 2004) whose functions are regulated by testosterone (Reckelhoff *et al.*, 2000). As the RAS has been suggested to stimulate the adrenergic nervous system in addition to COX-2 and Cyp4A, both testosterone dependent vasoactive pathways, it may be speculated that endothelial dysfunction is achieved by increased IR and oxidative stress, which upregulates the RAS and SNS, which in turn activates COX-2 and Cyp4A-dependent synthesis of vasoactive metabolites that attenuate endothelial NO/EDHF-mediated vasorelaxation, finally leading to hypertension. These multiple pathways and their potential interactions in mediating vascular complications secondary to insulin resistance are summarized in Figure 6.2

Oxidative stress also induces vascular cell death and subsequent remodeling, which may be pathological. Although not as severe as streptozotocin-induced diabetes, fructose-fed rats exhibit increased oxidative stress (Song *et al.*, 2005), which may increase iNOS expression and cause cell death. TNF- α , which is involved in inducing insulin resistance, is suggested to induce apoptosis by interfering with insulin signaling and the consequent signaling of antiapoptotic pathways (Goetze *et al.*, 2001; Hotamisligil *et al.*, 1996). It would be interesting to demonstrate cell death in FFR vasculature and the mechanisms involved. Additionally it would be interesting to determine whether blood vessels from insulin resistant rats undergo vascular remodeling and the role of matrix metalloproteinases (MMPs) in mediating this process. MMPs play an important role in vascular wall thickening and

vasoconstriction (Fernandez-Patron *et al.*, 1999; Fernandez-Patron *et al.*, 2000) in addition to being induced by various vasopressors such as norepinephrine (Briest *et al.*, 2003) and angiotensin 2 (Jimenez *et al.*, 2009; Walter *et al.*, 2008). Unpublished data by Nagareddy *et al.* (2009) have shown increased MMP 2, 9 and 7 activities in aorta/SMA from fructose-fed rats stimulated with phenylephrine. Furthermore, MMPs are elevated by impaired insulin signaling in cultured vascular smooth muscle cells (Nagareddy *et al.*, 2009; Unpublished data). MMPs are upregulated by various vasoconstrictors implicated in the metabolic syndrome such as angiotensin 2 (Guo *et al.*, 2008), and endothelin-1 (Felx *et al.*, 2006). However additional information is required with regard to the effects of COX-2 and 20-HETE on MMPs. It would be interesting to study the effects of the abovementioned agents and determine whether they are upstream, downstream or parallel to each other in influencing MMP activity in fructose-fed rats and whether this pathway could lead to vascular remodeling.

5.9 Limitations and future research directions

5.9.1 Limitations

In answering the research questions, we can identify several limitations of our studies described in this thesis.

1. We are creating an unnatural hormonal environment in the male body by increasing testosterone levels.
2. The literature on Cyp4A mRNA and protein expressions in rat seems to be unclear. Changes in Cyp4A1, 2/3 and 8 mRNAs have been reported in separate studies in diverse

disease models. No information exists with respect to the effects of metabolic syndrome on the expression of individual isoforms.

3. Similar to mRNA, isoform-specific protein expression has not been reported. The high sequence homology among isoforms poses a major hurdle to developing antibodies specific to each isoforms. Therefore we have used the commercially available antibody that recognizes Cyp4A1, 2/3. These antibodies have a very low signal to noise ratio, which results in high non-specific binding. Although we detected Cyp4A bands around ~51 kD by immunoprecipitation, there was significant background noise.
4. We encountered problems concerning the solubility and in vivo stability of HET0016. We followed the manufacturer's instructions and suspended the drug in 20% lecithin warmed to 70°C, which was sonicated in an ice bath. However, our consultations with Dr. Richard Roman (one of the pioneers in Cyp4A/20-HETE research) revealed that the drug has a short half life and must be dosed at least twice daily compared to our once a day dosing. We used this dosing protocol as the rats were injected for a total of 2 weeks, during which we did not want them to exhibit stress-induced artifact responses. Besides the drug was a gift sample and we had a limited quantity to work with, which did not allow for dosing twice a day.
5. 20-HETE levels are being measured in the plasma and may provide direct evidence for its role in the metabolic syndrome.

5.9.2 Caveats

We do not endorse surgical/chemical inactivation of testosterone or its replacement therapy in treating insulin resistance and hypertension. Our interest lies in using testosterone as a tool to investigate the vasoactive pathways it affects downstream. This could lead us to novel targets for developing antihypertensive drugs.

5.9.3 Future directions

1. Additional studies need to be conducted on the effect of Cyp4A inhibition on insulin resistance.
2. To determine if COX-2 expression is increased in the presence of phenylephrine. This would confirm our findings from vascular reactivity experiments.
3. To study the interplay between angiotensin 2, COX-2 and Cyp4A systems in fructose-fed rats.
4. To study the role of testosterone in influencing one or more of these pathways
5. To determine if increased oxidative stress induces endothelial and vascular cell death and identify the mechanisms involved.
6. To determine if remodeling occurs in fructose-fed rat vasculature and if so the role of cell death and MMPs in mediating this process.

6. SUMMARY AND CONCLUSIONS

Based on the available literature and our results we can summarize the following:

1. Feeding fructose induces insulin resistance in male rats, which is accompanied by endothelial dysfunction and increased blood pressure.
2. Testosterone is essential for the development of vascular complications and increase in blood pressure.
3. Testosterone acts on the androgen receptor and stimulates downstream vasoactive signaling pathways.
4. Cyp4A and cyclooxygenase-2 catalyzed mediators are testosterone dependent and play a key role in altered vascular reactivity secondary to insulin resistance as summarized in Figure 6.1.
5. Insulin resistance increases Cyp4A expression in the mesenteric arteries but not the aorta. Gonadectomy or Cyp4A-selective inhibition prevents its expression. Treatment with anti-androgen ameliorates the increase in Cyp4A expression.
6. Cyclooxygenase 2 is favored over COX-1 in mediating vasoconstrictor responses to agonists such as phenylephrine. Inhibition of COX decreases contraction and improves endothelium-dependent relaxation.

Overall, our results have identified potential testosterone-dependent pathways that are involved in inducing endothelial dysfunction and high blood pressure secondary to insulin resistance.

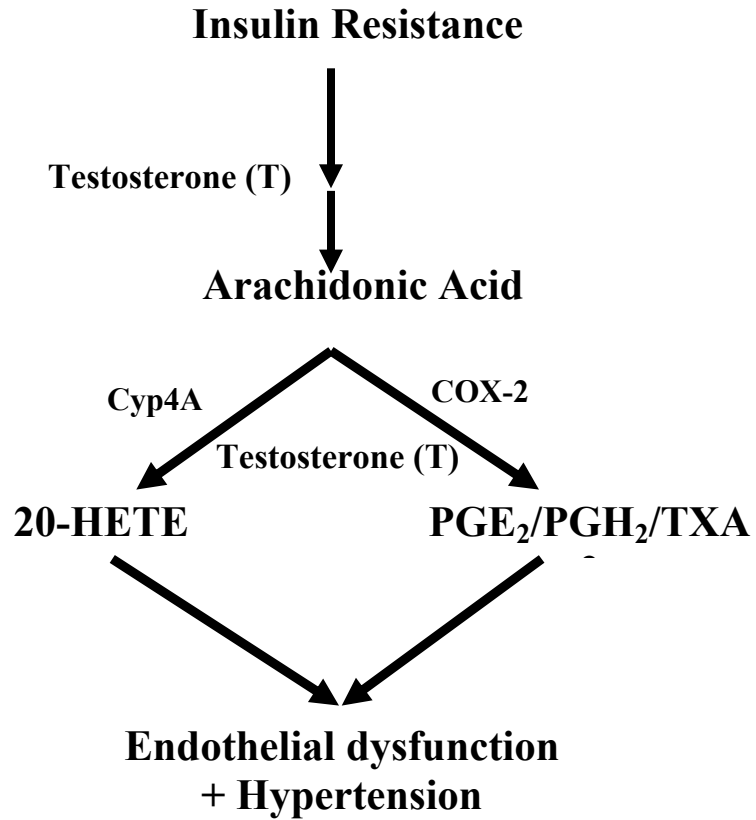


Figure 6.1: Schematic summary of the testosterone-dependent vasoactive pathways investigated in the present work that are involved in the development of endothelial dysfunction and hypertension secondary to insulin resistance. These pathways form a component of the bigger picture as shown in Figure 6.2.

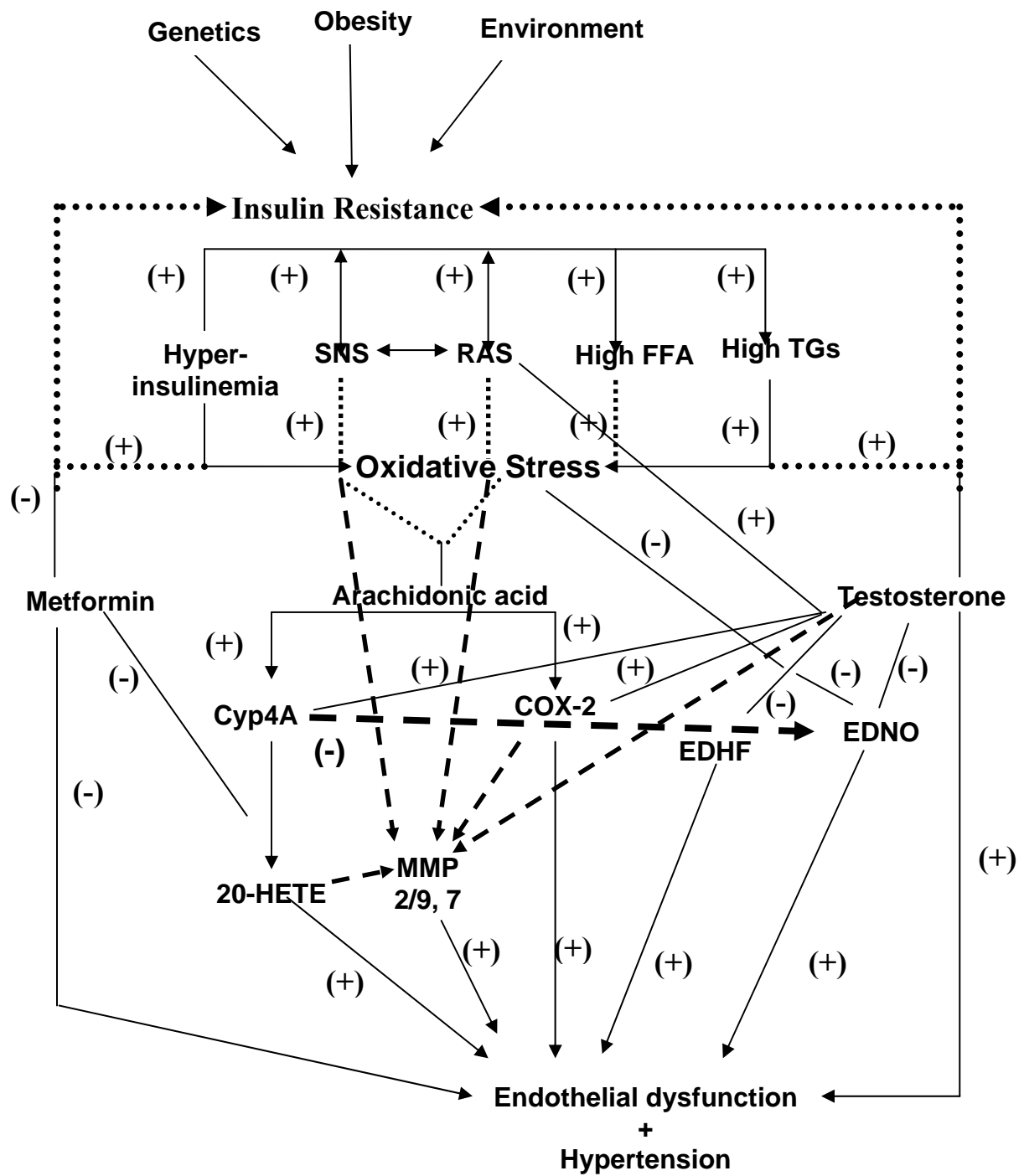


Figure 6.2: Schematic representation of the multiple pathways involved in the development of endothelial dysfunction and hypertension secondary to insulin resistance.

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The University of British Columbia

Animal Care Certificate

Application Number: A03-0063

Investigator or Course Director: John H. McNeill

Department: Pharmaceutical Sciences

Animals Approved:

Rats 200

Start Date: **July 1, 2002**Approval Date: **July 29, 2005**

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The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility