POTENTIAL ROLE OF SEX HORMONES IN ALTERED VASCULAR RELAXATION FOLLOWING INSULIN RESISTANCE

by

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ABSTRACT

Hypertension is one of the secondary complications associated with insulin resistance (IR). Previous studies from our laboratory have shown that female rats are protected from developing insulin resistance and hypertension, which was dependent on estrogen. Further, testosterone was suggested to regulate the development of hypertension secondary to IR. The present series of studies intended to extend these earlier findings on the influence of gender on the relationship between insulin resistance, hyperinsulinemia and hypertension. Experiments were aimed at examining two objectives. The first was to investigate and confirm the changes in the development of insulin resistance-induced hypertension in the absence and reimplantation of testosterone. To study the effect of sex hormones on insulin resistance and hypertension, we used the well-established fructose hypertensive rat (FHR) model. Male rats fed with fructose developed insulin resistance and hypertension. The loss of testosterone following gonadectomy prevented hypertension following fructose feeding. However gonadectomy did not prevent induction of insulin resistance. Replacing the testosterone reversed the fall in blood pressure due to gonadectomy without affecting IR. To test whether changes in blood pressure are reflected in vascular reactivity, we examined the relaxation responses to acetylcholine (ACh) in the mesenteric arteries of intact and gonadectomized fructose-fed rats. We found that relaxation was impaired in intact fructose-fed rats (F) and that the impairment did not occur in the gonadectomized fructose-fed rats (GF). To dissect out the specific contributions of endothelial vasodilators, we selectively inhibited nitric oxide (NO) synthesis using L-NAME. As EDHF (endothelium derived hyperpolarizing factor) relaxes blood vessels by opening the K_{Ca} (calcium sensitive potassium channel) function, we blocked EDHF action using a combination of charybdoxin and apamin, which individually block the large and the small K_{Ca} respectively. The tissues were then evaluated for changes in relaxation to ACh. In the mesenteric arteries of normal chow-fed rats, inhibition of either NO or EDHF attenuated the relaxation to ACh by nearly 50%. Inhibition of EDHF decreased the relaxation in F, but not GF, whereas inhibition of NO production decreased the relaxation in both the fructose-fed groups (F and GF). Relaxation following inhibition of EDHF was appreciable in GF as compared to F due to an increased NO-dependent response. Inhibition of NO abolished the vasorelaxant response due to an IR-induced loss in EDHF function. We conclude that gonadectomy prevents IR-induced hypertension in part by promoting NO-dependent vasodilation.
Based on the above results, we hypothesized that testosterone-dependent vasoactive agents are involved in inducing endothelial dysfunction and hypertension, secondary to IR. We focused on the androgen-dependent synthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) in the vasculature. Arachidonic acid hydroxylases belonging to the Cyp4A family of enzymes catalyze the production of 20-HETE. In the presence of 1-aminobenzotriazole (ABT), which inhibits Cyp4A, relaxation to ACh was improved in the mesenteric arteries of sham-operated male FHR. Treatment with ABT did not affect the relaxation to ACh in intact normal chow-fed (C) or gonadectomized groups (G and GFE), suggesting an elevated action of 20-HETE in the presence of testosterone in male FHR.

It is unclear as to whether it is the loss of estrogen or increase in testosterone that is responsible for the development of hypertension. Our second aim was to identify whether the development of insulin resistance and hypertension was gender dependent or whether a specific sex hormone regulated its development. In a separate series of experiments, intact and gonadectomized male fructose-fed rats were treated with estrogen implants, following which their insulin sensitivity improved. However, estrogen reduced the blood pressure only in the intact male fructose-fed rats (FE). Estrogen did not affect the blood pressure in gonadectomized fructose-fed rats (GFE). Furthermore, blood pressure in FE was higher than the estrogen implanted (GFE) or non-implanted gonadectomized rats (G). Estrogen decreased plasma testosterone in the intact rats. However, detectable amounts of testosterone were still present. In summary we suggest the presence of potential prohypertensive pathways, which are dependent on the presence of testosterone and are activated only following insulin resistance. We also suggest that the development of insulin resistance and hypertension are governed not by gender per se, but by the actions of specific sex hormones such as estrogen and testosterone.
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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
Kca : Calcium-sensitive potassium channel
CAD : Coronary Artery Disease
CE : Intact normal chow-fed and estrogen implanted
COX : Cyclooxygenase
CTX : Charybdotoxin
CVD : Cardiovascular Disease
CYP2C : Cytochrome P450 2C (Arachidonic acid epoxygenase)
CYP4A : Cytochrome P450 4A (Arachidonic acid hydroxylase)
EC50 : Molar concentration of an agonist, which produces 50% of the maximum possible response for that agonist.
ED70 : 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 fructose chow-fed
FE : Intact high fructose chow-fed and estrogen implanted
FHR : Fructose Hypertensive Rat
G : Gonadectomized and normal chow-fed
GF : Gonadectomized and high fructose chow-fed
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<td>Gonadectomized high fructose chow-fed and estrogen implanted</td>
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<tr>
<td>GFT</td>
<td>Gonadectomized high fructose chow-fed and testosterone implanted</td>
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<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HERS</td>
<td>Heart and Estrogen/progestin replacement study</td>
</tr>
<tr>
<td>20-HETE</td>
<td>20-hydroxyeicosatetanoic acid</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>ISI</td>
<td>Insulin sensitivity Index</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
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<tr>
<td>L-NAME</td>
<td>N⁰-nitro-L-arginine methyl ester hydrochloride</td>
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<td>L-NMMA</td>
<td>N⁰-G-monomethyl-L-arginine monoacetate</td>
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<td>NE</td>
<td>Norepinephrine</td>
</tr>
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<td>NO</td>
<td>Nitric Oxide</td>
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<td>NIH</td>
<td>National Institutes of Health</td>
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<td>NOx</td>
<td>Nitrite and nitrate levels</td>
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<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
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<td>OVX</td>
<td>Ovariectomized</td>
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<td>PCOS</td>
<td>Polycystic Ovary Syndrome</td>
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<tr>
<td>pD₂</td>
<td>Negative logarithm of the EC₅₀ value</td>
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<td>PE</td>
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<td>PGI₂</td>
<td>Prostacyclin</td>
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<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
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<td>PLC</td>
<td>Phospholipase C</td>
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<td>RAS</td>
<td>Renin angiotensin system</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay assay</td>
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<tr>
<td>Rₘₐₓ</td>
<td>Percent maximal response by a tissue to a given agonist</td>
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<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>s.c.</td>
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SEM : Standard error of the mean  
SHR : Spontaneously Hypertensive rat  
TG : Triglyceride  
TXA₂ : Thromboxane A₂  
VSM : Vascular smooth muscle  
WHO : World Health Organization  
WKY : Wistar Kyoto rat
LIST OF PUBLICATIONS

Publications/abstracts related to the present thesis

1. **Harish Vasudevan**, Prabhakara Reddy Nagareddy and John H McNeill Gonadectomy improves vascular responses to acetylcholine in fructose hypertensive rats Accepted for presentation at Experimental Biology 2005, San Diego USA.

2. **Harish Vasudevan**, Hong Xiang and John H McNeill Estrogen reduces insulin resistance and subsequent hypertension in male fructose hypertensive rats (Presented at the Canadian Therapeutics Congress held in June 2004)

3. **Harish Vasudevan**, Hong Xiang and John H McNeill Chronic treatment with estrogen improves insulin sensitivity in insulin resistant male rats (Experimental and Clinical Cardiology, 2004 9(1): 86) (Presented at the National Forum for Young Investigators in Circulatory and Respiratory Research held in May 2004).

Other publications/abstracts


3. Prabhakara Reddy Nagareddy, **Harish Vasudevan** and John H McNeill Oral administration of tungstate improves cardiac performance in STZ-diabetic rats (Presented at the Canadian Therapeutics held in June 2004).


Presentation related to the present thesis

• “Role Of Sex Hormones In Hypertension Following Insulin Resistance”
  **Harish Vasudevan** and John H McNeill; presented at the 4th Annual Merck Frosst Pharmacology Day, October 2004
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MY PARENTS
INTRODUCTION

The metabolic syndrome is comprised of a cluster of cardiovascular risk factors. These include abdominal obesity, hypertriglyceridemia, low levels of high-density lipoprotein (HDL), insulin resistance (IR) and hypertension. Though not cited in a specific order, obesity and insulin resistance are mentioned as the predominant factors contributing to the metabolic syndrome. Recently, the National Institutes of Health (NIH) have described the contribution of the metabolic syndrome to be of an equal magnitude to that of cigarette smoking in the development of cardiovascular disease (CVD). In addition, the metabolic syndrome has been suggested to be a predisposing factor in the development of Type 2 diabetes mellitus.

Recent studies in 2002 have estimated that up to 22% of all American adults suffer from the metabolic syndrome. This situation is expected to worsen by 2010 with up to 50 to 75 million patients with the metabolic syndrome in the United States alone, which is approximately 10-15% of the worldwide population that will be affected by the metabolic syndrome.

Insulin resistance and hypertension

Insulin resistance is a condition that results from a variety of uncharacterized factors. Resistance to insulin is developed, mainly in tissues such as the skeletal muscle, liver and adipose tissue. This is associated with a compensatory elevation in the insulin output, which is manifested as hyperinsulinemia. With time, as insulin resistance increases, the pancreas is unable to keep up with the increased demand, which results in attenuated insulin action, despite its presence in high concentrations. The patient is finally rendered diabetic by a relative state of hypoinsulinemia, induced thus. However, it is unclear as to whether IR precedes hyperinsulinemia or vice versa. Studies have shown that patients with hyperinsulinemia due to insulinoma are not necessarily insulin resistant. The problem requires further study. Insulin resistance and hypertension are two of the major factors contributing to the metabolic syndrome. Studies have shown that while one in every four adults suffers from hypertension in the US, the number of patients suffering from IR in the age group of 40 to 74 years is approximately 16 million. Not surprisingly, hyperinsulinemia and IR are often found to be associated with hypertension in both humans and in several animal models. From the information the hypothesis was developed that the metabolic impairments involved in inducing IR are directly related to the development of hypertension. 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. In addition to the studies demonstrating that both obese and lean hypertensive patients exhibit insulin resistance, several hypertensive rodent models exhibit similar defects in glucose metabolism and insulin action. These include the Dahl rat, the spontaneously hypertensive rat (SHR), the Milan hypertensive rat, and the fructose hypertensive rat (FHR). Although these hypertensive models are etiologically distinct, they share a common link in terms of defective glucose metabolism and insulin action. In normal rats, chronic treatment with insulin resulted in insulin resistance and hypertension over a period of 1-3 weeks.

**Animal models of insulin resistance, hyperinsulinemia and hypertension**

To understand the intricacies in the relationship between insulin resistance and hypertension, several animal models of insulin resistance or hypertension have been studied. Among them are rats that are genetically predisposed to hypertension, such as the SHR and the Dahl rat. In models of diet-induced hypertension, such as in the fructose hypertensive rat, consumption of a high carbohydrate diet induces insulin resistance, which precedes the development of hypertension. Several studies in both SHR and fructose hypertensive rats have shown that hypertension is at least in part secondary to insulin resistance and not the other way round. Clinical studies have shown that insulin sensitivity is significantly lower in the normotensive offspring of hypertensive patients as compared to the offspring of subjects with normal blood pressure and may thus precede the development of hypertension. The involvement of a genetic component in the association between insulin resistance and hypertension is therefore suggested.

1. **Spontaneously hypertensive rat:** The spontaneously hypertensive rat is one of the rodent models used to study the abnormalities in glucose metabolism and subsequent hypertension. Extensive studies have been conducted on the SHR to understand the mechanisms responsible for reduced insulin action and how this phenomenon contributes to hypertension. In comparison with its control, the Wistar-Kyoto rat (WKY), SHR has been shown to exhibit impairments in insulin receptor expression. Additionally downstream targets in insulin signaling such as (insulin receptor substrate) IRS-1 and glycogen synthase are also affected in SHR. Treating SHR with insulin sensitizers such as metformin resulted in a fall in
blood pressure (BP). \(^{35}\) Similar improvements in insulin action and blood pressure was observed following treatment with vanadium, suggesting that changes in insulin sensitivity determine the changes in BP. \(^{36}\)

2. *Fructose-fed hypertensive rat:* Fructose is a monosaccharide obtained from fruits and is also a component of sucrose. It is used commercially in high amounts as a sweetening substitute (fructose corn syrup) for glucose and sucrose in the preparation of desserts, condiments and carbonated beverages. \(^{37}\) First reported in 1987, \(^{22}\) feeding rats a high carbohydrate (fructose, 66%) diet for 6-8 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 \(^{38}\) or glucose. \(^{39}\) This concept is reflected in humans, where consumption of high amounts of fructose in the diet increases the risk of dyslipidemia, \(^{40,41}\) obesity, \(^{37}\) insulin resistance and heart disease in susceptible individuals. \(^{42}\) Similar to humans, fructose-fed animals also show elevated triglyceride levels. \(^{43}\) In the previous studies from our laboratory, body weights and plasma glucose levels were unchanged at the time of termination (6-8 weeks). \(^{43}\) However, there is evidence that long-term feeding with carbohydrate does produce obesity and hyperglycemia. \(^{44}\) The model mirrors the early sequelae observed in humans associated with insulin resistance, such as endothelial dysfunction \(^{26,45}\) and hypertension. \(^{46,47}\) 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. \(^{48}\) Our lab has previously demonstrated hypertension in SD rats, which had been fed with 66% fructose for 3 weeks. \(^{47}\) 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. \(^{46}\) This model provided the initial evidence which was instrumental in establishing the causal nature of the link between IR and hypertension. \(^{22,49,50}\) In high amounts, fructose bypasses metabolism by hepatic phosphofructokinase (PFK) in the glycolytic cycle. Phosphofructokinase is the rate-limiting enzyme in glucose metabolism. \(^{42}\) The metabolites of fructose block PFK activity and thus decrease hepatic glucose production resulting in insulin resistance. \(^{42}\) Further, high fructose feeding has been shown to increase the rate of *de novo* lipogenesis as compared to glucose. \(^{37}\) This effect may explain the development of hypertriglyceridemia in FHR. 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.\textsuperscript{47,51} The improvement in insulin sensitivity and hypertension observed thus were however reversible in fructose-fed rats subsequent to restoration of the hyperinsulinemic state.\textsuperscript{19} Studies have been reported with promising results using other insulin sensitizing agents such as thiazolidinediones.\textsuperscript{52} Similarly, insulin enhancing agents such as vanadium have been shown to prevent the development of hypertension in fructose hypertensive rats (FHR).\textsuperscript{53}

**Insulin resistance: Early secondary complications**

Most of the studies describing the development of hypertension following insulin resistance have mainly focused on the hemodynamic effects of insulin, which influence the changes in blood pressure. An early complication observed in FHR was an increased sympathetic discharge, which has been shown to contribute to hypertension.\textsuperscript{54} 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, although the patients were not insulin resistant.\textsuperscript{7,55,56} Previous studies from our laboratory have demonstrated a reduction in blood pressure in fructose-fed rats upon blocking the sympathetic activity by chemical sympathectomy.\textsuperscript{54} Decreasing sympathetic outflow using rilmenidine also prevents hypertension.\textsuperscript{57} Interestingly, fructose feeding has been shown to impair the vasorelaxant actions of insulin.\textsuperscript{58} Additionally, fructose hypertensive rats develop endothelial dysfunction as early as 2-3 weeks subsequent to fructose feeding.\textsuperscript{26} The alterations in specific endothelial vasoactive agents involved in the pathogenesis of hypertension in FHR are described in detail under the sub headings below.

**Insulin and endothelial function**

Under normal conditions, in addition to promoting glucose uptake, insulin has been shown to dilate the blood vessels in the skeletal muscle.\textsuperscript{59} Numerous studies have shown insulin-
dependent vasodilation as a reliable indicator of augmented muscle glucose uptake. It was shown that the activation of its receptor by insulin in normal physiological states triggers a cascade of pathways, one of which is the activation of protein kinase-B (PKB). Also known as Akt, this protein has been shown to upregulate endothelial nitric oxide synthase (eNOS) activity in the skeletal muscle vasculature. As a result, endothelial nitric oxide levels increased, resulting in vascular relaxation. In addition, studies have also reported a role for endothelium-derived hyperpolarizing factor (EDHF, explained in the following section) in insulin-evoked vasodilation in the mesenteric arteries. Thus, the endothelium is suggested to play a key role in the insulin-dependent changes in vascular tone. Treatment with L-NMMA, an inhibitor of nitric oxide synthase attenuated muscle glucose uptake in addition to reducing the blood flow.

Insulin resistance disrupts the equilibrium between endothelial vasoconstrictors and vasodilators. Following insulin resistance, in addition to impaired glucose uptake, the skeletal muscle blood vessels develop resistance to the vasodilatory effects of insulin. Endothelial dysfunction, one of the secondary outcomes of insulin resistance, contributes to the attenuation of insulin-induced vasorelaxation.

Mechanisms linking insulin resistance to hypertension

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. Katakam et al have demonstrated endothelial dysfunction at 2-3 weeks subsequent to insulin resistance in the isolated blood vessels of fructose hypertensive rats (FHR). This was characterized by attenuated vascular relaxation to the endogenous vasodilator acetylcholine (ACh). We have previously reported that endothelium-dependent relaxation to ACh is depressed in the mesenteric arteries from FHR. 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.

As previously mentioned, insulin relaxes the blood vessels under physiological conditions via the release of NO formed by the action of the constitutive endothelial nitric oxide synthase (eNOS). Insulin also induces endothelium-dependent vasoconstrictors, which are
counteracted by endothelial NO in states of normoinsulinemia. Following insulin resistance and hyperinsulinemia, insulin-induced vasodilation is impaired due to attenuated NO activity. This results in the unmasking of insulin-mediated vasoconstriction due to increased endothelin-1 action (vascular effects of endothelin-1 are described in following section). Indeed, our laboratory has previously examined this hypothesis and shown that arteries from insulin-resistant FHR are refractory to insulin-induced vasodilation.

In addition to NO, two other important endothelium-derived vasorelaxing factors are prostacyclin (PGI2) and a more recently discovered factor, endothelium-derived hyperpolarizing factor (EDHF). 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 the mesenteric arteries. Studies on VSM over the last decade have demonstrated an increasing significance of the role of EDHF with decreasing blood vessel size, suggesting the likelihood that EDHF is the major vasodilator (instead of NO) in resistance vessels such as the mesenteric and coronary arteries. It has been controversial as to whether the superior mesenteric artery (SMA) represents a true resistance vessel as compared to lower order mesenteric vessels. Studies by Christensen et al strongly suggest an equal if not decreased contribution of the SMA to vascular resistance in comparison with the lower order vessels. Thus, it is suggested that vasoactive pathways similar to those in the smaller blood vessels may be involved in regulating vascular tone in the SMA. 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 A). Arachidonic acid epoxygenases belonging to Cyp2C family of enzymes are mainly characterized as EDHF synthases. Several other candidates, namely anandamide, H2O2 and K+ ion 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). Irrespective of its chemical nature, EDHF opens the endothelial (calcium sensitive potassium channels) KCa channels in response to changes in endothelial intracellular [Ca2+]i levels. The efflux of K+ induced thus, hyperpolarizes the endothelium and subsequently the VSM resulting in relaxation. KCa function has been shown to be inactivated during insulin resistance (IR), thus disrupting endothelial hyperpolarization. Additionally, IR decreases Cyp2C expression and responses to
EETs\textsuperscript{90} in the rat mesenteric arteries.\textsuperscript{91} Therefore defects in the production of EDHF or EDHF-evoked $K_{Ca}$ function may lead to increased responses to vasoconstrictors.

**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.\textsuperscript{92} Insulin stimulates the production and release of ET-1 as well as the expression of its receptor, which promotes vasoconstriction.\textsuperscript{74,93-95} ET-1 has been shown to be involved in elevating BP in a number of experimental models of hypertension such as the stroke-prone SHR, the Dahl rats and angiotensin II-infused rats.\textsuperscript{96} 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.\textsuperscript{97,98} Moreover, our laboratory has previously shown that the vascular ET-1 content is higher in FHR as compared to normal chow-fed rats,\textsuperscript{97} in addition to altered reactivity of the mesenteric arteries to ET-1.\textsuperscript{73} An increase in the expression of both ET-1 peptide and its ETA receptor subtype has also been demonstrated in FHR.\textsuperscript{99,100}

Metabolites of arachidonic acid constitute the second class of endothelial vasoconstrictors. In addition to the formation of relaxing factors, endothelial arachidonic acid is also converted to potential vasopressor agents such as thromboxane A\textsubscript{2} (TXA\textsubscript{2}), (Figure A).\textsuperscript{101} Renal and/or vascular production of TXA\textsubscript{2} is increased in various hypertensive animal models including SHR\textsuperscript{102,103} and Dahl salt-sensitive rats.\textsuperscript{104} Our laboratory has previously shown that hypertension in FHR is accompanied by an increase in plasma TXA\textsubscript{2} levels. Treatment with the TXA\textsubscript{2} synthase inhibitor dazmegrel prevented the increase in BP. It was therefore suggested that hypertension in insulin resistant rats could be dependent on TXA\textsubscript{2} synthesis.\textsuperscript{46} The mechanism by which TXA\textsubscript{2} becomes elevated is not fully known. Two cyclooxygenase isoforms, the constitutively expressed COX-1 and inducible COX-2, act on arachidonic acid to yield prostaglandin endoperoxide H\textsubscript{2} (PGH\textsubscript{2}), which is converted downstream to TXA\textsubscript{2} by the action of thromboxane A\textsubscript{2} synthase. Preliminary data from our laboratory demonstrate that cyclooxygenase (COX) inhibition reduces norepinephrine (NE)-induced contraction in aorta from FHR but not in control rats, suggesting that there is enhanced production of COX-derived vasoconstrictor products, possibly TXA\textsubscript{2}, in vascular tissue of FHR.\textsuperscript{43} It was recently shown that COX-2 was overexpressed in the mesenteric arteries and thoracic aorta of fructose hypertensive
rats (FHR). This suggests an increased availability of intermediate arachidonate derivatives as substrates for the production of vascular TXA₂.

Other mechanisms suggested in support of insulin-induced vasoconstriction involve upregulation of the renin-angiotensin system (RAS) activity in the vasculature in addition to increased plasma levels of angiotensin-2 (AT-II) in FHR.
Figure A Schematic representation of the major pathways involved in the metabolism of arachidonic acid. The figure is adapted from the review by Sarkis and Roman.\textsuperscript{101}
Role of gender and sex hormones in hypertension and insulin resistance

In humans, while cardiovascular disease (CVD) affects both sexes, the risk of developing hypertension and other CVD is approximately twice in males as compared to age-matched females as per a study conducted by the World Health Organization. The difference in ratio is observed until the fourth and fifth decades, where the risk of CVD in women starts rising and nearly reaches to a 1:1 ratio with males by the seventh decade of their lives. The time indicated coincides with the development of menopause in women resulting in 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.

Effects of estrogen on hypertension: The hypothesized cardiovascular benefits of estrogen resulted in several clinical studies, which investigated the benefits and risks of estrogen replacement therapy. The results obtained so far have been unable to totally resolve the existing controversy surrounding hormone replacement therapy. Initial reports, most of which were short-term studies (3-6 months), indicated a lower incidence of CVD following estrogen replacement in postmenopausal women as compared to non-users. Further, these effects were observed when estrogen was used in lower doses. In later studies, with gradual increase in the dose of estrogen and duration of therapy (1-6 years), there was a significant increase in cardiovascular mortality in postmenopausal women. The Women’s Health Initiative (WHI) trial reported increased incidences of estrogen-induced thrombosis and subsequent development of myocardial infarction or stroke, some of which were fatal. Estrogen did not confer any protection in postmenopausal women with peripheral artery disease. The women in this study were aged 50 to 79 on average, and were followed up for 5.6 years. The recently concluded HERS (Heart and Estrogen/progestin Replacement Study) trial reported no significant benefit of hormone therapy in post-menopausal women with an established history of CVD. Similar conclusions were drawn from the HERS-II study, which was a follow-up of the HERS study. Thus, the above-mentioned and other studies indicate increased risk as compared to benefit in chronic estrogen replacement therapy.

Estrogen has also been used as an experimental tool in animals to understand the potential interactions between various vasoconstrictor and relaxant pathways. Under experimental conditions, the effects of estrogen have been studied in several strains of rats. Numerous studies
have demonstrated the presence of estrogen to exert a protective effect on the cardiovascular system of female rats in vivo. These positive effects were abolished following ovariectomy and were reinstated by estrogen treatment.\textsuperscript{119}

**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 normal rats, a 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. Secondly, most clinical studies are controlled for the gender variable. Studies to date have suggested a higher risk of coronary artery disease (CAD), myocardial infarction and hypertension in males with lower circulating levels of testosterone.\textsuperscript{120-123} An analysis of the Rancho Bernardo study, involving men between the ages of 30 to 79 confirms these findings.\textsuperscript{124,125} Both systolic and diastolic blood pressure inversely correlated with testosterone levels in these men.\textsuperscript{125} Thus, clinical studies with specific focus on blood pressure and CAD in the presence or absence of have suggested a protective role for testosterone in human coronary arteries.\textsuperscript{124} However this claim is unresolved, as males have been found to have higher blood pressure as compared to age-matched premenopausal women. 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.\textsuperscript{126} Indeed, isolated arteries from gonadectomized rats exhibited decreased pressor responses to various vasoconstrictors.\textsuperscript{127,128} Blocking the testosterone receptor in spontaneously hypertensive rats (SHR) using flutamide\textsuperscript{129} resulted in decreased blood pressure, in addition to increased vasorelaxation in vitro;\textsuperscript{130} thus indicating a receptor-mediated action of testosterone in facilitating the development of hypertension. Furthermore, gonadectomy of SHR resulted in a reduction in the renal angiotensinogen and renin mRNA levels, which was restored by testosterone implantation.\textsuperscript{131} Finally, results from Holla et al showed that knocking out the Cyp4A14 gene, an arachidonic acid monooxygenase, resulted in hypertension. In mice, this enzyme isoform is implicated in the formation of EET from arachidonic acid. Knocking out the enzyme resulted in increased levels of 20-hydroxyeicosatetraenoic acid (20-HETE; described in later sections) along with increased
expression of Cyp4A12 (catalyzes 20-HETE formation). The consequent upregulation of the prohypertensive arachidonate metabolite, 20-HETE, was abolished by gonadectomy.\textsuperscript{126} Thus experimental evidence suggests a potential role for testosterone in the development of hypertension.

**Role of sex hormones in insulin resistance and secondary hypertension**

However, the effects of gender differences on the interrelationship between insulin sensitivity and hypertension were unknown until recently. Recent evidence from our laboratory has demonstrated differences between males and females in the inter-relationship between hyperinsulinemia, IR, and hypertension. In fructose-fed rats, male rats developed significant hypertension and hyperinsulinemia after 9 weeks of fructose feeding, while female rats did not become hypertensive. Interestingly, they also failed to develop insulin resistance.\textsuperscript{132} To extend our findings on the relationship between insulin and BP in male and female rats, we treated both male and female rats chronically with exogenous insulin and measured BP and insulin sensitivity pre- and post-treatment. The results of this experiment demonstrated that chronic hyperinsulinemia produced insulin resistance and hypertension only in male rats, while the degree of insulin resistance was significantly lower in females. Further females failed to develop hypertension even in the presence of hyperinsulinemia.\textsuperscript{133}

**Role of estrogen in insulin resistance and hypertension:** To further investigate the hypothesis that estrogen is protective against the development of hypertension in states of hyperinsulinemia and insulin resistance, ovariectomized female rats were fed with fructose for 6-8 weeks. Insulin sensitivity was reduced after 8 weeks of fructose feeding, which was accompanied by elevated blood pressure.\textsuperscript{133} Most recently, unpublished results from our laboratory (Song and McNeill) have shown that chronic estrogen replacement in ovariectomized hyperinsulinemic female rats prevented the increase in blood pressure, in addition to improving insulin sensitivity. Collectively these data support the hypothesis that estrogen prevents the induction of insulin resistance and subsequent hypertension. The effects may be by direct insulin-like action or by indirectly enhancing insulin sensitivity. The specific pathways modulated by estrogen and the mechanisms by which these pathways are affected during hyperinsulinemia or insulin resistance require further study.

**Role of testosterone in insulin resistance and hypertension:** Our next question was that if estrogen played a protective role in the prevention of IR and hypertension in females, what were
the effects of androgens, especially testosterone on insulin resistance and subsequent hypertension.

Based on the current literature, testosterone was shown to be essential in rats for the development of hypertension. Its role in induction of insulin resistance is yet to be clarified. Testosterone has been strongly associated with the development of insulin resistance and hypertension in women with polycystic ovary syndrome (PCOS). This disease affects premenopausal women during their reproductive years, resulting in the loss of ovarian hormones. Further, such patients are hyperandrogenic and oligomenorrheic. Recent reports have made a strong case for the induction of endothelial dysfunction following PCOS, which has been associated with hyperandrogenism. In postmenopausal women, due to the loss of estrogen, a relative hyperandrogenic state along with elevated risk of IR and hypertension were observed. Similar hyperandrogenic states have been reported in pregnant women with preeclampsia. Hence, a second question arose as to the critical balance between estrogen and androgen that may be needed for the regulation of blood pressure. Thus, in addition to studies on the role of estrogen in hyperinsulinemic and insulin resistant animals, we have also been interested in understanding the role of androgens under insulin resistant conditions. Little information is available regarding the effects of androgens on the relationship between insulin sensitivity and blood pressure. Further, the studies conducted previously were focused only on the changes in blood pressure and not insulin sensitivity. In fructose-fed male rats, both the sham-operated and gonadectomized groups developed insulin resistance, but only the sham-operated rats developed hypertension. Therefore gonadectomized rats fed with a high fructose diet do not experience a rise in BP. Thus, it was established for the first time that testosterone is necessary for the development of hypertension following insulin resistance. 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. This indicated that testosterone may affect the development of hypertension in hyperinsulinemic/insulin resistant animals via the COX pathway and thus arachidonate metabolism. Based on our studies, we proposed that the interrelationship between hyperinsulinemia, insulin resistance, and hypertension was dependent on differences in gender. We hypothesized that the sex hormones estrogen and testosterone are protective and permissive, respectively to the induction of hypertension. Further studies are required to identify the specific aspects of the pathways that may be altered by hyperinsulinemia/insulin resistance and influenced by estrogen and testosterone.
Hormonal balance in men and women

The sex hormones, testosterone and estrogen are present in both males and females, which govern certain phenotypes associated with the opposite sex. As shown in Figure B, estrogen is present in all men (12-34 pg/ml) though it is significantly lower than in women (24-149 pg/ml). Similarly, testosterone levels in women (0.3-0.95 ng/ml) are lower compared to men (3-12 ng/ml). A large number of studies have investigated the individual contributions of estrogen and testosterone to the development of insulin resistance and hypertension, both clinically as well as in various animal models. However little is known as to how they affect insulin resistance and hypertension in the presence of each other. In other words, do estrogen and testosterone complement or counteract each other? Furthermore, how does the residual estrogen or testosterone influence insulin sensitivity and hypertension in gonadectomized male or female rats? A recent study by Fortepiani et al. showed that both 18 month-old ovariectomized (OVX) SHR and intact very old female (post estrous) SHR had significantly higher levels of testosterone, subsequent to the fall in estrogen levels. Young estrous rats, which served as controls with higher circulating estrogen, had significantly lower levels of testosterone. The mean arterial pressure (MAP) was significantly higher in males and 18-month OVX rats as compared to young estrous rats, which served as control. These results give an indication that the unmasking of testosterone-mediated effects following the loss of estrogen may be responsible for the increased MAP in these rats. Presently no studies have attempted to
investigate the interactions and interdependence of the sex hormones in males and females, and how the loss of one hormone influences the levels and effects of the other. Testosterone is converted to estrogen in the body by the action of aromatase.\textsuperscript{142,143} Interestingly, the expected increase in testosterone levels following the inhibition of aromatase and its influence on BP has not yet been studied.

**Role of sex hormones in the vasculature**

**Role of estrogen in the vasculature:** Estrogen elicits a multitude of effects that could potentially counter insulin resistance-induced vascular defects. As a steroid hormone, in addition to its effects on nuclear receptors to affect gene expression and regulation, non-genomic effects of estrogen have also been demonstrated in isolated vasculature.\textsuperscript{144,145} Acute application of estrogen causes relaxation in various vascular tissues\textsuperscript{144,146-149} involving multiple endothelium-dependent and independent\textsuperscript{150} pathways. Estrogen in the body has also been shown to attenuate the gene expression of endothelin converting enzyme (ECE) in vascular tissues.\textsuperscript{151,152} Ovariectomized female rats showed increased vascular ET-1 levels, which were reduced by treatment with both 17β-estradiol and phytoestrogens.\textsuperscript{151,153} Similar results were observed in postmenopausal women treated with phytoestrogens.\textsuperscript{154,155} Estrogen has also been shown to reduce the expression of vascular (angiotensin) AT\textsubscript{1} receptors.\textsuperscript{156,157}

In the isolated vasculature, estrogen achieves relaxation by exerting its effects on both the large and the small blood vessels. Estrogen-mediated relaxation in the large vessels such as the aorta is mainly dependent on the functional endothelial nitric oxide system.\textsuperscript{158,159} Although EDHF has been shown to be involved in relaxing large vessels, its contribution is secondary to that of NO.\textsuperscript{160} In ovariectomized rats, estrogen has been shown to enhance aortic endothelial sensitivity to insulin and NO.\textsuperscript{119} A second mechanism of action for estrogen is by increasing Ca\textsuperscript{2+} efflux via a direct interaction with calcium channels in the vascular smooth muscle (VSM).\textsuperscript{148,161} Thus the resultant decrease in [Ca\textsuperscript{2+}]\textsubscript{i} in the VSM contributes to the final relaxation process.

Estrogen has been shown to relax the mesenteric arteries by EDHF-dependent activation of large and small calcium-sensitive potassium channels (BK\textsubscript{Ca} and SK\textsubscript{Ca}). EDHF has been suggested as the predominant vasodilator in female rats compared to males.\textsuperscript{162} McCulloch et al. have demonstrated that EDHF-mediated vasorelaxation predominates over NO in females,\textsuperscript{163} resulting in differential regulation of vascular tone. The relative contribution of NO and EDHF
in the smaller vessels may therefore be contingent on gender. Previous studies on VSM have suggested an inverse proportionality of the size of the blood vessel to the contribution of EHDF in vasorelaxation. Fructose feeding-induced insulin resistance impaired EDHF and $K_{Ca}$ channel activity, resulting in attenuated vasodilatory responses. Thus it may be hypothesized that defects in production of EDHF or EDHF-evoked hyperpolarization in the smaller blood vessels lead to increased sensitivity to vasoconstrictors. These potential interactions are yet to be investigated.

**Role of testosterone in the vasculature:** The total effects of testosterone on endothelial vasoconstrictors are not yet clear. Studies have reported several pro- and antihypertensive pathways that could be directly or indirectly affected by the presence of testosterone in vivo. Plasma ET-1 levels are elevated in males as compared to females. However, gonadectomy was unable to affect endothelin receptor expression in blood vessels. Testosterone mainly affects arachidonic acid metabolism in the renal and peripheral vasculature. The 3 major metabolic pathways for arachidonic acid that result in the synthesis of vasoconstrictors include TXA$_2$ (cyclooxygenase-COX enzymes), 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] (lipoxygenase-LOX enzymes), and 20-hydroxy eicosatetananoic acid (20-HETE) (cytochrome P450 system). These pathways are shown in Figure A. In particular, COX-2 mRNA levels were upregulated in both the thoracic aorta and mesenteric arteries of male FHR and these levels were normalized upon gonadectomy. Higashiura et al and other groups suggest an upregulation in TXA$_2$ receptor density in the coronary and aortic vasculature in the presence of testosterone, though the changes during insulin resistance are yet to be ascertained. A pathway of interest is the recruitment of specific monohydroxylated arachidonic acid byproducts in the development of hypertension. Mainly characterized in the kidney and peripheral vasculature, these metabolites of arachidonic acid include (20-hydroxyeicosatetrananoic acid) 20-HETE, which has been implicated in several models of hypertension such as the Dahl salt-sensitive rat and spontaneously hypertensive rat (SHR). 20-HETE is a monohydroxylated derivative of arachidonic acid. It is synthesized mainly in the kidneys by the action of Cyp4A family of enzymes. Recently, a direct association has been demonstrated between the presence of testosterone and inhibition of renal Cyp4A14 (analog of rat Cyp4A2/3) expression in mice. The resultant increase in Cyp4A12 (analog of rat Cyp4A 8) led to elevated plasma 20-HETE levels and hypertension. Gonadectomy normalized Cyp4A12 expression and 20-HETE levels in the mice. Thus studies by Holla et al and others suggest a strong
likelihood that the presence or absence of testosterone is pivotal to affecting the function of vasoactive agents. However, little is known about the vascular crosstalk following gonadectomy in insulin resistant animals.

Contrary to its prohypertensive profile in vivo, testosterone relaxes both large and small blood vessels in vitro in an endothelium-dependent manner. As mentioned previously, following insulin resistance, the impairment in vascular relaxation to NO along with $K_{Ca}$ channel function contributes significantly to endothelial dysfunction and subsequent hypertension. Thus, it is likely that the removal of testosterone by gonadectomy may change the relative contributions of NO and EDHF in the mesenteric arteries, thus affecting the vasodilatory mechanism in the process. These changes may contribute to enhancing vascular relaxation, thus preventing IR-induced hypertension.

**RATIONALE, HYPOTHESIS AND OBJECTIVES**

In the present thesis, we continue our investigations into the intricate relationships between insulin and hypertension using sex hormones as a tool. Although gonadectomy has been shown to prevent hypertension in FHR, the changes occurring at the vascular tissue level that contribute to this effect are yet to be characterized. Since most of the studies in the mesenteric vasculature have focused on the interactions between NO and EDHF/$K_{Ca}$ channels and their contributions to vascular relaxation, our first objective was to look into the changes due to insulin resistance in the relative contributions of vascular NO and EDHF induced by the loss of testosterone. Prostacyclin, though an important vasodilator, does not significantly contribute to relaxing the mesenteric arteries. Thus, since NO and EDHF are the major vasodilators, we assumed that the relaxation observed following inhibition of NO would be due to $K_{Ca}$ action and vice versa.

Both male and females have testosterone and estrogen in their bodies. However it is unclear as to whether the IR-induced changes in blood pressure in either sex is due to a relative increase in estrogen or a decrease in testosterone levels. Based on the previous findings in which estrogen treatment was shown to lower blood pressure while testosterone was found to be associated with high blood pressure, our secondary objective was to investigate the influence of testosterone on estrogen levels when both were present in vivo. We were also interested in assessing the impact of the resultant changes in estrogen levels on the insulin sensitivity and blood pressure. We
hypothesized that: "Gender differences in the development of hypertension are, in part, a function of the interaction between nitric oxide and EDHF actions in fructose hypertensive rats."

Our secondary hypothesis was: The balance between testosterone and estrogen in male rats affects the induction of insulin resistance and subsequent development of hypertension.

To investigate these hypotheses, we designed experiments to answer the following questions:

1. What are the effects of gonadectomy on insulin resistance and hypertension?
2. How does gonadectomy affect the contributions of endothelial vasodilators, specifically nitric oxide and EDHF in normal and fructose-fed male rats?
3. Does chronic testosterone replacement in gonadectomized male fructose-fed rats reverse the antihypertensive effects induced by gonadectomy?
4. Does testosterone replacement in gonadectomized fructose-fed rats influence sensitivity to insulin?
5. What are the effects of estrogen supplementation on insulin sensitivity and blood pressure in gonadectomized male rats fed with fructose?
6. What are the effects of estrogen supplementation on insulin sensitivity and blood pressure in male fructose-fed rats with their testes intact?
7. Does the presence of testosterone in insulin resistant rats promote hypertension by positively affecting the action of prohypertensive arachidonic acid metabolites in the mesenteric arteries?
MATERIALS AND METHODS

General methodology

1. Assessment of insulin resistance/sensitivity

At the end of 6-7 weeks following fructose feeding, an oral glucose tolerance test (OGTT) was performed to assess changes in insulin sensitivity and glucose clearance in rats. After fasting the rats overnight (16 hours), they were orally gavaged with a 40% glucose solution (1g/kg body weight). Blood samples were withdrawn from the tail vein prior to and 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 until assayed.

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

\[
ISI = \frac{k}{\sqrt{[(FPG \times FPI) \times (MPG \times MPI)]}}
\]

The value of k was kept at 100 to enable us to obtain values between 1 and 15. 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. 0 (before glucose gavage) to 90 minutes (after glucose gavage)). These values serve as a composite index of hepatic as well as peripheral tissue sensitivity to insulin. 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, which is highly regarded as the standard for measuring insulin sensitivity.
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.\textsuperscript{43,176} This method is suitable and preferred for chronic measurement of blood pressure. This method has been validated in our laboratory in addition to others who have shown the measurement to be similar to the invasive intra-arterial method. 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. Isolated blood vessel preparation

The animals used in the hemodynamic studies were used to assess vascular smooth muscle (VSM) function. 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 (65 mg/kg) of pentobarbital (Somnotol\textsuperscript{TM}) administered intraperitoneally. Upon loss of foot and blink reflexes, the abdomen was opened and the superior mesenteric artery was excised. The tissue was placed in oxygenated ice-cold Krebs Ringer solution, the composition of which in mM was NaCl (118), KCl (4.7), CaCl\textsubscript{2} (2.5), MgSO\textsubscript{4} (1.2), KH\textsubscript{2}PO\textsubscript{4} (1.2), NaHCO\textsubscript{3} (25), Glucose (11.1), and EDTA (0.026). Prior to and after excision, the tissue was 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% O\textsubscript{2} and 5% CO\textsubscript{2} 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 wipes™ and the length and wet weights determined. The 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). Tissue sensitivity to agonist was calculated in terms of pD₂ (-log EC₅₀ - 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 Rₘₐₓ value. Both pD₂ and Rₘₐₓ were calculated by nonlinear regression analysis of the concentration response curves.¹³²,¹³³

4. Biochemical analyses

Following euthanasia by an overdose (60 mg/kg i.p.) of pentobarbital, blood was collected from rats by cardiac puncture. All other blood samples obtained during the studies were collected from tail vein. 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. 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 A₂. Upon centrifugation, the plasma was stored separately at –80°C until assayed. Plasma glucose was estimated with an automatic Beckman glucose analyzer II. Enzymatic colorimetric kits were used to measure plasma triglycerides. Plasma insulin was measured using commercially available radioimmunoassay kits from Linco Diagnostics Inc, USA, while 17β-estradiol and testosterone were measured in the plasma using RIA kits from MP Biomedicals, USA.
5. Plasma nitrite and nitrate analysis

Plasma was ultrafiltered though a 30kDa molecular weight cut-off filter (Ultrafree®-MC centrifugal filter units, Millipore Corporation, Bedford, MA). Nitrite and nitrate (NOx) levels in the plasma were determined using a commercially available colorimetric assay kit (Cayman chemical company, Ann Arbor, USA) based on the Griess reaction.

**Experimental protocols**

1. Effect of gonadectomy on the responses to a fructose diet in male rats

To confirm the possible involvement of testosterone in elevating the blood pressure and determine the changes in endothelium-dependent vascular relaxation of fructose-fed rats, we evaluated the effects of fructose diet in gonadectomized and sham-operated male Wistar rats. The rats were divided into 4 groups, namely sham-operated chow-fed control (C; n=10), sham-operated fructose-fed (F; n=10), gonadectomized chow-fed (G; n=10), gonadectomized-fructose-fed (GF; n=9). The rats were maintained on a 66% preformulated fructose diet. Prior to starting the fructose diet, basal systolic blood pressure was measured as previously described. Plasma was collected from the tail vein after a 5-hour fast for measurement of basal glucose, insulin and testosterone. During the course of the study, changes in blood pressure were assessed at 4 and 6 weeks. Body weights were measured at the start and termination of the experiment. At the end of 7 weeks, an oral glucose tolerance test was performed on the animals for changes in sensitivity to insulin. At termination, blood was collected via cardiac puncture for measuring testosterone and thromboxane A2.

2. Changes in the individual contributions of endothelial vasodilators (nitric oxide and EDHF/KCa) in normal male and gonadectomized rats fed with fructose

It has been previously shown in our laboratory that relaxation to ACh is attenuated in male fructose hypertensive rats. To examine the effects of gonadectomy on the vascular responses to acetylcholine (ACh) in fructose fed rats and determine the contribution of NO and EDHF in the vasculature, we used the same rats, which had been matched for age and duration of fructose feeding as described in protocol 2. On termination, the mesenteric arteries were isolated and
prepared as described above. Following equilibration and adjusting the tension to baseline, the tissues were challenged with 40 mM KCl. A concentration response curve to PE was constructed \((10^{-9} - 10^{-4} \text{M})\) to determine the \(ED_{70}\) dose. After washing out the PE, tissue rings were precontracted with the \(ED_{70}\) dose of PE and relaxation responses to increasing concentrations of ACh were obtained.

The following drugs were used to answer our questions.

**Effects of calcium-sensitive potassium channel blockers on ACh-induced relaxation**

To evaluate the changes in calcium-activated potassium channel-mediated vasodilation during insulin resistance and its specific contribution to the relaxation process, tissue rings were incubated using a combination of the large Ca-sensitive potassium channel inhibitor, charybdotoxin (CTX, \(10^{-8} \text{M}\)) and small Ca-sensitive potassium channel inhibitor, apamin (APA, \(0.25 \mu \text{M}\)) respectively. Relaxation to ACh was then studied on the rings precontracted with the \(ED_{70}\) dose of PE.

**Effects of N\(^\circ\)-Nitro-L-arginine -N-methyl ester (L-NAME) on ACh-induced relaxation**

After washing off the drugs, the tissues were incubated with the nitric oxide synthase (NOS) inhibitor L-NAME \((10^{-6} \text{M})\) for 20 minutes and responses to ACh in precontracted tissues were obtained as outlined above.

3. Effects of hormone replacement in normal and gonadectomized male fructose fed rats

3A. Effects of testosterone replacement in normal and gonadectomized male fructose fed rats

3B. Effects of estrogen replacement in normal and gonadectomized male fructose fed rats

We were interested in studying the changes in insulin sensitivity and blood pressure if we were to replace testosterone and estrogen separately in fructose-fed gonadectomized rats. Sham-operated and gonadectomized male Wistar rats were divided into 5 experimental groups namely, sham-operated chow-fed control (C; \(n=5\)), sham-operated fructose-fed (F; \(n=5\)), gonadectomized normal chow-fed (G; \(n=4\)) and gonadectomized-fructose-fed (GF; \(n=16\)). Subsequent to the measurement of blood pressure and plasma concentrations of glucose, testosterone and 17\(\beta\)-estradiol, the rats were started on a 66% fructose diet. To extend the
findings of our previous studies demonstrating the role of testosterone in abetting hypertension, we determined the effects of testosterone implantation on the blood pressure and insulin sensitivity. Estrogen was also evaluated for its effects on the same parameters in gonadectomized fructose fed rats. At the end of study week 4, blood pressure was measured in these rats, following which testosterone pellets (100 mg; 60 day release, Innovative research of America, Sarasota Fl) were implanted subcutaneously in one-half of the GF group (GFT, n=8) under halothane anaesthesia. Blood was withdrawn weekly for measurement of testosterone in the implanted rats. On completion of 2 weeks post-implantation, i.e. study week 6, the blood pressure was measured and an oral glucose tolerance test was performed. Forty-eight hours following the OGTT, the remaining gonadectomized rats being fed with fructose were implanted with a 0.5 mg estrogen pellet (60 day release, Innovative research of America, Sarasota Fl) (GFE, n=7). Implanting was carried out under halothane anaesthesia. The pellets were subcutaneously implanted using a trochar under aseptic conditions. One rat died due to halothane, but the procedure was successful in the remaining animals. Concentration of 17β-estradiol in the plasma was estimated at 3 and 10 days of implantation. Two weeks after implantation, i.e. study week 8, the blood pressure was measured in all the groups and an OGTT was carried out. The animals were terminated and blood was collected by cardiac puncture for analysis of plasma testosterone and 17β-estradiol.

4. Vascular effects of estrogen and testosterone implantation in fructose fed normal and gonadectomized male rats

Estrogen has been previously shown to induce vascular relaxation in male SHR and ovariectomized females. To determine any possible changes in vasorelaxation upon gonadectomy and subsequent testosterone or estrogen implantation, the same rats as described in study 4 were used in this experiment. After equilibration, the tissues were challenged twice with 40 mM KCl and a concentration response curve to PE was constructed (10−9 – 10−4M) to determine the ED70 dose. After 3 washings, tissue rings were precontracted with the ED70 dose of PE, subsequent to which relaxation responses to increasing concentrations of ACh were obtained. Tissues were then incubated for 20 minutes each with a mixture of 10−8M CTX and 0.25 μM apamin followed by 10−6M L-NAME. After incubation with each drug(s), precontraction to PE and subsequent concentration response curves to ACh were repeated.
5. Effects of gonadectomy on the contribution of vascular prohypertensive arachidonic acid metabolites

Testosterone has been implicated in regulating vascular and renal arachidonic acid metabolism by arachidonic acid hydroxylases (Cyp4A enzymes). The levels of 20-HETE, one of the prohypertensive byproducts of arachidonic acid hydroxylase action are reduced following gonadectomy. Replacement of testosterone in these animals restored the elevated 20-HETE levels in addition to the development of hypertension. Further Cyp4A expression was elevated in the presence of testosterone. Thus, we hypothesized that following IR, in the presence of testosterone, 20-HETE may play a role in decreasing vasorelaxation in FHR, which may be attenuated by gonadectomy. To determine if testosterone-dependent prohypertensive metabolites of arachidonic acid are involved in impairing the responses to ACh, the same groups of animals in study 4 were used. Subsequent to termination and obtaining the basal responses to ACh, the mesenteric arterial rings were incubated with 1-aminobenzotriazole (ABT), which is a nonselective suicide inhibitor of Cyp4A. After incubating for 20 minutes, the tissues were precontracted to ED70 dose of PE and the responses to increasing concentrations of ACh were recorded.

6. Effect of estrogen implantation in male rats fed with high fructose diet

It was previously demonstrated in our laboratory that ovariectomized female rats develop insulin resistance and hypertension, following fructose feeding. Treatment with estrogen has been shown to restore normal insulin sensitivity and blood pressure (Song et al, unpublished data). However, their effects on IR and hypertension are unclear with respect to males. To evaluate if estrogen implantation in male fructose fed rats produced effects similar to ovariectomized females, male Wistar rats weighing 300-350 gm were implanted with estrogen and simultaneously fed with 66% fructose diet for 7 weeks. The specific parameters of interest were blood pressure and markers of insulin resistance. The rats were divided into four experimental groups namely normal chow-fed control (C), control rats treated with estrogen (CE), fructose fed (F) and fructose-fed estrogen treated (FE). Estrogen pellets (0.5 mg for 60 day release) (Innovative Research of America, Sarasota FL) were used to provide estrogen in these rats i.e. the CE and FE groups. The estrogen pellets were implanted subcutaneously under halothane anaesthesia. The implantation was performed on the same day that the fructose
feeding was started. Prior to introduction of fructose, the basal blood pressure was measured in these animals by the procedure mentioned above. In addition, body weight and food intake were estimated. On the day of starting the fructose diet, the rats were fasted for 5-hours and blood was collected for measuring plasma glucose, insulin testosterone and 17β-estradiol. The rat chow in groups F and FE was replaced with a diet containing 66% fructose. Rats were allowed ad libitum access to food and water. The food consumption of the rats per kg body weight was assessed once a week, along with body weights. Owing to the unexplained death of 2 rats in the fructose-fed group treated with estrogen, plasma blood glucose was measured in the surviving rats for the following 2 weeks using Accucheck™ glucose strips (Roche).

At the end of 4 weeks of fructose feeding, changes in blood pressure were evaluated in these rats. This was repeated at weeks 5 and 6 after fructose feeding to monitor the development of hypertension.

At the end of study week 7, an oral glucose tolerance test (OGTT) was performed on the rats following an overnight fast. At termination, blood was collected via cardiac puncture for measuring triglycerides, 17β-estradiol, testosterone and thromboxane A₂.

In all of our studies, male Wistar rats were used. They were obtained from Charles River, Montreal, Canada. The animals were obtained at 6 weeks of age and were acclimatized in the faculty animal facility for one week prior to commencement of treatment. In case of the gonadectomized rats, the testes were surgically removed at 5 weeks of age and subsequently shipped when they were 6 weeks old. Until treatment was initiated, the animals were allowed ad libitum access to standard laboratory rat chow and drinking water. The maintenance of rats was in accordance with the guidelines outlined by the Canadian Council on Animal Care.

Reagents

The 66% high fructose diet was obtained from Teklad laboratory diets, Madison, Wi. KCl, phenylephrine, acetylcholine, charybdotoxin, apamin and 1-aminobenzotriazole were purchased from Sigma. 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. ABT was dissolved in 60% ethanol in Krebs Ringer.
Statistical Analyses

All data are presented as mean ± 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 curve maximum responses, one-way ANOVA was used. Mean values were considered significant at $P < 0.05$. The paired t-test was used to compare changes in vascular relaxation before and after a particular treatment. 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.
RESULTS

1. Effects of gonadectomy on the responses to a fructose diet in male rats

Prior to starting the fructose diet, plasma testosterone levels were measured in all the experimental groups. The gonadectomy of rats was successful, as testosterone was not detected in the plasma of the gonadectomized rats. Throughout the course of the study, testosterone levels were undetectable in gonadectomized animals. Testosterone was present only in the sham-operated groups. Upon termination, testosterone levels in the plasma of both sham-operated control (C) and fructose fed (F) rats did not change compared to their corresponding basal values (0 week, figure 1). At the end of 7 weeks, both gonadectomized and the sham-operated rats had significantly higher body weights compared to corresponding basal values (Table 1). However, the sham-operated rats weighed significantly more than the gonadectomized groups. Feeding the fructose diet did not influence the changes in weights. The animals were healthy and showed no external signs of discomfort. Feeding and drinking patterns were uniform in all the groups.

Throughout the study, none of the groups developed hyperglycemia (data not shown). Consistent with our previous studies, administration of glucose increased the insulin levels in all the groups as early as 10 minutes, which was sustained up to 60 minutes in the fructose-fed groups (F and GF; figure 2). Plasma glucose was elevated in all the groups at 10, 20 and 30 minutes following glucose ingestion, but was returning to normal at the 60 and 90 minute time points (figure 3). Comparison of insulin sensitivity index (ISI) values among the groups revealed attenuated insulin sensitivity in the fructose-fed animals (groups F and GF) (figure 4).

At the start of fructose feeding, all the rats had blood pressure values below 120 mm Hg. When blood pressure was measured in the rats 6 weeks following fructose feeding, there was a significant elevation in the blood pressure of sham-operated rats fed with fructose (F, 137 ± 1 mm Hg). In contrast, fructose diet failed to elevate the blood pressure in gonadectomized animals (111 ± 1 mm Hg) (figure 5). Gonadectomy by itself did not induce any change in the blood pressure of normal-chow fed (G) rats.

The level of plasma NOX at termination was unaffected in all the groups. Thus fructose feeding and resultant hypertension did not involve changes in plasma NOX levels (Table 5).
2. Changes in the individual contributions of endothelial nitric oxide and EDHF/KCa in normal male and gonadectomized rats fed with fructose

Following the challenge with KCl, the mesenteric arteries were contracted with increasing concentrations of PE (10^{-9} to 10^{-4} M). Experiments with male rats from study #1 were designed to study the effects of phenylephrine in the vasculature of fructose fed rats. Contrary to our hypothesis that insulin resistance and endothelial dysfunction would result in exaggerated responses to phenylephrine, the contraction to PE in fructose-fed sham operated rats (F) did not differ from the other groups. Both the gonadectomized groups (G and GF) exhibited robust contraction to PE, though there were no significant differences among the groups with increase in concentration (figure 6). An indicator of tissue sensitivity to drugs, pD$_2$ was calculated for PE. None of the groups differed significantly in their pD$_2$ values. Similarly there was no difference in the R$_{max}$ values in the four groups (Table 2).

The ED$_{70}$ dose of PE was determined based on the concentration response curve of PE as mentioned above. The experiments in this study were mainly aimed at investigating the changes in vascular relaxation to ACh in fructose fed male rats. Acetylcholine relaxed the arterial rings by up to 85% in both sham-operated as well as gonadectomized groups fed on normal chow (C and G). In concentrations from 10^{-7} M to 10^{-4} M the relaxation to ACh was impaired in the tissue rings of fructose-fed sham-operated rats (F) as compared to control (C) and gonadectomized (G) groups (figure 7a-b). GF group exhibited a relaxation profile similar to C and G groups. Thus the absence of testosterone improved relaxation to ACh following fructose feeding.

Subsequent to determining the responses to ACh, we were interested in studying the contribution of specific endothelial vasodilators, which were involved in mediating this Ach-induced vasorelaxation. Most of the studies on mesenteric arteries have reported a moderate to almost negligible role for PGI$_2$ in the vasodilatory process. Thus the two main endothelial agents involved in mediating vascular relaxation are nitric oxide and EDHF/KCa channels. With the hypothesis that the relaxation observed upon blockade of NO is due to EDHF action and vice versa, we evaluated vascular responses to ACh in the presence of specific NO or KCa channel inhibitors.
Effects of $K_{Ca}$ channel blockers on acetylcholine-induced vascular relaxation

Preincubation of the tissues with a combination of large Ca-sensitive potassium channel ($BK_{Ca}$) inhibitor, charybdotoxin (CTX, $10^{-8}$ M) and small Ca-sensitive potassium channel ($SK_{Ca}$) inhibitor, apamin (APA, 0.25 µM) resulted in a significant decrease in relaxation for all the groups compared to the responses before incubation. The fructose-fed sham-operated animals (F) exhibited significantly lower relaxation compared to the other groups (figure 8a). The area under the curve (AUC) for these responses was lower in F compared to other groups (figure 8b), suggesting a decrease in the contribution of NO. Incubation with CTX+APA significantly depressed the relaxation in C, G and GF groups as compared to the ACh-evoked relaxation observed prior to inhibition of $K_{Ca}$ (figure 10 a, c and d). However, despite the decrease, the relaxation in these groups was appreciable and significantly higher than those observed in F (figure 8b).

Effects of L-NAME on acetylcholine-induced vascular relaxation

Incubation with the nitric oxide synthase inhibitor $N^0$-nitro-L-arginine methyl ester (L-NAME, $10^{-6}$ M) for 20 minutes inhibited ACh-evoked relaxation in the mesenteric arteries of all groups. Responses to PE were exaggerated after incubation with L-NAME compared to contraction responses prior to L-NAME treatment. Relaxation was negligible in both the fructose-fed groups (F and GF) (figure 9 a-b). Similar to the results observed with CTX+APA, L-NAME decreased the relaxation in C and G groups compared to ACh-induced responses before treatment (figure 10a and c). However, the degree of relaxation in both the normal chow-fed groups (C and G) was significantly higher compared to F and GF (figure 9a-b).

The effects of each drug response were evaluated in individual groups. Selective inhibition of the $K_{Ca}$ channel or NO synthesis in the C group produced a significant decrease in relaxation to ACh. However the tissues showed up to 50% relaxation in either case. A similar pattern was observed with the gonadectomized controls (G). The relaxation of arteries from the GF group was decreased upon inhibition of $K_{Ca}$ channel, similar to C and G. But the inhibition of NO in GF resulted in a marked fall in relaxation resulting in negligible responses to ACh. In the sham-operated fructose fed rats (F), relaxation was diminished to a greater degree on inhibition of EDHF, which was significantly lower than that observed in GF group (figure 10 b, d).
3. Effect of hormone replacement in normal and gonadectomized male fructose fed rats

In this study, all the rats were healthy with no outward signs of discomfort or infection, particularly the rats implanted with hormone pellets.

3A. Effect of testosterone replacement in normal and gonadectomized male fructose fed rats

Testosterone treatment for 2 weeks did not affect the body weights in the fructose-fed rats. (data not shown). These results seem to contradict our results in Table 1. We observed that the gonadectomized rats had a lower body weight as compared to the sham-operated rats at the beginning of the study, which persisted throughout the study, resulting in lower weights upon termination. Thus in this study there seems to be no difference in the weight gain in all rats (C, F, G, GF and GFE). At 2 weeks after testosterone implantation (study week 6), all the fructose-fed rats were found to be hyperinsulinemic (figure 11) and insulin resistant (figure 13). The rats were however normoglycemic (figure 12). The insulin sensitivity indexes in the rats were similar to that in figure 4. Thus reimplantation of testosterone did not affect the insulin resistance in fructose-fed rats.

Before testosterone implantation, at 4 weeks of fructose feeding, there was no significant rise in the blood pressure. Two weeks after testosterone implantation, i.e. at study week 6, the gonadectomized fructose-fed rats (GFT) showed an increase in BP, while the control (C) and gonadectomized groups (G & GF) were normotensive (figure 14). The values of blood pressure in the GFT rats were comparable with those from F. Thus testosterone reversed the fall in blood pressure caused by gonadectomy. Some of the rats implanted with testosterone developed high BP, while the others did not. This could be due to the fact that the implants released high amounts of testosterone (Table 6), which was sustained only up to 2 weeks, after which there was no testosterone in the plasma. Further, the implants might have broken down in the body resulting in a marked release. Studies using reduced dose of testosterone need to be repeated.

3B. Effect of estrogen replacement in gonadectomized male fructose-fed rats

At the end of 3 weeks following estrogen implantation (study week 9), gonadectomized rats implanted with estrogen had a lesser weight gain compared to the other groups upon termination (figure 15).
However, the gonadectomized fructose-fed rats, which were treated with estrogen for 2 weeks did not become insulin resistant, as they had higher ISI compared to F, indicating normal insulin sensitivity (figure 18). Two weeks of treatment with estrogen decreased the insulin levels in the gonadectomized fructose fed rats (GFE). As a result, insulin levels in GFE were comparable with C and G groups, while it was significantly lower than the sham-operated fructose-fed rats (figure 16). Further, glucose levels in these rats were significantly lower in GFE than the remaining groups (figure 17).

Blood pressure measurements were repeated 2 weeks (study week 8) after implanting GF rats with estrogen- 0.5 g, s.c. - 60 day release. Estrogen did not affect the BP in GF rats (figure 19). The fructose-fed sham operated rats remained hypertensive at the end of 8 weeks of fructose feeding. As mentioned earlier, plasma testosterone being undetectable after 3 weeks of implantation, the GFT rats showed reduced blood pressures compared to values at study week 6. Prior to starting the fructose diet, gonadectomized rats had significantly higher plasma estradiol than the sham-operated males. To check the profile of estradiol release from the estrogen pellet, plasma estradiol levels were measured at 3 and 10 days following implantation and finally at termination. As shown in Table 7, there was a marked release of estradiol from the pellet on the 3rd day following estrogen implantation. 17β-estradiol values were greater than 3000 pg/ml at the end of 3 days. Ten days later, estradiol levels were significantly higher than basal levels although the 17β-estradiol levels had significantly decreased compared to the values on day 3. Estradiol was the highest in the estrogen-implanted group (GFE) compared to other groups until the study was terminated.

4. Vascular effects of estrogen and testosterone implantation in fructose fed normal and gonadectomized male rats

We were unable to involve the GFT group in the vascular reactivity experiments due to the loss of testosterone in the middle of the study. Phenylephrine (PE) induced robust contractions in all the groups, though no group was significantly different from the other (figure 20). Treatment with estrogen did not affect the responses to PE in gonadectomized rats. (Table 8)

Consistent with the previous study, acetylcholine induced relaxation by up to 85% in the mesenteric arterial rings of both sham-operated as well as gonadectomized groups fed on normal chow (C & G). All the tissues were precontracted with ED70 dose PE, prior to incubating with ACh. In a concentration from 10⁻⁶ M to 10⁻⁴ M, relaxation to ACh was severely impaired in
fructose-fed sham-operated rats (F) compared to control (C) and gonadectomized (G) groups (figure 21). Estrogen treatment did not significantly augment the relaxation as compared to non-implanted groups. Thus gonadectomy improved the relaxation responses to Ach, which was unaffected by estrogen.

**Effects of KCa channel blockers on acetylcholine-induced vascular relaxation**

Treatment with $10^{-8}$ M CTX and $0.25 \mu M$ APA resulted in a significant decrease in relaxation for all the groups compared to the responses before incubation. Upon KCa channel inhibition the relaxation to ACh in the estrogen-treated rats (GFE) was significantly decreased compared to C (figure 22a-b), although no variation was shown in comparison with G. The fructose-fed rats (F) showed the maximum reduction in relaxation among all the groups when the KCa channels were blocked suggesting reduced contribution of NO.

**Effects of L-NAME on acetylcholine-induced vascular relaxation**

Incubation with L-NAME ($10^{-6}$ M) for 20 minutes inhibited ACh-evoked relaxation in the mesenteric arteries of all groups. Responses to PE were exaggerated after incubation with L-NAME compared to contraction responses prior to treatment. Relaxation was nearly abolished in the all the fructose-fed groups (F and GFE), regardless of the presence and absence of testosterone. Estrogen implantation failed to alter this balance (figure 23 a-b). In comparison, the relaxation to ACh in normal chow-fed and sham-operated (C) as well as gonadectomized rats (G) was as high as 65%. Taken together the results suggest IR-impairment in the KCa channel function, which contributes to endothelial dysfunction.

5. **Effects of gonadectomy on the contribution of vascular prohypertensive arachidonic acid metabolites**

Incubation with $2 \times 10^{-4}$ M ABT for 20 minutes resulted in a significant decrease in the contraction to ED$_{70}$ concentration of PE. The relaxation pattern to ACh in F showed an appreciable improvement subsequent to incubation with ABT (figure 24 a-d). ABT did not affect the responses to ACh in C, G and GFE respectively.
6. Effects of estrogen implantation in male rats fed with high fructose diet

In this study, male fructose-fed rats having their testes intact were treated with estrogen implants to assess the role of sex hormone balance in regulating insulin sensitivity and blood pressure. On termination, male rats implanted with estrogen had significantly lower body weights compared to the non-implanted groups (figure 25). This was similar to our results from figure 15. There was no difference between the body weights of normal chow and fructose-fed groups at the end of the study. Thus fructose feeding \textit{per se} did not affect the changes in weights. Estrogen implanted rats consumed less food as compared to the untreated groups (figure 26). After significant weight loss in the 2\textsuperscript{nd} week following estrogen implantation, body weights in the estrogen implanted rats stabilized after the 3\textsuperscript{rd} week, although they were significantly lower than the control groups throughout the study. In this study, two animals in the fructose fed group being treated with estrogen (FE) died under unexplained circumstances in the 2\textsuperscript{nd} week of treatment. Analysis of blood glucose in the surviving rats revealed hypoglycemia, subsequent to which blood glucose levels were monitored weekly until normal. The rats were euglycemic by the end of study week 4 and survived to the end of the experiment.

Six weeks after fructose feeding and estrogen treatment, the animals were challenged with an oral glucose load of 1g/kg. Fructose-fed control (F) rats developed hyperinsulinemia within 10 minutes of ingestion of glucose, which was sustained till 60 minutes (figure 27). The glucose clearance profile was similar in all the groups over the entire 90 minutes of observation (figure 28). Estrogen (FE) prevented hyperinsulinemia in the fructose-fed rats and maintained insulin levels at par with controls. The insulin sensitivity index (ISI) was significantly lower in the fructose-fed group compared to the other groups, indicating the presence of insulin resistance in these animals. Impairment of insulin sensitivity in fructose fed rats was prevented by estrogen treatment as indicated by elevation of the insulin sensitivity index (figure 29).

At the end of 4 weeks following fructose feeding and estrogen implantation, the systolic blood pressure was higher in the fructose-fed rats (F) compared to the other groups. This pattern was repeated at the 5\textsuperscript{th} and 6\textsuperscript{th} weeks of fructose feeding, following which the fructose fed rats (F) developed hypertension (140 \pm 2 mm Hg). The fructose fed animals treated with estrogen (FE) showed an increase in BP at the end of 6 weeks (125 \pm 1 mm Hg), which was significantly lower as compared to F. Blood pressure was unchanged in both the control groups (C and CE) after 6 weeks (figure 30)
After 7 weeks of fructose feeding, the plasma triglyceride levels were measured at termination. Triglyceride (TG) levels were elevated in the untreated fructose-fed rat (F) as compared to the normal chow-fed rats, which is a characteristic of the FHR. Estrogen treatment did not affect plasma triglyceride levels in fructose-fed rats (FE), as shown in Table 9. Further the TG levels were unaffected in the normal chow fed rats (C and CE).

We had anticipated increased plasma estradiol levels at the end of the estrogen treatment. The plasma 17-β-estradiol levels were in fact elevated in CE and FE compared to C and F respectively (figure 31). However, there was a significant drop in the testosterone levels in the estrogen-treated groups at termination (figure 32). The ratio of testosterone to estradiol was compared in all groups. The testosterone to estradiol ratio was significantly higher in the fructose fed animals compared to the estrogen-implanted groups. Estrogen reduced the ratio in CE and FE respectively (figure 33).
Figure 1: Plasma testosterone levels were unchanged in male rats after feeding with fructose for 7 weeks. Fructose feeding did not alter the testosterone levels in sham-operated male rats (F, n=10) as compared to normal chow-fed controls (C, n=10). Testosterone levels were undetectable in the gonadectomized normal chow-fed (G, n=10) and gonadectomized high fructose-fed (GF, n=10) groups. Testosterone was measured using a commercially available RIA kit. All values are given as mean ± SEM.

Table 1: Body weights in sham and gonadectomized rats fed on normal chow or high fructose diet

<table>
<thead>
<tr>
<th>WEEKS</th>
<th>C</th>
<th>F</th>
<th>G</th>
<th>GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>BASAL</td>
<td>309 ± 3</td>
<td>305 ± 4</td>
<td>277 ± 4</td>
<td>273 ± 3</td>
</tr>
<tr>
<td>END</td>
<td>661 ± 20</td>
<td>639 ± 20</td>
<td>568 ± 16*</td>
<td>551 ± 13*</td>
</tr>
</tbody>
</table>

*P<0.05 G & GF vs C & F
Fig. 2 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=10) and gonadectomized high fructose-fed (GF, n=10) vs. normal chow-fed controls (C, n=10) and gonadectomized normal chow-fed (G, n=10). All values are given as mean ± SEM.

Fig. 3 Plasma glucose values in OGTT. Glucose levels were unchanged after OGTT. All groups, n = 10. All values are given as mean ± SEM.
Figure 4 Insulin sensitivity index (ISI) for control (C), fructose-fed (F), gonadectomized (G) and gonadectomized fructose fed (GF) rats. Absence of testosterone did not increase the ISI in fructose-fed rats (GF) compared to F. All groups, n = 10. * P<0.05 F & GF vs C & G All values are given as mean ± SEM.
Figure 5 Removal of testosterone in male rats prevented the development of hypertension following fructose feeding (GF), suggesting a pro-hypertensive role for testosterone. Systolic blood pressure was measured in the male fructose hypertensive rats, which were sham-operated (F, n=10) and gonadectomized (GF, n=10). Measurements were taken at 0 week (prior to start of fructose diet) and at 6 weeks (prior to termination). Fructose diet caused a rise in the BP of F. Blood pressure was unchanged in sham-operated normal chow-fed (C, n=10) and gonadectomized normal chow-fed (G, n=10) groups respectively. * P<0.05 F at termination (in white) compared to basal values (in black). All values are given as mean ± SEM.
Figure 6 Fructose feeding (F) does not alter contraction responses to phenylephrine (PE) in the mesenteric arteries. Superior mesenteric arteries were challenged with graded doses of PE ($10^{-9}$ to $10^{-4}$). Experiments were conducted in control (C), fructose sham-operated (F), gonadectomized (G) and gonadectomized-fructose fed (GF); all groups, n = 10. All values are given as mean ± SEM.

Table 2: $pD_2$ and $R_{max}$ values for responses to phenylephrine (PE)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>F</th>
<th>G</th>
<th>GF</th>
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</thead>
<tbody>
<tr>
<td>$pD_2$</td>
<td>6.77 ± 0.24</td>
<td>6.95 ± 0.11</td>
<td>6.78 ± 0.05</td>
<td>7.25 ± 0.15</td>
</tr>
<tr>
<td>$R_{max}$</td>
<td>4623 ± 416</td>
<td>5194 ± 510</td>
<td>6183 ± 567</td>
<td>5407 ± 388</td>
</tr>
</tbody>
</table>
Figure 7a Fructose feeding (F) reduces relaxation to acetylcholine in the mesenteric arteries, which is improved by gonadectomy (G and GF). Relaxation responses were obtained to acetylcholine (ACh, $10^{-9}$ to $10^{-4}$ M) after precontraction with ED$_{70}$ phenylephrine. * P<0.05 fructose sham-operated (F) vs. control (C), gonadectomized (G) and gonadectomized-fructose fed (GF); all n = 10. All values are given as mean ± SEM.

Figure 7b Area under the curve for relaxation to acetylcholine in sham-operated and gonadectomized fructose-fed rats. * P<0.05 fructose sham-operated (F) vs control (C), gonadectomized (G) and gonadectomized-fructose fed (GF); all groups, n = 10. All values are given as mean ± SEM.
Figure 8a Selective blockade of $K_{Ca}$ channel attenuated the relaxation in fructose-fed rats compared to C, G and GF, suggesting reduced involvement of NO. Relaxation responses were obtained to acetylcholine (ACh, $10^{-9}$ to $10^{-4}$ M) after inhibiting the $K_{Ca}$ channels by incubation with $10^{-8}$ M charybdotoxin (CTX) and 0.25 μM apamin (20 minutes) and precontraction with ED$_{70}$ phenylephrine. * P<0.05 fructose sham-operated (F) vs control (C), gonadectomized (G) and gonadectomized-fructose fed (GF); all groups, n = 5. All values are given as mean ± SEM.

Figure 8b Area under the curve for selective blockade of $K_{Ca}$ channel in sham-operated and gonadectomized fructose-fed rats. * P<0.05 fructose sham-operated (F) vs control (C), gonadectomized (G) and gonadectomized-fructose fed (GF); all groups, n = 5. All values are given as mean ± SEM.
Figure 9a Selective inhibition of nitric oxide synthesis using L-NAME showed that $K_{Ca}$ channel function is impaired in FHR, and in gonadectomized rats fed with fructose. Relaxation responses to ACh were estimated after incubating with $10^{-6}$ M L-NAME (20 minutes) and precontracting with ED70 PE. * $P<0.05$ gonadectomized (G) vs control (C), fructose-fed (F) and gonadectomized-fructose fed (GF); all groups, $n = 5$. All values are given as mean ± SEM.

Figure 9b Area under the curve for selective inhibition of nitric oxide synthesis using L-NAME in gonadectomized rats fed with fructose. * $P<0.05$ gonadectomized (G) vs control (C), fructose-fed (F) and gonadectomized-fructose fed (GF); all groups, $n = 5$. All values are given as mean ± SEM.
Table 3: \( pD_2 \) and \( R_{Max} \) values upon inhibition of \( K_{Ca} \) channels (n=5)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>F</th>
<th>G</th>
<th>GF</th>
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<tbody>
<tr>
<td>( pD_2 )</td>
<td>6.27 ± 0.11</td>
<td>6.02 ± 0.15*</td>
<td>6.8 ± 0.21</td>
<td>6.53 ± 0.23</td>
</tr>
<tr>
<td>( R_{Max} )</td>
<td>48.3 ± 3.5</td>
<td>30.3 ± 6.6**</td>
<td>52.8 ± 5.6</td>
<td>51.8 ± 7.2</td>
</tr>
</tbody>
</table>

* P<0.05 F vs G  
** P<0.05 F vs C, G and GF

Table 4: \( pD_2 \) and \( R_{Max} \) values upon inhibition of NO synthesis (n=5)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>F</th>
<th>G</th>
<th>GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>( pD_2 )</td>
<td>6.46 ± 0.19</td>
<td>5.88 ± 0.27</td>
<td>6.50 ± 0.19</td>
<td>6.52 ± 0.05</td>
</tr>
<tr>
<td>( R_{Max} )</td>
<td>43.5 ± 9.10</td>
<td>18.2 ± 6.0</td>
<td>69.6 ± 6.7*</td>
<td>37.3 ± 7.8</td>
</tr>
</tbody>
</table>

*P<0.05 G vs C, F and GF
Fig. 10 a-d: Comparison of area under the curve (AUC) in individual study groups, i.e. C, F, G and GF. In all graphs, relaxation to ACh in the absence of inhibitors CTX+APA or L-NAME is higher than the relaxation in their presence. In all the groups, * P<0.05 (PE+ACh) vs (CTX+APA) and (L-NAME); In GF, @ P<0.05 (CTX+APA) vs L-NAME). All groups, n = 5. All values are given as mean ± SEM.
Table 5: PLASMA NITRITE & NITRATE LEVELS (n=6) (All values are given as mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>F</th>
<th>G</th>
<th>GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA NITRATE &amp; NITRITE (NOx) LEVELS (µM)</td>
<td>28.75 ± 1.97</td>
<td>17.77 ± 3.55</td>
<td>34.90 ± 4.89</td>
<td>28.83 ± 6.10</td>
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</table>

Figure 11: Plasma insulin levels were the highest in F, GF and GFT compared to C and G. Testosterone implantation in gonadectomized rats subsequent to fructose feeding. * P<0.05 sham-operated fructose-fed (F), gonadectomized fructose-fed (GF) & gonadectomized fructose-fed treated with testosterone (GFT) vs. sham-operated normal chow-fed (C), & gonadectomized normal chow-fed (G). All groups, n=5. All values are given as mean ± SEM.
Figure 12 Plasma glucose levels were unchanged in all the groups C, F, G, GF and GFT. Testosterone implantation in gonadectomized rats subsequent to fructose feeding. All groups, n=5. All values are given as mean ± SEM.

Figure 13 Insulin sensitivity index (ISI) for control (C), fructose-fed (F), gonadectomized (G), gonadectomized fructose fed (GF) and gonadectomized fructose testosterone-implanted fed (GF) rats. Treatment with testosterone did not change the ISI in fructose-fed rats (GFE) compared to F. * P<0.05 F, GF & GFT vs C & G All values are given as mean ± SEM.
Figure 14 The fall in blood pressure induced by gonadectomy was reversed subsequent to implantation with testosterone and fructose feeding (GFT). Fructose diet in the presence of testosterone caused a rise in the BP of male rats (F, n=5) and GFT (n=8). * P<0.05 F (in white) and GFT (in grey) vs. C, G and GF. All values are given as mean ± SEM.

Figure 15 Body weights at termination. Estrogen-treated gonadectomized rats that were fructose-fed (GFE) weighed less compared to untreated controls (C, F & G) throughout the study. * P<0.05 GFE vs. C, F and G. All values are given as mean ± SEM.
Figure 16 Plasma insulin levels were the highest in sham-operated high fructose-fed (F) compared to sham-operated normal chow-fed (C, n=5), gonadectomized normal chow-fed (G, n=4) and gonadectomized fructose-fed, treated with estrogen (GFE). Estrogen decreased plasma insulin levels (see inset) in GFE (n=7), as compared to F, (n=5). * P<0.05 F vs. C, G & GFE. All values are given as mean ± SEM.

Figure 17 Plasma glucose levels were decreased in GFE compared to C, F and G. Estrogen decreased plasma glucose levels (see inset) in gonadectomized rats subsequent to fructose feeding. * P<0.05 GFE vs C, F and G. All values are given as mean ± SEM.
Figure 18 Insulin sensitivity index (ISI) for control (C, n=5), fructose-fed (F, n=5) gonadectomized (G, n=4) and gonadectomized fructose fed plus estrogen (GFE, n=7) rats. Estrogen increased the ISI in gonadectomized fructose-fed rats (GFE) compared to F. * P<0.05 F vs C, G & GFE All values are given as mean ± SEM.

Figure 19 Implantation of estrogen for 2 weeks in gonadectomized male rats (GFE, n=7) did not affect blood pressure. Fructose diet caused a rise in the BP of sham-operated male rats (F, n=5). * P<0.05 F vs. C, G, GFE and GFT. All values are given as mean ± SEM.
TABLE 6: PLASMA TESTOSTERONE LEVELS (ng/ml)
(All values are given as mean ± SEM.)

<table>
<thead>
<tr>
<th>TIME (Weeks)</th>
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<th>G</th>
<th>GFE</th>
<th>GFT</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>2.56 ± 0.4</td>
<td>2.87 ± 0.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1</td>
<td>NOT ESTIMATED</td>
<td>&gt; 20*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>1.38 ± 0.4</td>
<td>2.19 ± 0.2</td>
<td>ND</td>
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<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>2.8 ± 1.3</td>
<td>3.05 ± 0.7</td>
<td>ND</td>
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<td>ND</td>
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</tbody>
</table>

- P< 0.05 GFT vs C, F, G and GFE (at the same time period)
- ND: Not detectable

TABLE 7: PLASMA ESTRADIOL LEVELS (pg/ml)
(All values are given as mean ± sem.)

<table>
<thead>
<tr>
<th>TIME (Days)</th>
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<th>F</th>
<th>G</th>
<th>GFE</th>
<th>GFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>66.7 ± 3.3</td>
<td></td>
<td></td>
<td>87.2 ± 3.9 *</td>
<td></td>
</tr>
<tr>
<td>AFTER IMPLANTATION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>90.8 ± 6.5</td>
<td>104.9 ± 2.3</td>
<td>96.6 ± 3.9</td>
<td>&gt;3000 *</td>
<td>88.3 ± 4.5</td>
</tr>
<tr>
<td>10</td>
<td>83.2 ± 2.9</td>
<td>88.7 ± 3.5</td>
<td>85.4 ± 2.9</td>
<td>2773.6 ± 91.5 *</td>
<td>92.4 ± 2.5</td>
</tr>
<tr>
<td>30</td>
<td>39.9 ± 5</td>
<td>74.9 ± 10.6</td>
<td>56.2 ± 9.6</td>
<td>161.4 ± 31.5 *</td>
<td>80.6 ± 15.6</td>
</tr>
</tbody>
</table>

* P< 0.05 GFE vs C, F, G and GFT (at the same time point)
Figure 20 Fructose feeding (F) does not alter contraction responses to phenylephrine (PE) in the mesenteric arteries. Superior mesenteric arteries were challenged with graded doses of PE ($10^{-9}$ to $10^{-4}$) control (C, n=5), fructose-fed sham-operated (F, n=5), gonadectomized (G, n=4), and gonadectomized-fructose fed + estrogen treated (GFE, n=7). All values are given as mean ± SEM.

Table 8: pD$_2$ and R$_{Max}$ values for responses to phenylephrine (PE) in gonadectomized fructose-fed rats treated with estrogen (All values are given as mean ± sem.)

<table>
<thead>
<tr>
<th></th>
<th>pD$<em>2$ &amp; R$</em>{Max}$</th>
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<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>pD$_2$</td>
<td>6.4 ± 0.15</td>
</tr>
<tr>
<td>R$_{Max}$</td>
<td>8631 ± 836</td>
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</table>
Figure 21 Fructose feeding (F) reduces relaxation to acetylcholine in the mesenteric arteries, which is improved by gonadectomy (G and GF). Estrogen implantation (GFE) did not affect the vasorelaxation induced by gonadectomy. Relaxation responses were obtained to acetylcholine (ACh, $10^{-9}$ to $10^{-4}$ M) after precontraction with ED$_{70}$ phenylephrine. * P<0.05 fructose sham-operated (F, n=5) vs. control (C, n=5), gonadectomized (G, n=4) and Gonadectomized-fructose fed and estrogen treated (GFE, n=7). All values are given as mean ± SEM.
Figure 22a Selective blockade of $K_{Ca}$ channel attenuated the relaxation in sham-operated fructose-fed rats (F, n=5) compared to C (n=5) and G (n=4), indicating reduced NO activity. Relaxation responses were obtained to acetylcholine (ACh, $10^{-9}$ to $10^{-4}$ M) after inhibiting the $K_{Ca}$ channels by incubation with $10^{-8}$ M charybdotoxin (CTX) and 0.25 μM apamin (20 minutes) and precontraction with ED$_{70}$ phenylephrine. *P< 0.05 F vs. C and G; # P<0.05 C vs. GFE. All values are given as mean ± SEM.

Figure 22b Area under the curve for selective blockade of $K_{Ca}$ channel in sham-operated and gonadectomized rats treated with estrogen and fed with fructose. *P<0.05 fructose sham-operated (F, n=5) vs. control (C, n=5) and gonadectomized (G, n=4). @ P<0.05 gonadectomized-fructose fed plus estrogen (GFE, n=7) vs. C. All values are given as mean ± SEM.
Figure 23a Selective inhibition of nitric oxide synthesis using L-NAME showed that $K_{Ca}$ channel function is impaired in FHR, which remain unchanged in fructose fed rats that were gonadectomized. Estrogen does not affect IR-induced impairment in $K_{Ca}$ channel function in males suggesting a NO-mediated pathway. Relaxation responses to ACh after incubating with $10^{-6}$ M L-NAME (20 minutes) and precontraction with ED$_{70}$ PE. * P<0.05 gonadectomized (G) and control (C) vs. fructose-fed (F) gonadectomized-fructose fed (GF) and gonadectomized-fructose fed treated with estrogen (GFE). # P<0.05 C vs. F and GFE. All values are given as mean ± SEM.

Figure 23b Area under the curve for selective inhibition of nitric oxide synthesis using L-NAME in gonadectomized rats treated with estrogen and fed with fructose. * P<0.05 gonadectomized (G, n=4) vs. control (C, n=5), fructose-fed (F, n=5) and gonadectomized-fructose fed and estrogen treated (GFE, n=7). All values are given as mean ± SEM.
Figure 24a Relaxation to ACh is unchanged in control rats by inhibition of Cyp4A enzyme using 1-aminobenzotriazole. Relaxation responses were obtained to acetylcholine (ACh, $10^{-9}$ to $10^{-4}$ M) after inhibiting Cyp4A by incubation with $2 \times 10^{-4}$ M ABT (20 minutes) and precontraction with ED$_{70}$ phenylephrine. Normal chow-fed sham-operated untreated in the absence of ABT (C, open squares) vs. C in the presence of ABT (dark triangles). (n=5). All values are given as mean ± SEM.

Figure 24b IR-induced impairment in relaxation to ACh is improved by inhibition of Cyp4A enzyme using 1-aminobenzotriazole. Relaxation responses were obtained to acetylcholine (ACh, $10^{-9}$ to $10^{-4}$ M) after inhibiting Cyp4A by incubation with $2 \times 10^{-4}$ M ABT (20 minutes) and precontraction with ED$_{70}$ phenylephrine. *P<0.05 fructose-fed sham-operated in the absence of ABT (F, open squares) vs. F in the presence of ABT (dark triangles). (n=5). All values are given as mean ± SEM.
Figure 24c Relaxation to ACh is unchanged in gonadectomized rats following inhibition of Cyp4A enzyme using 1-aminobenzotriazole. Relaxation responses were obtained to acetylcholine (ACh, 10^{-9} to 10^{-4} M) after inhibiting Cyp4A by incubation with 2 x 10^{-4} M ABT (20 minutes) and precontraction with ED_{70} phenylephrine. Gonadectomized normal chow-fed in the absence of ABT (G, open squares) vs. G in the presence of ABT- (dark triangles). (n=4). All values are given as mean ± SEM.

Figure 24d Relaxation to ACh in gonadectomized rats, which were fed on fructose and treated with estrogen. Inhibition of Cyp4A enzyme using 1-aminobenzotriazole did not significantly change the relaxation compared to values before treatment with ABT. Relaxation responses were obtained to acetylcholine (ACh, 10^{-9} to 10^{-4} M) after inhibiting Cyp4A by incubation with 2 x 10^{-4} M ABT (20 minutes) and precontraction with ED_{70} phenylephrine. Fructose-fed gonadectomized + estrogen in the absence of ABT (GFE, open squares) vs. GFE in the presence of ABT (dark triangles). (n=7). All values are given as mean ± SEM.
Figure 25 Body weights at termination. Estrogen treated normal chow-fed male rats (CE, n=10) & (FE, n=8) high fructose-fed rats weighed less compared to untreated normal chow-fed controls (C, n=9) & high fructose-fed rats (F, n=10) throughout the study. * P<0.05 C & F vs. CE and FE. All values are given as mean ± SEM.

Figure 26 Daily food consumption was higher in untreated controls (C & F) compared to estrogen treated groups (CE & FE). * P<0.05 C & F vs. CE & FE. All values are given as mean ± SEM.
Fig. 27 Plasma Insulin levels were the highest in F compared to other groups, while CE had least insulin levels. Estrogen decreased insulin levels in both the normal chow-fed male rats (CE, n=10) & high fructose-fed rats (FE, n=8) compared to untreated high fructose-fed rats (F, n=10). * P<0.05 F vs. C, CE and FE; @ P<0.05 F vs. CE. All values are given as mean ± SEM.

Fig. 28 Plasma glucose values in OGTT. No significant changes in glucose levels were observed. All values are given as mean ± SEM.
Figure. 29 Insulin sensitivity index (ISI) for control (C n=9), control-estrogen treated (CE, n=10), fructose fed (F n=10) and fructose fed estrogen treated (FE n=8) rats. Estrogen improved ISI in FE compared to F though less than CE. F had the least ISI. * P<0.05 FE vs. F; # P<0.05 FE vs. CE; @ P<0.01 CE vs. C; + P<0.001 CE vs. F; x P<0.05 F vs. C. All values are given as mean ± SEM.
Figure. 30 Systolic blood pressures for control (C n=9), control-estrogen treated (CE, n=10), fructose fed (F n=10) and fructose-fed estrogen treated (FE n=8) rats. Systolic blood pressure was measured using the tail-cuff method prior to and post-treatment. * P<0.05 FE vs. F; + P<0.01 F vs. C & CE (n = 10 in all groups). All values are given as mean ± SEM.

Table 9: Changes in plasma triglyceride (TG) levels
(All values are given as mean ± SEM.)

| PLASMA TRIGLYCERIDE (TG) LEVELS (mM) |
|---|---|---|---|
| C | CE | F | FE |
| 0.6 ± 0.1 | 0.5 ± 0.1 | 2.0 ± 0.4* | 1.6 ± 0.2* |

* P<0.0 F & FE vs C & CE
Figure 31 Plasma 17β-estradiol at week 8 in male rats treated with estrogen. * P<0.05 FE vs. F; @ P<0.01 C vs. CE; # P<0.001 C vs. F & FE; +P<0.001 CE vs. F & FE. Estrogen levels were highest in FE. All values are given as mean ± SEM.

Figure 32 Plasma testosterone at week 8 in control (C n=9), control-estrogen treated (CE, n=10), fructose fed (F n=10) and fructose fed estrogen treated (FE n=8) rats. * P<0.001 FE vs. F; #P<0.05 C vs. F & FE; @P<0.001 CE vs. F. All values are given as mean ± SEM.
Figure 33 Ratio of plasma testosterone to estrogen in control (C n=9), control-estrogen treated (CE, n=10), fructose fed (F n=10) and fructose fed estrogen treated (FE n=8) rats. * P<0.01 FE vs. F; @ P<0.01 F vs. C; # P<0.001 C vs. CE & FE. All values are given as mean ± SEM.
DISCUSSION

General Overview

Insulin resistance (IR) is the initial stage in the metabolic syndrome. The interaction of genetic and environmental factors has been suggested to induce resistance to insulin. Following insulin resistance, metabolic abnormalities such as hyperinsulinemia and elevated plasma triglycerides are observed. These effects are observed as early as 2-3 weeks in fructose hypertensive rats (FHR). Feeding a high fructose diet induces insulin resistance, hyperinsulinemia, hypertriglyceridemia and hypertension. Though unclear in humans, studies on animals and isolated cells have suggested several pathways, which may be involved in inducing insulin resistance following high fructose feeding. One theory deals with decreased 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. A second hypothesis deals with an early reduction in circulating leptin levels associated with lower insulin levels subsequent to chronic high fructose feeding. The compensatory rebound response by the endocrine system may be manifested by hyperinsulinemia and hyperleptinemia. Recent evidence by Huang et al have supported this theory, wherein male SD rats, fed with fructose for 8 weeks, developed hyperinsulinemia and hyperleptinemia. The endothelium in FHR becomes dysfunctional over 2-3 weeks secondary to insulin resistance as previously mentioned. This could be due either to the loss in endothelium-dependent relaxation or increased synthesis or responses to endothelial vasoconstrictors. Reports have been published supporting changes in the functions of both endogenous vasodilators and vasoconstrictors. It is unclear as to which is the key pathway influencing IR-induced development of endothelial dysfunction. Hypertension has been closely associated with insulin resistance. Insulin resistant rats become hypertensive over 3-7 weeks depending on the duration of fructose feeding and the animal strain. In humans as well as animal hypertensive models such as the SHR, females are less susceptible to developing hypertension compared to males. A similar profile was observed in the induction of insulin resistance, where female rats fed with fructose did not develop insulin resistance and hyperinsulinemia in comparison with age-matched males. As a consequence the association between insulin resistance and hypertension was linked to differences in gender. The importance of sex hormones in the development of hypertension was shown in ovariectomized rats, which developed insulin resistance and hypertension subsequent to feeding with...
The sex hormones estrogen$^{43,133}$ and testosterone$^{105}$ have been separately shown to prevent and permit, respectively, the development of hypertension following insulin resistance. Our laboratory recently showed that gonadectomized fructose-fed rats did not develop hypertension despite the presence of insulin resistance.$^{105}$ However, it is still unclear which specific vascular mediators are involved, and are influenced by sex hormones under normal and insulin resistant states.

In an effort to extend the understanding on the role of testosterone in mediating hypertension in FHR, we designed the present series of experiments. The objectives were:

1. To study the relationship between insulin resistance and hypertension in male fructose fed hypertensive rats. We confirmed the previous results from our laboratory demonstrating the absence of hypertension in gonadectomized insulin-resistant rats.

2. To understand the changes in the contributions of endothelial vasodilators following insulin resistance and how are they modulated by the presence or absence of testosterone in the body. Individual contributions of nitric oxide (NO) and endothelium derived hyperpolarizing factor (EDHF) to vasorelaxation were determined in intact and gonadectomized fructose-fed rats.

3. To investigate potential prohypertensive pathways, which are dependent on testosterone. Previous studies have indicated the normalization of prohypertensive arachidonate metabolites following gonadectomy.

**Effects of testosterone on insulin sensitivity and hypertension**

**Effects of testosterone on insulin sensitivity**

The male sex hormone, testosterone has been the subject of extensive research. In addition to its classical role as a steroidal sex hormone in males, testosterone is also involved in other physiological processes. Of those, 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.$^{134}$ Improving the insulin sensitivity with metformin reduced both the blood pressure and androgen levels in these patients.$^{184,185}$ Our laboratory has recently demonstrated the development of fructose-induced insulin resistance to be independent of testosterone.$^{105}$ In our experiments, we studied the effects of testosterone on insulin levels and insulin sensitivity in fructose-fed rats. In our fructose-fed
rats (F and GF), we found sustained hyperinsulinemia and attenuated insulin sensitivity as compared to normal chow-fed rats (C and G). However, all rats were normoglycemic (figure 3). Thus fructose feeding induced insulin resistance, which is a characteristic of this model. Insulin resistance was induced in both the sham-operated and the gonadectomized rats, as indicated by a decreased insulin sensitivity index (figure 4). Thus, irrespective of the presence or absence of testosterone (figure 1), male rats developed resistance to insulin. However, the effects of high concentrations of testosterone on insulin sensitivity were unclear. Supraphysiological levels of testosterone in rats were shown to depress insulin sensitivity. However in humans low-dose testosterone (replacement dosages) may increase insulin action, whereas high-dose testosterone (pharmacological dosages) appears to decrease insulin action. In our studies, plasma testosterone, regardless of its presence in physiological or supraphysiological levels (Table 6), did not affect the induction of insulin resistance subsequent to fructose feeding (figure 13). Additionally, testosterone levels were unchanged in the sham-operated groups (figure 1) whether fed on normal chow (C) or on high fructose chow (F). This indicates that fructose feeding did not affect testosterone levels. 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. In addition, rats implanted with testosterone for 2 weeks had impaired insulin sensitivity. The depression in insulin sensitivity in the presence of supraphysiological levels of testosterone (GFT) was similar to the sham-operated (F) and gonadectomized (GF) fructose-fed groups. Our results therefore show insulin sensitivity to be independent of testosterone levels in fructose-fed male rats.

**Effects of testosterone on insulin resistance-induced hypertension**

The results from our studies have reproducibly demonstrated increased blood pressure in fructose-fed rats with intact testes. In fructose-fed insulin resistant rats the presence of testosterone, whether endogenous or following implantation in gonadectomized rats permitted the development of hypertension (figures 5 and 14). These observations confirm previous results from our laboratory indicating that testosterone is associated with the development of IR-induced hypertension. As plasma testosterone levels were unchanged in the FHR compared to normal chow-fed groups, we suggest that IR-induced hypertension does not affect circulating testosterone levels. Rather the mere presence of testosterone is sufficient for inducing hypertension in fructose-fed rats. Here, we speculate 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 studies, where blocking the androgen receptor in SHR by the anti-androgen flutamide resulted in a fall in blood pressure. However, the involvement of 5-dihydrotestosterone, the active metabolite of testosterone may be ruled out as inhibiting 5α-reductase (which converts testosterone to 5-dihydrotestosterone) did not reduce hypertension in SHR.\(^{129}\) Hence, we infer that the development of hypertension follows induction of insulin resistance. Presently no studies using androgen antagonists have been reported in animals with subphysiological levels of testosterone i.e. (< 1 nM/ml). Such an experiment may reveal the presence of a threshold level for testosterone action. While most studies in whole animals support the prohypertensive role of testosterone,\(^{105,126,129}\) its hemodynamic effects have been controversial under clinical settings. In men with coronary artery disease, testosterone may prove beneficial.\(^{190}\) Alternatively, several other studies have demonstrated an increased risk of hypertensive symptoms in males with low circulating testosterone levels.\(^{120-123}\) In contrast, women with polycystic ovary syndrome are insulin resistant, hyperandrogenic and are at a higher risk for developing hypertension.\(^{134}\) Though insulin-sensitizers such as metformin have been shown to reduce blood pressure,\(^{185,191,192}\) no studies using anti-androgenic drugs have been reported. Further studies need to be conducted to clarify these ambiguities.

**Role of testosterone on vascular reactivity**

Endothelial function in isolated blood vessels is measured by the degree of relaxation produced upon precontracted tissue by incrementally graded doses of acetylcholine (ACh).

Most of the studies on hypertension and vascular reactivity have been conducted on male rats. Furthermore the effects of endogenous testosterone on endothelial function and responses to vasoconstrictors are unclear. In addition, the outcomes of gonadectomy on responses to vasoconstrictors such as PE and NE in insulin-resistant male rats are unknown. We studied the responses to PE in the isolated mesenteric arteries of control sham-operated normal chow-fed (C), sham-operated fructose-fed (F), gonadectomized normal chow-fed (G) and gonadectomized fructose-fed rats (GF). PE did not significantly affect the contraction in any of the groups (figure 6). Further, \(ED_{50}\) values were unchanged in any of the groups (Table 2). Previous studies by Verma et al have reported no difference in responses to NE in the mesenteric arteries of control
or fructose-fed insulin resistant rats. Thus our results agree with the previously reported findings.

Similarly, the effects of gonadectomy on vascular relaxation are still unclear. A study by Martinez et al reported no change in the relaxation to ACh in the aorta of gonadectomized rats as compared to intact male Wistar controls. Presently, there are no reports indicating the involvement of gonadectomy in influencing endothelial relaxation following insulin resistance. We studied the changes in vascular relaxation to ACh in the mesenteric arteries from intact and gonadectomized rats fed with fructose. On comparing the responses of the tissues to $10^{-9}$ M – $10^{-4}$ M ACh, endothelium-dependent relaxation was significantly decreased in the sham-operated fructose-fed rats (F) compared to other groups (figure 7). This is in agreement with the previous reports from our laboratory and other groups indicating depressed vasorelaxation following insulin resistance. Endothelium-dependent relaxation was as high as 85% (of the contraction value to ED70 dose of PE) in both the normal chow-fed groups, i.e. C and G. In the gonadectomized fructose-fed rats (GF), relaxation to ACh was significantly higher as compared to F and of a similar degree as C and G groups (figure 7 a-b). Thus our study is the first to report a beneficial effect of gonadectomy in the vasculature of insulin resistant rats. Our data suggest that subsequent to insulin resistance, the presence of testosterone is essential for the activation and maintenance of certain prohypertensive agents in the mesenteric arteries. Though we do not have results regarding the androgen receptor function in insulin resistance, we believe that the attenuated endothelial function in males may be due to downstream effects of testosterone receptor activation. To our knowledge, only one study has reported the induction of vasorelaxation by blocking the androgen receptor in the aorta and intestinal vessels of male Sprague-Dawley rats. We plan to examine the role of androgen receptor blockade on hypertension and associated endothelial function in our subsequent studies.

**Effect of testosterone on endothelial vasodilators**

As explained in the earlier sections, differences in gender directly influence cardiovascular function. Under normal physiological conditions, the main endothelial vasodilators, nitric oxide and EDHF are responsible for mediating vasorelaxation. Based on previous reports, which indicated an insignificant role for prostacyclin in relaxing rat mesenteric arteries, we hypothesized that NO and EDHF/KCa are the two major vasorelaxant pathways involved. Inhibiting one of them would depress responses to ACh, with the resultant relaxation reflecting
the function of the other vasodilator. Responses to both NO and EDHF are impaired in the vasculature of male rats following insulin resistance. We found impaired endothelium-dependent relaxation in the isolated mesenteric arteries of male fructose-fed rats.

We inhibited the vascular NO synthesis using L-NAME in order to determine the extent to which relaxation is EDHF-dependent. Our results from protocol # 2 and 4 demonstrate impaired relaxation to ACh in fructose-fed rats following inhibition of NO (figure 9 a-b and 23 a-b). Interestingly, L-NAME abolished the NO-dependent relaxation in the gonadectomized fructose-fed rats to a similar degree as the relaxation in sham-operated fructose fed rats. This suggests that the impairment in EDHF action secondary to insulin resistance is unaffected by gonadectomy (figure 9 a-b and 23 a-b). Further, the responses to ACh in the normal chow-fed rats (C and G) were significantly higher in the presence of L-NAME as compared to the fructose-fed rats (F and GF). Thus EDHF action in both sham-operated and gonadectomized rats was unaffected by the absence of insulin resistance. In the presence of L-NAME, tissues from G group exhibited increased relaxation at higher concentrations of ACh as compared to C, suggesting enhanced EDHF action. That insulin resistance-induced changes are responsible for the loss of EDHF action may be confirmed by treating male fructose-fed rats with insulin sensitizers such as metformin or thiazolidinediones, following which changes in vascular reactivity and EDHF action are evaluated. Our data suggest that in normal rats, the presence of testosterone reduces EDHF-dependent vasorelaxation by the action of certain testosterone-dependent agents, which are rendered ineffective following gonadectomy. However, these results do not provide information as to whether they play a role in promoting hypertension. As all the groups were matched for age prior to the study, we may rule out age as a factor.

To determine the NO-dependent relaxation, we inhibited EDHF in rat mesenteric arteries using the combination of large and intermediate Ca-sensitive potassium channel inhibitor, charybdoxin (CTX, 10^-8 M) plus small Ca-sensitive potassium channel inhibitor, apamin (APA, 0.25 μM). Loss of EDHF action was reflected by depressed ACh-evoked relaxation in all the groups, which was in agreement with previous reports. In the sham-operated fructose-fed rats, responses to ACh were significantly lower compared to the other groups. CTX+APA-induced inhibition of K_c a depressed the relaxation to a lesser degree in the gonadectomized fructose-fed group (GF) compared to F (figure 8 a-b and 22 a-b). Since EDHF action was blocked, the observed relaxation reflects a nitric oxide-dependent vasorelaxation process.
It was unknown whether gonadectomy caused a change in the levels of NO or improved responses to NO, which improved the relaxation to ACh in GF. The levels of nitrite plus nitrate (NOx) in the plasma of GF show no significant changes compared to C, F and G respectively (Table 5). Thus the gonadectomy-enhanced the vascular responses to NO as shown in figure 8a-b are not due to changes in NO levels. However, experiments need to be performed using NO donors such as sodium nitroprusside in order to determine the specific changes in the contributions of NO to vascular sensitivity in intact and gonadectomized rats.

Whether one or another vasorelaxant is more important in the mesenteric arteries of male rats is unclear. Studies in SHR have suggested identical contributions of both NO and EDHF to relaxing isolated mesenteric arteries. Our studies, in part, agree with these findings as there is a similar extent of involvement of NO and EDHF in control rats (C) (figure 10 a). Abdullah and Docherty have shown that NO-mediated relaxation is predominant over EDHF/KCa, as L-NMMA significantly inhibited the relaxation to ACh in the mesenteric arteries of male rats as compared to responses following inhibition of the KCa channels. In gonadectomized rats, our results support a preferential improvement in the NO-mediated vasodilation (figure 8, 10 c-d). This suggests that the insulin resistance-induced decrease in endothelium-dependent vasorelaxation occurs only in the presence of testosterone. This results in a shift in the balance between the actions of endothelial vasoconstrictors and vasodilators. Gonadectomy prevents this potential shift towards vasoconstrictor action by promoting NO-dependent vasorelaxation. Studies need to be carried out to determine these putative testosterone-dependent vasoactive agents and their influence on endothelial vasodilators.

**Testosterone-dependent vasoconstrictor action**

Testosterone has been implicated in promoting the action of several vasoconstrictors. In addition to reports on testosterone-dependent regulation of angiotensin-II, previous studies have strongly supported the role of testosterone as a modulator of prohypertensive metabolites of arachidonic acid. Thromboxane A2 (TXA2) and 20-HETE are the major testosterone-dependent arachidonate derivatives, that have been implicated in the induction of hypertension. Reports in cultured rat aortic smooth muscle cells have demonstrated a testosterone-dependent regulation of TXA2 receptor function. We have reported elevation of TXA2 following insulin resistance in FHR. In addition, our laboratory has recently shown upregulated mRNA levels of COX-2 in FHR, which was prevented in gonadectomized fructose-fed rats. Thus, we
believe that the induction of COX-2 in pathological states such as insulin resistance may augment the levels of intermediate endoperoxides required for the production of TXA₂.

20-Hydroxyeicosatetraenoic acid (20-HETE) is the other major vasoconstrictor, which is regulated by the presence of testosterone. A strong correlation has been established between the presence of testosterone and 20-HETE function.²⁰⁶,²⁰⁸,²⁰⁹ 20-HETE is converted to vasoconstrictor endoperoxides in the kidneys by COX-2.¹⁰⁵ Evidence supporting this interaction was demonstrated in rat aorta by Escalante et al.,¹⁰¹,¹⁹⁹ who showed that indomethacin inhibited the contraction responses to 20-HETE. Information on vascular 20-HETE activity following insulin resistance could also shed light on the decreased NO and EDHF-dependent vasorelaxation as 20-HETE has been previously shown to inhibit NO²⁰⁰,²⁰¹ and EDHF/K_{Ca}²⁰² activity in the renal vasculature. Therefore, we investigated the role of 20-HETE in depressing the relaxation to ACh in intact and gonadectomized fructose-fed rats. By using 1-aminobenzotriazole (ABT), which inhibits arachidonic acid hydroxylase, synthesis of 20-HETE was blocked. Prior to treatment, relaxation to ACh was reduced in the sham-operated fructose-fed rats (figure 7 and 21). ABT reversed this effect, resulting in a significantly higher ACh-induced relaxation (F) compared to responses prior to treatment (figure 24 b). This suggests the involvement of 20-HETE in the decreased vasorelaxation in these animals. The gonadectomized fructose-fed rats did not exhibit any change in the relaxation to ACh following treatment with ABT. Also no significant differences were observed in the C and G groups of rats (figure 24 a and c). Thus, our results suggest elevated activity of Cyp4A-catalyzed vasoconstrictors such as 20-HETE in male fructose-fed rats, which is attenuated by gonadectomy. However, this data is insufficient to conclude that 20-HETE is involved in mediating insulin resistance-induced hypertension. Additional experiments need to be carried out to determine the vascular reactivity to 20-HETE in isolated blood vessels of intact and gonadectomized male fructose-fed rats. Secondly, the protein levels of renal and vascular Cyp4A isoforms and their gene regulation need to be evaluated and quantified under the above-mentioned experimental conditions. We can get a clear picture of the contribution of 20-HETE to depressed endothelial vascular relaxation by studying the changes in blood pressure of insulin resistant mice, in which the Cyp4A12 gene has been knocked out. This is feasible as a mouse model has been developed in which the Cyp4A14 gene has been knocked out, resulting in elevated plasma 20-HETE levels and subsequently hypertension.²⁰⁶

In the present set of experiments, there is no direct evidence to suggest that 20-HETE may be involved in suppressing endothelial vasorelaxation. However, previous reports have been
indirectly supportive of a role for 20-HETE in the attenuation of endothelial vasorelaxation. Under normoinsulinemic conditions, Cyp4A expression is present in the kidney. The onset of diabetes induced renal Cyp4A while Cyp2C expression was depressed. Insulin treatment restored the normal expression of the enzymes. It could be hypothesized that a similar process may be occurring following insulin resistance, which results in the induction of Cyp4A and subsequent increase in 20-HETE function. 20-Hydroxyeicosatetraenoic acid has also been shown to inhibit NO and Kc function in the renal vasculature. In addition, vascular 20-HETE activity has been shown to be dependent on the activation of AT-II and phospholipase C (PLC). Thus, it would be interesting to study AT-II levels and changes in AT1-receptor expression subsequent to insulin resistance and how testosterone affects their potential outcomes on 20-HETE action. In addition, studies evaluating the influence of testosterone on the changes in plasma 20-HETE levels following insulin resistance and its effects on endothelial vasodilation need to be performed. As the synthesis of 20-HETE is testosterone dependent, these results will enable us to understand some of the prohypertensive pathways influenced by testosterone and how they contribute to endothelial dysfunction and hypertension following insulin resistance.

**Sex hormone regulation in the development of insulin resistance and hypertension**

Little is known regarding the metabolic and cardiovascular outcomes of hormonal balance in the body in terms of the development of insulin resistance or hypertension. This is including cases where the loss or replacement of either testosterone or estrogen in whole animals shifts the physiological equilibrium between the hormones. The existing literature supports a gender-dependent difference in the development of insulin resistance and hypertension. It should be noted that both males and females have small amounts of estrogen and testosterone respectively, which are essential for executing various physiological processes. Thus it is unclear whether insulin resistance and hypertension in males are caused by the relative absence of estrogen or the presence of testosterone. We therefore studied the effects of estrogen implantation in male rats with the following aims.

1. To study the effects of estrogen implantation on insulin resistance and hypertension in male FHR. Almost all studies on the beneficial effects of estrogen are on female rats. We were
interested in assessing the outcomes of estrogen on insulin sensitivity and hypertension in male FHR.

2. To study the effects of estrogen implantation on insulin resistance and hypertension in gonadectomized fructose-fed rats.

3. To determine the role of hormonal balance in the development of insulin resistance and hypertension in male rats. Clinical and experimental evidence suggest opposite roles for testosterone and estrogen. However, it is unclear whether it is the presence of testosterone or the absence of estrogen, which influences the development of hypertension secondary to insulin resistance.

Effects of sex hormone balance on the induction of insulin resistance and subsequent hypertension in male rats

Male fructose hypertensive rats (FHR) developed insulin resistance, which was similar to that in ovariectomized (OVX) female fructose-fed rats. In the male fructose-fed rats implanted with estrogen, insulin sensitivity was elevated (figure 29). Unpublished results from our laboratory (Song and McNeill) indicate a similar effect of estrogen in fructose-fed female OVX rats. Estrogen treatment lowered the food intake (figure 26) and consequently the weight gain in male rats (figure 25). Treatment with estrogen significantly reduced the testicle size in male rats, suggesting decreased testicular activity. In a study by Valigora et al, chronic estrone injection (an estrogen analog) over 10 weeks in SHR reduced plasma testosterone levels from 1.2 ng/ml to 0.1-0.2 ng/ml. Our results show similar reduction in the testosterone levels following estrogen treatment (figure 32). However, in both the cases, plasma testosterone was detectable and quantifiable. Estradiol levels were elevated in the estrogen-implanted rats compared to non-implanted groups (figure 31). However, we also saw an increase in the plasma estradiol levels in the unimplanted fructose-fed male rats (F) as compared to normal chow-fed rats (C). It should be noted that even this relative "increase" in estradiol was unable to prevent insulin resistance secondary to fructose feeding. This result was surprising since it was in contradiction to our hypothesis that estrogen enhances insulin sensitivity. To date, we have not found any studies, which have investigated the effects of diet-induced insulin resistance on changes in plasma estradiol levels. While we can speculate that the high concentration of fructose present in the diet affects the synthesis of estradiol from testosterone in males, we have no data concerning this issue. Additional studies are needed to look at the changes in the levels and actions of
estradiol under states of excess carbohydrate feeding. Secondly, potential changes in the bioavailability of estrogen need to be determined in the presence of chronic fructose feeding. Plasma triglycerides were unchanged in the estrogen treated and fructose fed rats (Table 9). The data agree with studies from our laboratory and other groups, which have shown unchanged or, in some cases, increased plasma triglyceride levels in the presence of estrogen, following fructose feeding.43,207

Changes in the sex hormone balance in males and females may be a predisposing factor for hypertension. In studies in our laboratory on ovariectomized rats, insulin resistance induced hypertension,133 which was reversed by estrogen. However, plasma testosterone was not measured in these studies. Previous studies have shown elevated testosterone levels in ovariectomized rats in comparison with estrous controls.141 Similar increases in testosterone levels have been reported in women suffering from PCOS134 or preeclampsia140 in addition to postmenopausal women with or without CVD.139 Reintroduction of estrogen reduced the blood pressure and androgen levels in each case. However it is unclear whether the increased estrogen levels or the concomitant decrease in testosterone mediated the fall in blood pressure. In the present study, insulin resistant male rats developed hypertension similar to OVX rats, which was reduced by estrogen (figure 30). However, the blood pressure in fructose-fed rats treated with estrogen (FE) was significantly higher than that of normal chow-fed groups (C and CE). In comparison with gonadectomized fructose-fed rats, where the blood pressure values were 100-110 mm Hg, estrogen induced a fall in the BP of intact males (125±1 mm Hg; protocol # 6), which was still higher than that observed in GF (protocols #1 and 3). These results support our hypothesis that only a complete loss of testosterone in the plasma or its action prevents the development of hypertension. In other words, even the presence of physiological levels of testosterone is sufficient for the induction of hypertension following insulin resistance. In studies on SHR, the estrone-induced decrease in testosterone to 0.1-0.2 ng/ml did not reduce the blood pressure,206 contrary to gonadectomy, which lowered the blood pressure in SHR.131 Thus the blood pressure lowering effects of estrogen in male FHR could merely be an outcome of the estrogen-induced improvement in insulin action, as it has been previously shown that improving the insulin sensitivity lowers blood pressure in both experimental35,47 and clinical setings.192,208 Studies need to be conducted to validate the importance of testosterone to the development of hypertension. One such experiment would be to treat ovariectomized (OVX) fructose-fed rats with flutamide. Since fructose induces insulin resistance and hypertension in OVX rats, concomitant blockade of the testosterone receptor could shed light on the contribution of
testosterone to hypertension. Based on these and other results, we may understand more as to how sex hormones counteract each other’s effects on the hemodynamics and vascular reactivity.

**Effects of estrogen implantation on the development of insulin resistance and hypertension in gonadectomized rats**

We conducted protocol # 3 and 4, where gonadectomized fructose-fed rats were implanted with estrogen pellets (GFE). Though basal plasma 17β-estradiol was higher in gonadectomized rats compared to sham-operated groups, fructose feeding induced insulin resistance in the gonadectomized rats (GF). Insulin sensitivity was improved (figure 18) only when supraphysiological levels of estrogen were present (Table 7). Studies on women affected with polycystic ovary syndrome (PCOS) support our results. These women who were implanted with estrogen analogs had improvement in insulin sensitivity along with reduction in testosterone levels. Furthermore, in our studies the presence of testosterone in the sham-operated rats did not affect plasma estradiol levels compared to the gonadectomized groups. Thus it could be speculated that there exists a threshold level of plasma estrogen in both males and females, below which, the rats become susceptible to developing insulin resistance. A possible second hypothesis is that the low number of estrogen receptors present in males as compared to females prevents increased estrogen action.

In the absence of testosterone, estrogen reduced the plasma insulin levels in GFE compared to GF in response to an oral glucose load (figure 16). In female rats, estrogen has previously been shown to improve insulin sensitivity. Gonadectomized rats implanted with estrogen showed no change in the blood pressure (figure 19). Confirming previous studies from our laboratory, the results from protocol # 1 and 3 have demonstrated normal blood pressure in gonadectomized rats even after fructose feeding (figures 5, 15 and 19). We suggest that estrogen does not affect the testosterone-dependent decrease in blood pressure. However, additional studies need to be conducted to verify if the vasorelaxant pathways recruited by estrogen in males are similar or different as compared to those in females.
Vascular responses in gonadectomized-estrogen treated fructose-fed rats

In our studies, vascular responses to PE were unaffected by estrogen implantation in the mesenteric arteries of gonadectomized fructose-fed rats (GFE) (figure 20). In previous reports, estrogen did not change the contractile responses to noradrenaline in the aorta of gonadectomized rats. However, no studies of a similar nature have been reported in mesenteric arteries. Estrogen is known to inhibit the synthesis and action of ET-1 in OVX rats. Additionally, endothelial NO is upregulated in the presence of estrogen. Both by direct action and indirect enhancement of insulin activity, estrogen induces increased endothelial NO. Responses to ACh were unchanged in the GFE group (figure 21). Interestingly, our results showed a decrease in the NO-dependent relaxation to ACh in the gonadectomized fructose-fed rats plus estrogen (GFE) as compared to the sham-operated chow-fed controls (C) (figure 22 a-b). We are unable to explain this effect. Immunohistochemical analysis of the vascular eNOS and relaxation responses to NO donors such as sodium nitroprusside may help us verify the present results. Taken together, the presence of estrogen in gonadectomized male rats does not significantly influence blood pressure or endothelium-dependent vasodilation. Thus, estrogen did not produce any additive effects in improving the endothelium-dependent vasorelaxation.

On the other hand, vascular K<sub>Ca</sub>-dependent relaxation was significantly impaired following fructose feeding (figure 23 a-b). Estrogen treatment did not affect this impairment. The results are partly in agreement with the hypothesis that NO is the major vasodilator in the mesenteric arteries of male rats. In a study by Cignarella et al, estrogen did not improve relaxation in the aorta of gonadectomized male rats. Our results in the mesenteric arteries are in agreement with this information. However, our results contradict the finding of impaired relaxation following gonadectomy. Treatment with the Cyp4A inhibitor, 1-aminobenzotriazole (ABT) did not alter the relaxation in the vascular tissues of GFE as compared to basal relaxation with ACh (figure 24 d). This suggests the non-involvement of estrogen in influencing vascular tone in gonadectomized rats. Additional mechanistic studies are required to assess the specific changes affecting the contribution of nitric oxide in the mesenteric arteries.
Limitations and future research directions

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 or decreasing testosterone or estrogen levels respectively.

2. We have been unable to elucidate the role of residual levels of estrogen or testosterone in the body subsequent to removal of the ovaries or testes.

3. We have only assessed the contributions of testosterone and estrogen to the development of insulin resistance and hypertension. We have not investigated the role of progesterone in influencing these effects. Nor have we investigated the involvement of 5-dihydrotestosterone (active metabolite of testosterone) in the development of insulin resistance and hypertension. However, based on previous reports, we may rule out 5-dihydrotestosterone as a potential modulator of hypertension.

4. Problems were encountered in the hormonal profiles following implantation. Rats showed discomfort at the site of implantation subsequent to introduction of the 100 mg testosterone pellet. Secondly, contrary to its claim of releasing testosterone in 60 days, all the testosterone was released from the pellet within 2 weeks. The rapid release resulted in a sharp rise and fall in testosterone over a short time. A similar profile was observed in the estrogen-implanted rats. Plasma estradiol levels were as high as 3000 pg/ml within the first ten days, but the levels were significantly decreased (161.4 ± 31.5 pg/ml) at termination (3-4 weeks post-implantation). Although we have found that the mode of blood collection, i.e. tail vein or cardiac puncture significantly affects the plasma estradiol levels, the release patterns were unpredictable. Experiments using chronic testosterone injection need to be conducted to confirm the role of testosterone in insulin resistance and hypertension.

5. Our vascular reactivity studies were relatively preliminary and exploratory in terms of mechanism. Studies on the mesenteric arteries using specific inhibitors of Cyp4A isoforms or nitric oxide signaling need to be done. Additionally, effects of sex hormones on vascular reactivity need to be studied at the protein and genetic levels. We plan to estimate the protein expression and mRNA levels in the kidney and mesenteric arteries, using western blotting and real time reverse transcriptase-polymerase chain reaction (RT-PCR) techniques respectively.
6. 20-HETE levels need to be measured in the plasma along with changes in the expression of various Cyp4A isoforms implicated in the synthesis of 20-HETE.

Caveats

- At no point in our studies do we advocate or endorse estrogen replacement therapy as beneficial in insulin resistance and hypertension, due to their long-term cardiovascular side effects.
- We also do not support manipulation of hormone levels and their action in normal men or women in order to treat insulin resistance and hypertension. Our interest lies in exploiting these hormones as a tool to investigate the pathways they affect. This could lead us to novel targets for developing antihypertensive drugs.
SUMMARY AND CONCLUSIONS

Sex hormones in insulin resistance and hypertension

1. We were interested in extending the previous findings from our laboratory with respect to the dependence of insulin resistance and hypertension on gender. Experiments were outlined in animals and isolated blood vessels to understand the involvement of testosterone in the development of hypertension following insulin resistance. In addition to confirming that gonadectomy prevents insulin resistance-induced hypertension, but not insulin resistance itself, we demonstrated that replacing the lost testosterone restores hypertension. Further we showed that the presence of testosterone during insulin resistance impairs vascular reactivity in the mesenteric arteries of male rats. Gonadectomy prevented the loss in vascular relaxation by enhancing the contribution of NO, though additional studies are required.

2. Our second part attempted to resolve which hormone; estrogen or testosterone dictates the predisposition to hypertension. In intact male fructose-fed rats, estrogen reduced high blood pressure, which was higher than gonadectomized fructose-fed rats. Plasma testosterone was reduced but not lost in the intact animals. However, the presence of estrogen in intact males reduced the blood pressure and insulin sensitivity, whereas in gonadectomized animals there was no change in the blood pressure despite improvement in insulin sensitivity. Estrogen implantation did not change the vascular reactivity in comparison with untreated gonadectomized rats. Hence we showed that estrogen might not significantly influence vascular relaxation in the absence of testosterone. But there exists a “threshold” level for estrogen, below which, it does not prevent insulin resistance and hypertension.

3. We showed that insulin resistance-induced impaired vasorelaxation in male rats involves the action of 20-HETE. Blocking its formation resulted in significant enhancement in the vasodilation, which was not seen in intact normoinsulinemic or gonadectomized rats.

Based on these results, it is concluded that indeed there is a sex hormone-dependent link in the interrelationship between hyperinsulinemia, insulin resistance and hypertension. Both males and females develop hypertension, but only in the presence of at least small amounts of testosterone. Rather than gender, we suggest the development of hypertension to be testosterone dependent (figure C). Estrogen antagonizes testosterone actions in addition to reducing its levels in the body. Further studies are required at the endocrine level to characterize how do they mutually

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regulate each other in physiological and pathophysiological states. The evidence in humans regarding the role of sex hormones in insulin resistance and hypertension can at best be described as controversial. The results of our studies are promising enough to encourage an extensive mechanistic approach so that hormone-dependent prohypertensive pathways such as increase in hydroxyl arachidonic acid derivatives may be identified. In chronic high blood pressure, tight control should be maintained over the changes in metabolic parameters. Our studies, if extrapolated to humans may result in novel target-based treatment strategies for controlling blood pressure.

Figure C: Mechanisms linking insulin resistance and hyperinsulinemia to hypertension and the potential role of sex hormones in influencing these effects. (+/-) signs in parenthesis indicate increase or decrease in effect. (?) indicates that effects of hormones (T and E) are unknown in insulin resistant conditions. (see text for details).
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