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Association of Insulin Actions with Blood Pressure in Rodent Models of Obesity and Diabetes

Margaret Ann Maher Abel
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Margaret Ann Maher Abel entitled "Association of Insulin Actions with Blood Pressure in Rodent Models of Obesity and Diabetes." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Michael B. Zemel, Major Professor

We have read this dissertation and recommend its acceptance:

Michael Karlstad, Roland Bagby, Mary Ann Handel

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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ASSOCIATION OF INSULIN ACTIONS WITH BLOOD PRESSURE IN RODENT MODELS OF OBESITY AND DIABETES

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Margaret Ann Maher Abel
May 1995

DEDICATION

In memory of my sister,

Mary Ellen Maher Reese

We can only wonder what you would have accomplished
if given the chance.

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ABSTRACT

Obesity, insulin resistance and hypertension are risk factors for cardiovascular disease which occur in association more than individually in the general population; this clustering of symptoms is called Syndrome X. The association between hypertension and insulin resistance in this syndrome has traditionally been attributed to the effect of increased circulating insulin on sympathetic nerve activity and renal sodium retention. However, it has been shown that insulin exerts a direct vasodilatory action on the vasculature, which may be impaired in insulin resistant states. Studies in our laboratory have revealed that this vasodilatory effect is, in part, due to insulin stimulation of calcium pumps involved in extrusion of calcium from the cytosol. It is reported herein that recovery from agonist-induced intracellular calcium transients is impaired in vascular smooth muscle from insulin deficient streptozotocin-induced diabetic rats and insulin resistant obese Zucker rats. The obese Zucker rat is a genetic model of obesity, insulin resistance and hypertension carrying two mutant fatty (*fa*) alleles. Siblings carrying one or zero copies of the *fa* allele have been considered lean. However, there appear to be intermediate phenotypes in heterozygous lean animals which may be sensitive to environmental manipulations. The interaction of genetics and environment is an important area for study, as many disease processes are influenced by both. Consequently, studies were conducted to assess the effects of high fat feeding on rats carrying zero, one or two copies of the *fa* allele. Results indicate the presence of gender, genotype, and diet interactions. The *fa* allele does not appear to be recessive, and male animals carrying one copy of *fa* are more susceptible to the deleterious consequences of high fat feeding than female siblings or those carrying zero copy of *fa*. Hence, the heterozygous lean Zucker rat appears to be an appropriate model to study diet-gene interactions with respect to cardiovascular risk.

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PART 1

INTRODUCTION

I. INTRODUCTION

Cardiovascular disease is a prevalent problem in industrialized societies. Several large epidemiological studies since the 1970s have shown links among risk factors of cardiovascular disease and mortality (1 - 3). In the largest study thus far, 1.8 million Norwegians exhibited a mortality curve for cardiovascular disease that was U-shaped when considering body mass index (BMI), with lowest risk at a BMI of 23 and a 2% increase in mortality with each kilogram increase in body weight (3). Data from the San Antonio Heart study showed that 287 subjects out of 2930 had high blood pressure and many of these also exhibited non-insulin dependent diabetes or impaired glucose tolerance and obesity. (4). Several other epidemiological, cross sectional and longitudinal studies demonstrate the same clustering of risk factors accompanying cardiovascular disease (3).

In 1988, Reaven described a clustering of risk factors associated with cardiovascular disease which he called Syndrome X. The risk factors included hyperlipidemia, hypertension, and hyperinsulinemia (5). Obesity, localized to the abdominal body cavity and associated with androgen levels in men and women, has also been frequently observed (6). In addition, cellular insulin resistance has been associated with non-insulin dependent diabetes mellitus (NIDDM), hyperlipidemia and hypertension (6).

The inter-relationships of these risk factors, as well as cause and effect questions, have been the focus of many recent studies. These coexisting conditions are likely influenced by both genetics and environment. The contribution to pathology of various abnormal genes has been demonstrated in both human and animal studies of obesity,

insulin resistance and hypertension (7 - 10). In addition, environmental factors such as high fat or salt diets and exercise are known to modulate one or more of the clustered risk factors (11 - 21).

Many diseases result from factors in an organisms environment, imposed on a background of genetic susceptibility. Thus, there is a continuum of pathological observations influenced by a number of genes and variable environments. The interaction of genes with environment in cardiovascular disease has been studied by few; therefore, more attention is currently being focussed on the contributions of each, and how they may be modulated together, or separately, to reduce morbidity and mortality.

The association of hypertension with insulin resistance has generally been attributed to increased sympathetic activity and renal sodium retention occurring secondary to hyperinsulinemia in obesity and NIDDM (6). However, recent studies suggest that insulin instead has a vasodilatory action which is directly impaired at the target tissue level (22 - 27). The target tissue of insulin, in this case, is not a traditionally accepted insulin effector such as skeletal muscle, adipose or liver, but is instead vascular smooth muscle.

Increased blood pressure may be caused by increased total peripheral resistance which is determined by vascular smooth muscle contraction. The state of contraction of smooth muscle is, in part, dependent on the intracellular calcium concentration (28) which has been shown in several studies to be affected by insulin (29 - 38). Insulin has been shown to play an important role in intracellular calcium regulation in erythrocytes and vascular smooth muscle cells (29 - 38). Consequently, insulin effects on vascular tone in light of variable genetic and environmental backgrounds is a rich area for study.

II. RATIONALE

It is known that insulin causes vasodilation and that insulin affects calcium metabolism in cultured vascular smooth muscle cells (VSMC). However, it is not clear if calcium metabolism is functionally altered in VSMC from insulin resistant or deficient animals. Therefore, the intracellular calcium responses of enzymatically isolated VSMC from insulin resistant (obese Zucker) and deficient (streptozotocin-induced diabetic) rats to pressor agents must be compared with VSMC from control animals. Freshly isolated cells maintain in vivo characteristics representative of the disease state which may not be present in cultured cells, however, damage may occur as a consequence of the enzymatic digestion procedure.

The interactions of genetics and environment in Syndrome X are not understood, however, there are animal models which can be used to further investigate these relationships. The obese Zucker rat (*fa/fa*) is a well known genetic model of obesity, insulin resistance and hypertension. However, it is not possible to genotype these animals and thus the effects of one mutant gene in lean heterozygote (*Fa/fa*) siblings have not been examined. The study of early development and environmental interactions with the *fa* allele must be examined in offspring from a Brown Norway/Zucker cross which can be genotyped. Studying the effects of a single loci against varied genomic background is a limitation of this model, however, statistical procedures utilized here are aimed at controlling for genetic variability by accounting for litter effects. This model may be useful for studying the relationships among obesity, insulin resistance and hypertension from the molecular level to the whole animal.

III. OBJECTIVES

The purpose of this dissertation is threefold: 1) to help elucidate the actions of insulin on vascular smooth muscle, which may alter cellular contractile activity and thus peripheral vascular resistance, in VSMC from insulin resistant (Zucker obese rats) and insulin deficient (streptozotocin-induced diabetic rats) animals; 2) to investigate the effect of dietary fat on the development of hypertension associated with obesity and insulin resistance; 3) to determine if there exists a gene dosage effect of the *fa* allele which may be modulated by environment (dietary manipulations) in a new animal model.

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PART 2

LITERATURE REVIEW

I. VASCULAR TONE AND TOTAL PERIPHERAL RESISTANCE IN HYPERTENSIVE STATES

Blood flows through the body carrying oxygen and nutrients to the tissues and carbon dioxide and wastes away from the tissues. For tissues to remain healthy, a fairly constant rate of blood flow must be maintained. According to Ohm's law:

$$Q = \frac{\text{pressure difference}}{\text{resistance}}$$

the rate of flow (Q) through any conduit is determined by the driving force (pressure difference) behind the fluid inside the conduit and the hindrance (resistance) to flow resulting from the friction of moving molecules against each other and the walls of the conduit. The length and radii of the conduits of a system and the viscosity of the fluid moving through the system are the three factors which influence resistance (1). The circulation is a closed circuit of conduits containing arteries, arterioles, capillaries, venules, and veins. The sum of the resistances of all systemic blood vessels is the total peripheral resistance. At a constant blood pressure, if the total peripheral resistance increases, then blood flow to the tissues will decrease. Therefore, compensatory increases in blood pressure must accompany increases in total peripheral resistance to maintain a constant rate of blood flow and thus homeostasis at the tissue level (2).

There are many factors which regulate the total peripheral resistance exerted by the circulatory system and manage to keep it at a level of pressure and flow which is satisfactory for tissue gas and chemical exchange. However, when regulation is dysfunctional and total peripheral resistance increases, arterial pressure must rise to

maintain tissue blood flow. The consequence of this compensatory mechanism is the disease state, hypertension (1).

A. Determinants of total peripheral resistance

From a purely physical perspective, the total peripheral resistance is determined by blood viscosity, length and ramification of the vasculature and most importantly, the vessel radii (2). The viscosity of blood in the average human being is determined primarily by the hematocrit, or proportion of red blood cells in the blood. Red blood cells typically comprise about 40% of the blood volume. In polycythemia (as occurs at high altitudes) and anemia (accompanying some nutrient deficiencies) the red blood cell concentration rises and falls, respectively and may significantly affect the resistance to blood flow. These conditions are typically acute, treatable and do not contribute to the pathogenesis of hypertension in the general population (3).

The length and ramification of the circulatory system is, at any given time, constant. Acute changes in this component are not under consideration in altering resistance, however angiogenesis does occur over the long term in some organisms for various reasons. When angiogenesis does occur, for instance with increased adiposity, capillary density is rarefied and vascular resistance appears to increase (4, 5).

The radii of the vessels of the circulatory system are not constant. Vessels undergo both acute changes in diameter (constriction and dilation) and long term changes in diameter (atherosclerotic lesions). Small decreases in the radii of resistance vessels, produce large changes in resistance because, according to Poiseuille's Law and the concept of laminar flow, resistance is inversely proportional to the fourth power of the radius (2, 6). Although larger arteries and veins contract and dilate to some extent,

the major site of regulation of total peripheral resistance in the systemic circulation is at the arteriole and metarteriole. The state of constriction and relaxation of the smooth muscle of these vessels found embedded within tissues is largely the determinant of total peripheral resistance. In addition, constriction or relaxation of selective arterioles controls resistance and thus pressure and blood flow to the tissue or organ the arteriole feeds (7). Therefore, when one tissue bed has increased resistance, another must have decreased resistance if arterial pressure is to remain constant (1).

B. Regulation of vascular resistance

The major regulating mechanism for vascular resistance is vessel diameter control (2). Small arteries and arterioles are innervated by the sympathetic nervous system originating at the vasomotor center in the medulla. There is constant tonic stimulus of contraction of the smooth muscle of the vasculature. Increased sympathetic nervous activity will result in increased constriction while reduced sympathetic input will result in dilation (8).

Another regulator of vessel diameter, intrinsic or myogenic tone in smooth muscle, in some small arteries and arterioles has been shown to exist independent of innervation or circulating factors. Cells simply depolarize and contract in response to increased intravascular pressure. The mechanism of this response is unknown, but activation of specific potassium channels (possibly by simple mechanical stretch due to pressure) that cause depolarization and calcium influx is suspected (9, 10).

Furthermore, there are numerous chemical regulators of vascular tone. Hormones, such as vasopressin, angiotensin II and epinephrine are circulating vasoconstrictors that bind to membrane receptors and, acting through cellular second

messengers, elicit excitation-contraction coupling in smooth muscle cells. Other circulating hormones, such as atrial natriuretic peptide act by similar pathways but result in relaxation and vasodilation (11).

Other chemical substances, such as arachidonate or prostaglandins, act in a paracrine or autocrine fashion and may result in contraction or relaxation by acting on membrane proteins and eliciting signal transduction (11). In addition, excessive metabolic end products or substrates (carbon dioxide and oxygen) in surrounding tissues may have a tremendous local effect on arteriolar radii and thus resistance. Hypoxic tissues vasodilate in an attempt to increase blood flow and thus oxygen delivery. These effects may be direct or due to vasodilator substances released from compromised tissues (11).

C. Derangements of vascular tone and total peripheral resistance in hypertension

The blood flow rate (cardiac output) is equal to the mean arterial pressure divided by the total peripheral resistance. Therefore, if either total peripheral resistance or cardiac output increases the arterial blood pressure increases as well (1).

Cardiac output (blood flow rate from the heart) equals the stroke volume times the heart rate. Heart rate is controlled primarily by an intrinsic rate from the sinoatrial node with further input from the autonomic nervous system, through the cardioacceleratory and inhibitory centers of the medulla. Mild tachycardia, in exercise for example, may contribute to an increased cardiac output and blood pressure, but excessive tachycardia may decrease cardiac output because there is little time for ventricular filling between beats and stroke volume is compromised (12).

Stroke volume is dependent on venous return, nervous influence on contractility, and the structural capabilities of a given heart. As reflected in the Frank-Starling mechanism, the heart has the intrinsic capability to pump, within physiological limits, the incoming blood from the venous system. Regardless of the afterload (aortic pressure) the cardiac output is dependent on the preload (end-diastolic volume) (13).

Some investigators suggest that increases in the total peripheral resistance are immediately corrected by properly functioning kidneys and thus do not lead to long term increases in blood pressure. Increased renal blood pressure results in pressure natriuresis and diuresis with subsequent decreases in blood volume and thus venous return (1). Others maintain that decreasing the extracellular volume with pharmacological agents (diuretic therapy) does not lower hypertension adequately in every individual, while those drugs which decrease total peripheral resistance (Ca^{2+} channel blockers, angiotensin converting enzyme inhibitors, and alpha-receptor antagonists) may (14). Laragh and others maintain that hypertension is clinically and endocrinologically heterogenous, and thus requires individualized pharmacological intervention. However, the common factor in all diastolic hypertensive states is vasoconstriction which may be renin-angiotensin-mediated (high renin) or sodium-volume-related (low renin) (15).

Cellular calcium abnormalities may occur in both low and high renin hypertensive states. In the low-renin type, abnormal calcium regulation between intracellular and extracellular sites may result in increased dependence of blood pressure on extracellular calcium and enhanced sensitivity to calcium channel blockers (16). In contrast, high-renin hypertensives exhibit reduced sensitivity to therapeutic treatment with calcium channel blockers, and thus calcium dysregulation occurs between the cytosol and

intracellular storage sites (16). Regardless, in both cases increases in intracellular calcium may lead to increased vasoconstriction and consequent increases in total peripheral resistance (11).

II. CELL CALCIUM AND EXCITATION-CONTRACTION COUPLING IN VASCULAR SMOOTH MUSCLE

There are many determinants of vascular smooth muscle tone. Among these, stretch of smooth muscle cells opens ionic channels, as does voltage changes on the extracellular surface. In addition, multiple chemical substances and hormones such as norepinephrine, angiotensin II and vasopressin act on cell surface receptors to cause entry of ions through specific channels or release of ions from intracellular stores. Numerous other proteins are undoubtedly involved in modulating the sensitivity to ion concentrations, although these mechanisms are not clearly understood (11).

A. Excitation contraction coupling

The excitation-contraction mechanism in smooth muscle is very different than that in skeletal or cardiac muscle. In smooth muscle increased intracellular calcium binds to calmodulin and, together, these activate a myosin light chain kinase which phosphorylates the myosin light chains in the contractile apparatus. Both heads of the myosin molecule must be phosphorylated for the molecule to have Mg^{2+}-ATPase activity. Phosphorylation of the myosin light chains apparently increases the flexibility of the myosin-actin junction allowing for actin-activated Mg^{2+}-ATPase activity. Cleavage of high energy phosphate bonds of the ATP allows contraction to occur. The myosin heads may then be dephosphorylated by a protein phosphatase. Thus, increases in cytosolic calcium, activation of myosin light chain kinase, phosphorylation of myosin, followed by interaction with actin, results in production of force and shortening. Conversely, decreases in cytosolic calcium and dephosphorylation produce relaxation (17).

Like other muscle types, vascular smooth muscle contraction is a calcium-dependent cellular event (11). However, the status of intracellular calcium is not the only determinant of contractile activity. Although poorly understood, there are many interactions between the thick (myosin) and thin (actin, tropomyosin, caldesmon and calponin) filament proteins and sarcoplasmic protein kinases and phosphatases. (18) Some of these proteins are suspected to modulate the sensitivity of the contractile apparatus to calcium by phosphorylation events. For instance, protein kinase C (which is activated by diacylglycerol in the phosphoinositol second messenger system) phosphorylates a site on the myosin light chain kinase causing a lowered affinity for calcium-calmodulin molecules and a resultant decrease in myosin light chain phosphorylation (18).

B. Calcium concentrations in the body fluids

Calcium is an essential ion in excitable cells, in addition to its second messenger actions in all cells. Therefore, the concentration gradients of calcium between extra- and intracellular spaces are tightly controlled by homeostatic mechanisms. Hypocalcemia results in tetany, while hypercalcemia results in lethargy, diuresis, arterial hypertension, and other serious symptoms, each of which can lead to death. Three main calcitrophic hormones which control the concentration of calcium are parathyroid hormone, calcitriol and calcitonin (19).

Parathyroid hormone is released from the parathyroid gland and acts, via cAMP, primarily on the bone and kidney; the primary stimulus for its release is decreased ionized calcium in plasma (20). In bone, parathyroid hormone stimulates osteoclast activity causing demineralization and release of calcium into the plasma. In the kidney,

parathyroid hormone activates 1-alpha-hydroxylase which hydroxylates carbon 25 of calcidol (1-OH-D_3) to form calcitriol (1,25-$(OH)_2$-D_3) in the proximal tubule. It also stimulates phosphodiuresis and favors the reabsorption of calcium (20).

Calcitriol is a metabolite of Vitamin D_3 which acts as a steroid hormone on the intestinal cells to increase calcium binding protein synthesis and thus calcium absorption. It also has a permissive effect with parathyroid hormone in bone. The combined actions of calcitriol and parathyroid hormone result in increased calcemia (21).

In contrast, calcitonin is produced and released by the parafollicular C cells of the thyroid when plasma calcium levels increase above normal. It acts, via cAMP, to inhibit osteoclast activity in bone and to increase calcium excretion in the kidneys. Several gastrointestinal hormone stimulate secretion of calcitonin. Maintenance of extracellular calcium levels by the aforementioned hormones is critical to the function of excitable cells (22).

Levels of these circulating endocrine factors are altered in some forms of hypertension. Parathyroid hormone levels are increased in low-renin, salt sensitive humans and animals (19). Calcitriol, in excess, appears to increase intracellular calcium and exert a pressor effect in low-renin hypertensives, as well. Thus, therapeutic efforts for increasing plasma calcium and reducing the circulating levels (dietary calcium supplementation) of these hormones are suitable for some patients (19). Again, the major factor determining vascular smooth muscle contraction is intracellular calcium levels, and the effects of calcium regulatory hormones on intracellular calcium levels are less clear than the effects on extracellular concentrations.

Cytosolic calcium concentrations in vascular smooth muscle and other cells are maintained within a rather narrow range around 10^{-7} M. Extracellular calcium

concentrations are approximately 10^{-3} M, while intracellular compartmentalized calcium concentrations are approximately 10^{-5} M. Therefore, there are concentration gradients from outside to inside the cell and from intracellular compartments to the cytosol (23). The three major components to be considered in myoplasmic regulation of cell calcium are calcium influx, extrusion and sequestration (24); and these will be discussed in detail below.

C. Mechanisms of calcium influx

Calcium entry in vascular smooth muscle has been studied both by measuring the ionic flux of the isotope ^{45}Ca and by patch clamp techniques. Calcium enters the vascular smooth muscle cell cytosol from the extracellular space by four distinct mechanisms. The leak pathway, voltage-gated channels, stretch sensitive (myogenic) and hormone-receptor operated channels are present on the plasmalemmal membrane. There are distinct differences in calcium channels in vascular smooth muscle cells compared to those in cardiac muscle cells (25).

Passive leak of calcium occurs in a unidirectional mode in the absence of depolarization, stretch or agonist stimulation. The channels responsible for this leak have not been clearly identified, but are blocked by increasing hydrogen ion concentrations or polyvalent cation addition. The leak of calcium into the cytosol is compensated by constant sequestration and extrusion so that intracellular calcium concentrations remain fairly stable, however, if these homeostatic mechanisms are altered, leak may play a role in smooth muscle activation (26).

The voltage-gated channels, which are the best understood, are members of a gene super family which includes voltage dependent sodium and potassium channels and

are inhibited with dihydropyridines and augmented by Bay K8644 (25). There appear to be distinct types of these channels in different muscle tissues; most are activated by potassium depolarization and are dependent on extracellular calcium. There is a slow, sustained, high threshold or L type current (plasma membrane dihydropyridine receptor) which is distinguished from a fast, transient, low threshold or T type current (25, 27). The evidence suggests increased activity in L type channels in hypertensive animals (27).

Activation of stretch sensitive channels may be dependent on the presence of endothelium, suggesting some intermediary substance. These are sensitive to diltiazem but not dihydropyridines. Receptor operated channels appear to be directly operated by ATP and are insensitive to dihydropyridines (11).

Calcium can also enter the cytosol through second-messenger-operated channels present on the sarcoplasmic reticulum membrane. The first channel is an inositol triphosphate (IP_3) receptor-operated channel and is inhibited by heparin (25) and may be modulated by calcium itself (26, 28). It requires the presence of guanosine triphosphate and is therefore thought to be activated through a guanosine triphosphate-binding protein (29). The ryanodine receptor, another intracellular release calcium channel, is found in smooth muscle in much lower levels than in striated muscle (25). The mode of activation for this receptor is unknown; however it does appear to be involved in calcium-stimulated calcium release (11, 26) and modulated by cADPr (28).

D. Mechanisms of calcium efflux and sequestration

Calcium is extruded from vascular smooth muscle cell cytosol to the external environment or internal compartments via active transport. Two distinct Ca^{2+}-ATPases exist on the plasmalemmal and sarcoplasmic reticulum membranes. In addition, calcium

is extruded from the cell by a Na^+-coupled exchanger on the plasma membrane a mitochondrial pump-leak activity of calcium (26).

Metabolic inhibition of smooth muscle is accompanied by increases in cytosolic calcium, as the major source of calcium efflux is a plasma membrane Ca^{2+}-ATPase. It is a 130 kilodalton protein which is stimulated by calmodulin and inhibited by vanadate. Unlike the Na^+-K^+-ATPase this pump is insensitive to ouabain. It is suspected that prostaglandins and oxytocin inhibit the activity of this pump, and thereby cause contraction (30). The mRNA of this pump is increased with treatment of smooth muscle cells with insulin (31).

Although the plasmalemmal Na^+-Ca^{2+}-exchanger has not yet been purified it is an antiport known to be dependent on Na^+ and Ca^{2+} gradients and membrane potential. The stoichiometry of the pump is $3Na^+$:Ca^{2+} in and out, respectively. Under physiological conditions this pump does not appear to be critically involved in calcium homeostasis; however, in hypertensive states it may become more important (33).

The sarcoplasmic reticulum Ca^{2+}-ATPase is a 100 kilodalton protein with 2 isoforms (11). The calcium storage capacity of the sarcoplasmic reticulum is enhanced by the calcium-binding proteins calsequestrin and calreticulin which are found on the luminal surface. Agents which inhibit the plasmamembrane Ca^{2+}-ATPase, such as cyclopiazonic acid and thapsigargin, and thereby lead to the depletion of intracellular stores, cause an increase in calcium influx from the plasmamembrane. The mechanism for this influx has not been identified, but appears to require GTP hydrolysis and some intracellular message which may be sequestered with calcium in a given compartment (28).

Calcium influx, efflux and sequestration may be influenced by a myriad of extracellular and intracellular substances, such as insulin. These factors may control the transcriptional or translational expression, activity and turnover of calcium regulating proteins and thus the contractile state of the cells.

III. RELATIONSHIPS AMONG OBESITY, INSULIN RESISTANCE AND HYPERTENSION: SYNDROME X

Obesity, insulin resistance and hypertension occur together more than individually in the general population. This is so much so that Reaven, in 1988, described a syndrome which he called "X" and included the following pathological conditions: dyslipidemia, hyperinsulinemia and hypertension (33, 34). Kaplan subsequently observed that android shaped obesity, characterized by increased visceral fat and androgen levels, was also frequently present along with these risk factors for cardiovascular disease. He termed these clustered symptoms "the deadly quartet": upper-body obesity, glucose intolerance, hyperlipidemia and hypertension (35).

The aforementioned risk factors of cardiovascular disease do not always occur together, but in various combinations (36 - 56). Thus, hypertension is part of a syndrome which includes abnormalities of carbohydrate and lipid metabolism and insulin sensitivity, sometimes independent of obesity (36, 38, 39). Several studies have shown that insulin-mediated glucose disposal is significantly reduced in both nonobese and obese subjects diagnosed with hypertension when compared to controls (36 - 38). In addition, basal insulin, total cholesterol, triglycerides and free fatty acids are significantly increased in both obese and nonobese hypertensives (36). Moreover, hyperinsulinemic euglycemic clamp studies demonstrate significant blood pressure elevations in individuals with reduced insulin sensitivity (37). Thus, insulin resistance, independent of obesity, is associated with hypertension.

Insulin resistance, with compensatory hyperinsulinemia, has been said to be the factor which links obesity and hypertension (33 - 36, 42 - 46, 49, 53, 56). Indeed,

individuals with diagnosed non-insulin-dependent diabetes (NIDDM) are frequently obese and hypertensive (35). The major cause of morbidity and mortality from NIDDM is coronary artery disease (CAD). The etiology of CAD associated with NIDDM is the focus of research in many physiology and nutrition laboratories. The perturbations present when insulin is absent or target tissues are resistant to insulin effects include acidosis, arteriosclerosis and general wasting (33 - 35). Therefore, it is important to understand the growth promoting and metabolic actions of insulin in the normal individual in comparison to those in pathological insulin states.

A. Insulin signal transduction

The insulin gene is located on the short arm of chromosome 11 in humans, encoding 3 exons with a total of 1430 base pairs (57). The biologically active insulin molecule consists of two polypeptide chains connected by two disulfide bridges. It is produced from a high molecular weight precursor, preproinsulin, and processed in the rough endoplasmic reticulum of pancreatic β cells (58). Proteolytic cleavage results in equimolar quantities of insulin and C (connecting) peptide. The bridged portion of the molecule is conserved, but the other amino acids vary considerably among different species. Circulating glucose is the major stimulus for the apparently independent processes of insulin synthesis and secretion (59).

The 120 kilobase insulin receptor gene is located on human chromosome 19 and contains coding regions for an α and β subunit as well as a signal sequence (60). The promoter region is GC-rich and lacks TATA or CAAT boxes. Three transcriptional activation sites have been identified and contain putative transcriptional activator Sp1 binding regions (61). Transcription of the insulin receptor is upregulated in part by a

glucocorticoid response element in the 5'-flanking region, and interestingly, there is a reciprocal insulin responsive element on the glucocorticoid receptor (62). Proteolytic processing of a 1382 preproreceptor results in a 731 amino acid α subunit which is external to the plasma membrane, and a 620 amino acid β subunit with 194 extracellular amino acids, 23 spanning segment amino acids and 403 cytoplasmic domain amino acids. Two α and two β subunits form disulfide linked heterotetramers which are glycosylated on the extracellular side. The cytoplasmic domain has tyrosine kinase activity and is thus the proposed "first step" in the insulin signalling cascade described below (61).

The insulin signal transduction pathway is not yet clearly defined. The multiple cellular actions of insulin in various tissues make it unlikely that there is simply one pathway or mechanism of action (62). The insulin receptor belongs to a family of tyrosine kinases which transfer the gamma phosphate of ATP to tyrosine residues on other proteins or autophosphorylate (63). Insulin simultaneously increases the phosphorylation of some proteins (ribosomal S6, ATP citrate lyase, calmodulin) and the dephosphorylation (glycogen synthase, pyruvate dehydrogenase, hormone-sensitive lipase, Glut4) of others. The protein phosphatase inhibitor okadaic acid inhibits many of the actions of insulin, yet potentiates insulin-stimulated glucose transport (64).
It is thus proposed that the tyrosine kinase activity initiated by insulin binding to the insulin receptor is the first step in a phosphorylation cascade that has the potential of affecting numerous cellular proteins (65).

Immediately following a meal, the blood glucose rises and is the main stimulus for the rapid secretion of insulin. Amino acids and gastrointestinal hormones potentiate the release of insulin to a glucose stimulus. Insulin release is a biphasic phenomenon, with preformed, stored insulin released immediately and a sustained release of freshly

synthesized insulin secreted within an hour. Insulin circulates unbound with a plasma half-life of 6 minutes and is destroyed by insulinase in the liver or kidneys (66). Steroid hormones, such as cortisol, progesterone and estrogen increase insulin production and may, in large prolonged doses, result in β cell overuse and malfunction. Growth hormone has an effect on insulin metabolism as well, possibly related to insulin-like growth factor I (IGF-I) cross reactivity with the insulin receptor (67).

B. Classical actions of insulin

Since its discovery and extraction from the pancreas by Banting and Best in 1922, insulin has been known primarily for its effects on metabolism (68). Insulin action has historically been considered pathway specific and tissue specific. Lipogenesis, glycogenesis and glucose utilization are among the traditional metabolic pathways that insulin, in concert with other chemical substances, regulates. The traditionally accepted target tissues of insulin are adipose, liver and skeletal muscle (65).

In adipose tissue, insulin increases amino acid, potassium and glucose uptake (via Glut4), lipogenesis (via activation of lipoprotein lipase and fatty acid synthase), and protein synthesis. It decreases lipolysis (via inhibition of hormone-sensitive lipase), and proteolysis. Indirectly, insulin increases the utilization of glucose as an energy-producing substrate for the rest of the body and thus acts as a "fat sparing" hormone. In the absence of insulin, an accumulation of fatty acids in the plasma results in shifting utilization of other metabolic substrates to ketone bodies. In addition, the absence of insulin's combined actions with the liver results in increased plasma lipoprotein concentrations (66).

In striated muscle insulin also increases amino acid, potassium and glucose uptake (via Glut4) and protein synthesis. In addition, it stimulates glycogen synthesis and inhibits proteolysis (66).

In the hepatocytes, insulin increases potassium uptake, glycogen, protein and fatty acid synthesis and inhibits glycogenolysis, gluconeogenesis and proteolysis. In contrast to adipose tissue and skeletal muscle, insulin does not directly stimulate glucose transport in hepatocytes. Instead there is an indirect increase in glucose transport as a result of insulin activation of glucokinase, the enzyme responsible for phosphorylation and temporary trapping of glucose in the cell. Insulin also inhibits phosphorylase and stimulates phosphofructokinase and glycogen synthase, resulting in hepatic glucose storage in the postabsorptive state (66).

The overall widely accepted actions of insulin are present in the absorptive state and lead to anabolism and storage of metabolic substrates for later periods of macronutrient deprivation. Glucagon is the counter hormone secreted by the pancreas, during fasting, and affects the liver with actions which oppose those of insulin.

C. Non-classical actions of insulin

In recent years, recognition of insulin's actions on cellular activities, other than metabolic substrate regulation, has led to a different view of the hormone. Insulin is now regarded as a growth factor which contributes to the normal growth and maturation process (68). Insulin modulates the production of lipogenic and glycolytic enzymes, as well as structural cellular proteins, possibly via protein phosphorylation cascades (65). In addition, regulation of intracellular calcium, and other ions, through transcriptional control of membrane ATPases appears to be one of insulin's important roles (69, 70).

D. Theories of insulin association with hypertension

Hypertension in insulin resistant states has generally been attributed to a compensatory increase in circulating insulin (55). The observed hyperinsulinemia may be due to increased pancreatic secretion of insulin, due to lack of negative feedback as result of failure to lower blood glucose (55, 71). Another explanation for hyperinsulinemia is impaired hepatic extraction, not pancreatic secretion, as fasting C-peptide levels are comparable in nonhypertensive versus hypertensive NIDDM subjects, while insulin is up to 70% higher in hypertensives (71, 72). Excess insulin has been shown to increase sympathetic nerve activity and renal sodium reabsorption, both of which may lead to hypertension. However, many of these effects of insulin have been demonstrated with supraphysiological doses that do not reflect the plasma insulin status of hyperinsulinemic individuals (73). In contrast, recent evidence suggests that insulin elicits a direct vasodilatory response from the vasculature (74 - 79) which is due, in part, to insulin regulation of calcium regulatory proteins (31, 80 - 91).

1. Hyperinsulinemia and sympathetic activation

Hyperinsulinemia, with or without obesity, has been associated with hypertension in several studies (33 - 36, 42 - 46, 49, 53, 56, 73, 92 - 97). While metabolic responses to insulin in target tissues are abnormal in insulin resistant states, it appears that insulin sensitivity of the sympathetic nervous system remains intact (98). There appears to be a fundamental relationship between macronutrient intake and sympathetic activity (93). Fasting and caloric restriction have been shown to suppress sympathetic activity in a number of tissues, such as heart and brown adipose tissue, while overfeeding stimulates sympathetic activity (93). Insulin-mediated glucose metabolism in central neurons within

the ventromedial hypothalamus is implicated in this phenomenon, as hypoglycemia, 2-deoxyglucose, and streptozotocin-induced diabetes lowers sympathetic activity. Moreover, plasma norepinephrine levels are increased with euglycemic insulin infusion in normal humans (96).

Although many studies have shown that hyperinsulinemia increases sympathetic nerve activity (92 - 97) other have not (99). Insulin injection into the hypothalamus actually decreases sympathetic nerve firing rate (99). Furthermore, few studies have found insulin to raise blood pressure when infused in normal persons or animals (73), although insulin infusion has been shown to increase blood pressure in some rat strains (97). Conversely, high blood pressures are sustained under ganglionic blockade in hyperinsulinemic, insulin resistant Zucker obese rats (100).

In normal humans, insulin infusion has been shown to exert a vasodilatory effect with decreases in vascular resistance and blood pressure (74, 76 - 77). It has been suggested then that hyperinsulinemia may only cause increases in blood pressure in insulin resistant subjects, due to selective tissue resistance. In other words, the vasodilatory actions of insulin are resistant, while the kidney and sympathetic nervous system remain responsive (73). In support of this notion, insulin increases skeletal muscle blood flow and decreases local vascular resistance more than it increases systemic vascular resistance; these effects are blunted in obesity.

The paradoxical results of insulin effects on sympathetic activity and blood pressure may represent species differences (73), as well as environmental versus genetic etiology (93). It is clear that insulin increases circulating catecholamines in many species (93). However, the role of hyperinsulinemia and insulin activation of sympathetic nerves in hypertension has not been established. It is possible that impairment of insulin's

vasodilatory actions in insulin resistance may be permissive to insulin's stimulation of sympathetic activity.

2. Renal Sodium Retention

Insulin has been shown to have an antinatriuretic effect on the kidney in nonphysiological concentrations in normal, obese and diabetic individuals (69, 73, 101 - 103). In addition, physiological hyperinsulinemia induced during oral glucose tolerance tests results in reduction of sodium excretion in both normo- and hypertensives (101). It is not yet clear whether insulin exerts a greater antinatriuretic effect in hypertensives, independent of hyperinsulinemia.

Renal blood flow and glomerular filtration rate remain relatively normal in obese hypertensives, but increase during development of obesity-induced dog models of hypertension (73). However, there is a parallel shift in the pressure natriuresis curve so that sodium balance is achieved at a higher blood pressure (73). The increase in sodium and chloride reabsorption appears to be a defect somewhere past the proximal tubule (103). Interestingly, plasma renin activity in obese hypertensives and the dog model of obesity induced hypertension is elevated, despite a decrease in sodium excretion and expansion of the extracellular fluid volume. It has been suggested that the chloride concentration at the macula densa is decreased, causing renin release by juxtaglomerular cells and thus failure to suppress angiotensin II formation (73, 103). If this is the case, the sodium and chloride reabsorption must occur in the loop of Henle sections and may be related to increased interstitial pressures compressing both the loop and vasa recta, thereby slowing tubular flow rate and allowing more time for sodium reabsorption in these sections (73).

The cause of increased sodium reabsorption and a shift in the pressure natriuresis in kidneys of obese hypertensives remains unclear. Theoretically, it could be altered renal hemodynamics that go unrecognized because the kidney adjusts in the long term, tubular resistance to insulin actions, or as Hall et al. suggest, a structurally mediated decrease in medullary flow rates (73, 103).

3. Cation transport

The role of ions in regulating insulin actions, and conversely insulin regulating ion transport has been the focus of several recent studies (31, 69, 80 - 91, 104 - 107). Inheritance of abnormal calcium regulatory mechanisms presents strong evidence that cation transport in at least some hypertensives is a primary problem. Platelets from offspring of hypertensives exhibit higher baseline calcium levels than controls. (105) In addition, erythrocyte membrane calcium ATPase activity in offspring with hypertension in their background is lower (106).

There is a well documented relationship between blood pressure and ion concentration homeostasis of sodium and calcium, as the intracellular concentration of both cations is increased in hypertensive subjects (70, 73). It is estimated that 50% of hypertensives are salt sensitive. Acute administration of insulin increases renal sodium reabsorption (69). When persons with genetic predisposition for hypertension consumed high salt diets, those that developed hypertension had higher platelet calcium levels and lower plasma ionized calcium than those that remained normotensive (105). The regulation of these ions may be related through the renin-angiotensin system, with concurrent alterations of both sodium and calcium excretion (104). There is evidence

that suggests that renal handling of calcium is impaired in hypertensives with hypercalciuria observed in particular in low renin conditions. (107)

Zemel and others have reported decreased calcium ATPase and associated increases in erythrocyte calcium content in elderly and diabetic hypertensives when compared to non-diabetic hypertensives and age matched controls (80, 81, 87). However, other ions were not significantly different between groups (81). Furthermore, insulin has been found to increase ^{45}Ca efflux in aorta from normal rats in a dose-dependent fashion (88). In precontracted endothelial-vascular strips, insulin increased the rate of relaxation, but not in the presence of a calcium ATPase inhibitor (88). The decrease in vascular reactivity of normal tissues with insulin treatment is associated with a decreased intracellular calcium responses to various vasoactive agonists.

In cultured rat vascular smooth muscle cells (A7r5), insulin incubation resulted in increased expression of plasma membrane and sarcoplasmic reticulum calcium ATPases (31). In addition, the rate of recovery to baseline calcium following agonist stimulation is markedly increased in insulin treated cells (86, 91). Inhibition of agonist-stimulated contraction of primary cultured vascular smooth muscle cells also occurs with exposure to physiological doses of insulin (89).

A series of studies using the insulin resistant obese Zucker rat as a model for NIDDM associated with hypertension have further elucidated the role of insulin in vascular smooth muscle calcium regulation, vascular reactivity and blood pressure (83). Obese Zucker rats consistently have higher conscious blood pressures than lean controls (83, 87). In addition, aortic strips from obese animals exhibit exaggerated vascular reactivity to agonists and depolarization compared to strips from lean controls (87). Erythrocyte calcium ATPase activity and calcium content are decreased (83) similar to

human diabetic hypertensives (81). Moreover, ^{45}Ca efflux in aortic vascular smooth muscle is significantly reduced in obese versus lean animals (83). Rats made insulin deficient with streptozotocin injection also exhibit hypertension during stress. Moreover aorta from these animals are more reactive to vasoactive agonists and exhibit delayed ^{45}Ca efflux (84, 90).

All in vitro effects of insulin observed in the above studies, and by others, require 1-2 hours of incubation and are inhibitable by treatment with cyclohexamide, a protein synthesis inhibitor (85). This strongly suggests that insulin regulation of intracellular calcium is at either the transcriptional or translational level.

Calcium appears to play some role in the more classical actions of insulin signalling as well. Youn et al. observed that subcontractile increases in intracellular calcium increased glucose transport in skeletal muscle (108). In contrast, Draznin et al. found that large increases in intracellular calcium resulted in decreased glucose transport in adipose tissues (109). However, the same group later described a bell-shaped relationship of glucose transport with increasing intracellular calcium concentrations. It appears that the optimal range for calcium stimulation of transport was 140-370 nM (110).

The relationship of intracellular calcium and insulin signal transduction in glucose transport in vascular smooth muscle has been studied in our laboratory. We have demonstrated that the insulin responsive glucose-transporter GLUT4, is present and that insulin stimulated glucose transport occurs in vascular smooth muscle (111). In addition, data from this laboratory links phosphorylation of glucose and a non-metabolizable glucose analogue to insulin regulation of calcium metabolism. Incubation of cells with glucose or a phosphorylated analogue in combination with insulin resulted in increased

expression of plasma-membrane and sarcoplasmic reticulum calcium ATPases, and increased extrusion of cytosolic calcium following agonist induced transients. These results do not occur in glucose-free media or with an analogue which is merely transported into the cell and not phosphorylated (112).

Considering the above evidence, hypertension seen in insulin resistant states may result from decreased expression of these calcium pumps. This may lead to an overall increase in the concentrations of intracellular calcium in vascular smooth muscle and the observation of increased vascular reactivity to vasoactive agents (83).

Other less studied explanations for the associations among obesity, insulin resistance and hypertension, have recently been recognized or have not received as much attention (4, 5, 73). Rarefaction of the vasculature with increasing tissue mass, as in obesity, has been proposed to result in insulin resistance from a prereceptor standpoint (5). Decreased density of tissue feeding vessels results in inadequate perfusion of nutrients to the tissues as well as increased total peripheral resistance (4, 5). However, this theory does not adequately explain the relationship of insulin resistance and hypertension when obesity is not present.

E. Pharmacological evidence

There is quite a bit of pharmacological evidence that insulin resistance and hypertension are related, and that calcium is involved, but cause and effect remain unclear (113 - 119). It is quite a challenge to treat the diabetic hypertensive, as many older hypertensive medications have diabetogenic effects (113). Thiazide diuretics, commonly used in volume expanded hypertensives, increase blood glucose and cholesterol. In addition, they decrease potassium and may increase the incidence of

impotence, although no direct relation to coronary heart disease has been demonstrated. Beta-blockers, used to attenuate sympathetic responses, increase blood glucose and inhibit the catecholamine adjustments to insulin-induced hypoglycemia. They also may impair responses to hypoglycemic drugs and have been reported to have adverse effects on limb blood flow. Another widely used antihypertensive therapy, angiotensin converting enzyme inhibition does not appear to be diabetogenic and may actually result in increased insulin sensitivity (113).

Interestingly, the calcium channel blockers have antidiabetic effects. Nitrendipine has been shown to increase 2-deoxyglucose in adipocytes from elderly obese hypertensives (114) and diltiazem has been shown to increase insulin receptor tyrosine kinase activity (115). Furthermore, glucose-induced insulin secretion is calcium dependent, while liver mobilization of glucose is also calcium dependent (glycogenolysis and gluconeogenesis), so these drugs may reduce hyperinsulinemia and hyperglycemia along with blood pressure when used by diabetic hypertensives (116).

Conversely, some antidiabetic drugs studied produce decreases in blood pressure. Thiazoledinedione compounds, such as pioglitazone or ciglitazone have produced attenuation of blood pressure in Dahl salt sensitive, 1-kidney, 1-clip hypertensive rats and Zucker obese rats (117, 118). In addition, in cultured vascular smooth muscle cells, ciglitazone attenuated sustained platelet-derived growth factor stimulated increases in intracellular free calcium (118). Metformin a biguanide which increases insulin sensitivity, reduced blood pressure in nondiabetic lean hypertensives (119) and spontaneously hypertensive rats (120).

Thus pharmacological evidence from antihypertensive and antidiabetic medications strongly suggests a link between altered insulin states and hypertension

(113 - 120). In some studies, calcium is clearly involved in this relationship (114 - 116, 118). The effects of anti-obesity agents, such as fenfluramine, on blood pressure, insulin sensitivity and calcium regulation have not been widely studied.

F. Experimental models of obesity, insulin resistance and hypertension

In most models of experimental hypertension there is an obvious insult to the kidney affecting renal blood flow or tubular absorption (73). Compensatory changes in blood pressure result in kidney vital functions appearing normal, while cardiac and vascular abnormalities become exaggerated. Moreover, in many models of genetic or experimentally-induced hypertension, the hypertension follows the kidney in cross-transplantation studies. Thus transplantation of kidneys from a spontaneously hypertensive rat to a normotensive rat results in hypertension in the normotensive rat. Conversely, transplantation of kidneys from a normotensive rat to a spontaneously hypertensive rat results in normalization of blood pressure (121). These experiments support the kidney as the primary organ responsible for long term blood pressure control. Many of these experimental models exhibit other metabolic abnormalities related to impaired kidney function and to compensatory circulatory changes. However, they do not exhibit obesity or hyperinsulinemia and are thus inadequate models for the study of Syndrome X.

There are fewer models of experimentally induced obesity. Lesions of certain areas of the brain, such as the ventromedial hypothalamus, in the rat produce hyperphagia and obesity accompanied by hyperinsulinemia (122). This mechanism of obesity induction was shown to be dependent on neural pathways to the pancreas, suggesting autonomic nervous system and insulin involvement (123). In experiments

where two rats are parabiosed, allowing exchange of circulating factors, when one has a ventromedial hypothalamic lesion it becomes obese and the partner loses fat and lean body mass (124). Many other metabolic abnormalities accompany the obesity induced by hypothalamic lesions and appear to be secondary to the observed increase in insulin secretion (125). However, animals with ventromedial hypothalamic lesions have lowered sympathetic activity and do not appear to be hypertensive, and consequently are also inadequate models for the study of Syndrome X.

The only well known experimentally induced model for Syndrome X, which includes obesity, dyslipidemia, hypertension and hyperinsulinemia is high fat feeding. Epidemiological data show a strong correlation between body weight and blood pressure (126). There is a great deal of evidence describing this relationship, and although insulin is thought to play a role, the cause and effect relationships are not clear (33 - 36, 38, 42 - 46, 49, 53, 56, 126). In 1939, Wood and Cash, developed an experimental model of obesity-induced hypertension produced by high fat feeding in the dog (127). A recent resurgence of research in this area, by Rocchini, Hall and colleagues (128, 129) has resulted in further characterization of this model. Blood pressure increases in parallel with weight gain and is associated with tachycardia and increased cardiac output in dogs fed high fat (129). Renal blood flow, GFR and filtered sodium load are increased during the development of obesity in this model (129). Thus, a parallel shift toward higher blood pressures of the pressure natriuresis curve is observed, with no change in slope (130). Although renal changes have been the main focus of the groups studying this model, but insulin sensitivity and the effects of insulin resistance on peripheral vascular resistance have been investigated at the systemic level (131).

Obesity-induced hypertensive dogs are hyperinsulinemic and exhibit the impaired glucose tolerance characteristic of Syndrome X (131). Additional insulin infusion does not cause the typical decrease in peripheral vascular resistance observed in normal dogs (131, 132), suggesting that the vasculature of these dogs is resistant to the vasodilatory actions of insulin. Furthermore, chronic hyperinsulinemia in these animals does not further raise the blood pressure, suggesting that insulin does not increase blood pressure by increasing sympathetic output (131). Unfortunately, the manifestations of vascular insulin resistance in vitro, such as insulin's effects on intracellular calcium regulation, have not been investigated in this valuable model which mimics Syndrome X.

Unlike dogs, inbreeding of rats for medical research allows interesting observations to be made as different strains react to high fat feeding. High fat diets have been reported to increase and decrease plasma insulin in rats. Sprague Dawley rats fed high fat diets are hypoglycemic, hypoinsulinemic and exhibited lower body weights, with increased fat pads weights. In addition, fat feeding decreases adipocyte glucose transport and basal (GLUT1) and insulin stimulated (GLUT4) glucose transporter concentrations (133). There were major flaws in this study, as the energy percent of protein in the diet was significantly different (26% normal versus 15% high fat). Energy intake was comparable between both groups, but extremely high fat diets may be relatively unpalatable and induce steatorrhea (133). In another study, Sprague Dawley rats fed high dietary fat, combined with sucrose, exhibited decreased glucose tolerance and insulin sensitivity; however these effects were not attributed to high fat intake, as increased sucrose alone had the same effects. Furthermore, increased dietary fiber or exercise did not improve insulin resistance (134). In a subsequent study, skeletal muscle glucose transporter expression was elevated in high fat fed rats compared to high

carbohydrate or normally fed rats. Again, the high fat fed rats were hypoinsulinemic with lower blood glucoses than control animals. (135)

In contrast, others have found that hyperglycemia, hyperinsulinemia and weight gain accompany high fat feeding in normal rats (136 - 139). Blood pressures in Sprague Dawley rats fed high polyunsaturated fats and high saturated fat diets were increased 8% and 17%, respectively. Rats fed the saturated fat diet had fasting insulin levels 38% above control (136). Two rat strains, one that becomes obese (S 5B PL) versus one that does not (Osborne-Mendel) with high fat feeding, were compared on high fat diets and both exhibited decreased sensitivity to insulin; however, there was no significant difference between strains (138). In addition, inbred Fischer 344 female rats fed a high fat (39.5%), high sucrose (39.5%) diet for two years were heavier, insulin resistant, hyperinsulinemic, hyperlipidemic and hypertensive in comparison with siblings fed low fat (6%), complex carbohydrate diets. Viscoelasticity (a measure of clotting activity) was higher in the high fat sucrose fed rats. The risk factors clustered in most of the high fat sucrose fed animals, although not every animal exhibited increases in every risk factor. Moreover, in the low fat, complex carbohydrate group there was no aggregation of and few increases in risk factors (139).

At the molecular level, concentrations and activity of some enzymes involved in lipid biosynthesis and lipolysis appear to be directly affected by the amount of fat in the diet in rats. In Sprague Dawley rats, increased dietary fat results in liver and adipose fatty acid synthase activity suppression (140). The suppression was more exaggerated on high saturated than unsaturated fat diets, while adipose FAS was suppressed equally (140).

It appears that the genetic background of some animals, like people, make them more susceptible to deleterious consequences of high fat intake. In addition, the methodologies of studying the effects of high fat vary widely. Following is a list of questions which must be asked when comparing the literature on high fat feeding. What was the percentage of fat used? What was the composition of fats fed? Did the animals gain weight? If not, was satiety value or palatability of the diets different? Did the animals exhibit steatorrhea? Were micronutrients adjusted for caloric value per gram of diet? Was there cholesterol in the fat diet? All of these factors may dramatically change the outcomes of high fat diet studies and thus should be carefully considered.

G. Genetic influences on obesity, insulin resistance and hypertension

Causative links of both genetics and environment to obesity, hypertension, and Syndrome X have been demonstrated. From an evolutionary perspective, the thrifty genotype hypothesis maintains that the ability to store scarce fuels during periods of starvation leads to obesity, associated dyslipidemia and hypertension in "western" lifestyles (141). For instance the Pima Indians, Nauruans and Mexican Americans exhibit varying combinations of increased risk factors of Syndrome X based on a combination genetic and environmental influences (142, 143)

Syndrome X represents a complicated pathological picture. Individuals exhibiting the symptoms of Syndrome X are a heterogenous group; there are undoubtedly several genes whose mutations result in this condition. Specific candidate genes studied thus far include: apolipoprotein-D (144), lipoprotein lipase (145), low-density-lipoprotein receptor (146), insulin (147) and the insulin receptor gene (148). It should also be noted that the phenotypes expressed in many people exhibiting Syndrome X are undoubtedly

produced by a combination of expression patterns of different genes and thus may be described as quantitative traits.

1. Human pedigree and twin studies

There are well known associations of genetics and obesity in humans (149). When both parents are obese the probability of offspring obesity has been found to be as high as 80%. The probability is surely affected by environment as eating habits and activity levels are learned from a very early age in most societies (149). However, adoption studies show the weights of adopted children to be more like their biological than adoptive parents. Furthermore, comparison of over 600 pairs of identical and fraternal twins reared together or separately showed that even when reared apart, identical twins were two times as likely to have similar weights than fraternal twins (150, 151).

Most studies of human genetic transmission have used body mass index (BMI) as the determinant of obesity. Heritability levels of 20-30% for BMI in nuclear and adoptive families are described, while estimates of 60-90% heritability are observed between twins (149, 150). BMI transmission assessed across generations in a Canadian population with several types of relatives adjusted for age and gender was 35%, however, genetics could account for only 5% of transmission (149). Bone structure, percent body fat and percent body mass are not considered when using the BMI as an obesity index. A more sensitive analysis of body composition, utilizing under water weighing, performed with nine representative relations revealed a 55% transmissible effect, with 25% of the variance attributed to genetics (149).

Total body fat is a function of energy intake, energy expenditure, the thermic effect of food, and nutrient partitioning. There does not appear to be a significant genetic effect for habitual energy intake, although a 20% genetic effect on food preference for dietary fat and carbohydrate has been reported (149). Resting metabolic rate (RMR) accounts for approximately 70% of daily energy expenditure; the fat free mass is the best predictor for RMR. Residual variance after RMR is adjusted for age and gender may reflect heritable differences. The RMRs of monozygotic twins are more highly correlated than in dizygotic twins, with 40% of the variance attributable to inheritance. In the only study thus far addressing the heritability of the thermic effect of food, genetic effects were estimated to be 30% or more (149). Studies of activity level contribution to energy expenditure have not revealed significant genetic effects, as separating environmental effects has been difficult in humans. Lastly, a single report of genetic transmission of nutrient partitioning characteristics described a genetic transmission of 20% (149).

Human pedigree and twin studies of the general population examining all factors of Syndrome X have not been conducted. However, there is a heritable syndrome referred to as "familial dyslipidaemic hypertension (FDH)" which is similar to Syndrome X exhibiting characteristics of hyperlipidemia, insulin resistance, central obesity and hypertension . There appear to be several different gene effects resulting in FDH in specific subsets, but the metabolic results are similar and significantly influenced by diet and physical activity (152). Other extreme cases of insulin resistance result from single gene mutations (such as mutations in the insulin receptor) in humans, however people suffering from Type A syndrome or leprechaunism, are not likely to reproduce (153). The study of NIDDM inheritance is a more general analysis of inheritance of insulin

resistance. The concordance of dizygotic twins with NIDDM has been shown to be 25%, while concordance between monozygotic twins is reported as high as 95% (154).

Numerous studies have been done to compare the relationships between insulin sensitivity and/or blood pressure in different races. The Pima Indians are an especially interesting example of a distinct human population which exhibit obesity and insulin resistance when employing the westernized lifestyle (141). They have been shown to have higher fasting insulin and lower rates of glucose disposal with insulin infusion, although mean blood pressure was not significantly correlated with fasting plasma insulin in this population (142).

Lastly the genetic component of familial resemblance of blood pressure has been studied across different ethnicities, within and between families, in adoptive families and in twins. Whether probands are hypo-, normo- or hypertensive, blood pressures of family members are significantly more similar than by chance (155). Obesity also aggregates in families and has a marked influence on blood pressure after adolescence Studies of different ethnicities are confounded by environmental factors, such as degree of "westernized lifestyle" and socioeconomic status. These factors are accompanied by differences in the major factors for determining blood pressure: sodium intake, physical activity, obesity and psychological stress. Studies comparing the effects of African ancestry versus Caucasian ancestry on blood pressure distribution show significant associations between blood pressure and darker skin color (156, 157); however, there were no differences seen when these variables were compared in a socially homogenous community (158).

Within and between family studies have consistently shown significant correlations of blood pressure in first degree relatives approximately 0.22 for systolic

pressure and 0.18 for diastolic pressure, and support the notion that blood pressure is a quantitative trait. Siblings generally exhibit higher correlations than parents and offspring (155). Adoption studies have also supported a genetic component in the transmission of blood pressure, as there is a higher correlation between parents and natural childrens than between parents and adopted childrens blood pressures. In addition, natural siblings have higher blood pressure correlations than adoptive siblings (159). However, the correlations between adoptive or natural children within a household are higher than those between parents and natural children; thus, environmental factors clearly exert a strong influence (160). The coaggregation of weight and blood pressure within families is especially notable (159, 160).

In a comprehensive historic study of twins, Stocks (161) found correlations of 0.81 for systolic pressure in monozygotic twins, while same-sex dizygotic twins and same-sex siblings exhibited systolic pressure correlations of 0.44 and 0.45, respectively. When other correlates affecting blood pressure such as height, were accounted for, genetic heritability of blood pressure was estimated to be 20-30% (161).

There are clearly genetic components in obesity, diabetes and hypertension. Syndrome X is a newly described clustering of risk factors which encompass, in many cases, the above polygenic diseases. Confounding factors in the human population, such as household environment, socioeconomic status or psychological stress, make genetic contribution and gene-environment interactions very difficult to study. Advances in molecular biology, as well as, single or polygenic mutant animal models will allow more sensitive analysis of the physiological factors affecting body weight, insulin sensitivity and blood pressure.

2. Animal genetic models

Genetic obesity was recognized by Cuenot in 1905 in the yellow mouse (162). There are now many well known rodent models of genetic obesity; in mice and in rats. The obese phenotypes of these models differ in onset, severity, and accompanying pathologies such as insulin resistance. Chromosomal locations of seven mutants have been established, although only mouse *ob* and *yellow* have been cloned. The function of the protein products of these genes are currently under investigation. It is suspected that the primary defects in these genetic obesities lie in the central nervous system and elicit the symptoms of hyperphagia, hyperinsulinemia, autonomic outflow abnormalities and altered regulation of the hypothalamohypophysial-adrenal axis (162).

There are several genetic mouse models which may mimic Syndrome X in humans. These mouse mutants are all obese, dyslipidemic and insulin resistant. It is not currently known whether these animals exhibit hypertension. The autosomal recessive mutants are Diabetes (*db*), Obese (*ob*), Tubby (*tub*) and Fat (*fat*) loci are located on chromosomes 4, 6, 7, and 8 and share homology with regions on human chromosomes 1, 7, 11, and 16, respectively. The Yellow (A^{y}) dominant mutant is located on mouse chromosome 2, with a human homologue on chromosome 20 (163). Recent molecular advances coupled with conventional parabiosis experiments are leading to a more comprehensive understanding of genetic obesities in the mouse.

The mouse obese (*ob*) gene, whose 167 amino acid protein product (which is probably a hormone since it contains a signal sequence) regulates the size of the body's fat stores and whose mutation result in gross obesity and NIDDM, was recently cloned (164). In some mutants no protein product is formed, while in others a defective protein product is overproduced indicating interruption of feedback inhibition. The likely site of

action is hypothalamus which controls food intake and energy expenditure (122, 126, 164). The mouse gene shares 84% homology with the human gene (164). From cross-circulation experiments the protein product of *ob* is suspected to be a circulating factor. The *ob/ob* mice do not have the ability to produce a suspected satiety signal (165). These animals exhibit impaired reproductive abilities which may be related to defects imparted by the mutant alleles on steroid actions or impaired steroidal actions on allelic expression.

Mice carrying mutations of the *db* gene, which has not yet been cloned, have similar, if not identical obesity-diabetes syndromes as *ob/ob* mice. These animals fail to respond to the same satiety signal that *ob/ob* mice cannot produce (165). Fascinating parabiosis experiments have shed light on the interacting functions of the *ob* and *db* genes in mice. When an *ob/ob* mouse is parabiosed to a lean littermate the *ob/ob* mouse loses some weight but does not become lean. When a *db/db* mouse is parabiosed to a lean littermate the littermate stops eating. When an *ob/ob* mouse is parabiosed to a *db/db* mouse the *ob/ob* mouse stops eating. Apparently the *db/db* mouse secretes a satiety signal to which it cannot respond but that the *ob/ob* mouse does not produce but can respond to (165). It also suggests that the *db* protein product is the receptor through which *ob* acts (166). The *db/db* mouse does not exhibit impaired reproductive capacity as observed in the *ob/ob* mouse (162).

The viable yellow mouse (A^{vy}/-) carries a mutation near the locus of the agouti gene which results in obesity, insulin resistance and a yellow coat color. The agouti protein gene has been mapped to mouse chromosome 2 and cloned. It codes for a 131 amino acid protein with a signal sequence (166, 167). Normally, agouti is involved in determining coat color as it regulates different pigment production (eumelanin versus

phaeomelanin) in the environment of the mouse hair follicle in a paracrine fashion (166, 167). The lethal yellow mutation is the result of a 170 kilobase mutation in a gene called *Raly* which has the same transcriptional orientation as agouti and maps proximal to the agouti gene. In mutant viable yellow A^{vy} mice the agouti protein is ectopically expressed in nearly every tissue tested (167). Thus the agouti protein is active in general metabolism only when mutated in the mouse and produces marked, obesity, insulin resistance, impaired responses of adrenergic receptors and tumor production. Interestingly, recent studies in our laboratory indicate that ectopic expression of the agouti protein may result in altered intracellular calcium metabolism in skeletal muscle, which may be a manifestation of insulin resistance (168).

The human homologue for the agouti protein has recently been cloned and is located on human chromosome 20q11.2; it exhibits 80% homology to the mouse agouti gene. The human agouti gene produces a 132 amino acid protein with a signal sequence and is closely linked to *MODY* (maturity onset diabetes of the young) which is normally active in adipocytes and testis, not muscle or liver (167).

Although it is easier and less costly to study genetic factors in smaller rodents, the study of physiological processes is typically easier in larger animals. There are several genetic rat models available for the study of genetic obesity, insulin resistance or hypertension. The Zucker obese rat is by far the most studied rat model which approximates Syndrome X in humans. This double mutant for the fatty (*fa*) allele exhibits many if not all of the symptoms associated with syndrome X; they are hyperlipidemic, hyperinsulinemic, hypertensive and obese (169 - 173).

The location of the fatty gene is on rat chromosome 5 (174). The gene is flanked by the interferon alpha (*Ifa*) gene and the facilitative glucose transporter (*Glut1*),

similar to the *db* gene loci on mouse chromosome 4. *Ifa* and *Glut1* are linked to *fa* in the gene order *Ifa-fa-Glut1*; the same occurs in the *db* mouse (*Ifa-db-Glut1*), suggesting that *db* and *fa* are mutant alleles of a homologous gene. (174) Therefore, we may speculate that the *db* and *fa* may code for the receptor of the protein encoded by *ob*.

Attempts to characterize the development of obesity and insulin resistance have led to the following pathological time line. Obesity is present in obese Zucker rats at age 3 to 5 weeks when compared to lean (*Fa/Fa*) siblings (175). Hyperinsulinemia appears as early as 3 to 4 weeks (169), with increased insulin concentration and glucose stimulated release (172). Hypertriglyceridemia and glucose intolerance are present by age 11 to 13 weeks (169, 176). While some investigators have found hyperglycemia in obese Zucker rats, others have not (177, 178). Nonetheless, skeletal muscle glucose transport is lower in obese animals (179 - 181). Obese Zucker rats are also hyperphagic to a described "breakpoint", which may be reached with the maximal possible size of each individual animal. The magnitude and duration of hyperphagia is influenced by dietary components; animals on a high fat diet reach this breakpoint sooner (182).

The obese Zucker rat has been extensively studied as a model of hypertension. Blood pressure studies of obese versus lean Zucker rats are conflicting; however, with direct arterial measurements in conscious, unrestrained animals it has been consistently demonstrated that obese Zucker rats are hypertensive by as early as six weeks of age (83, 87, 100, 170, 183 - 185). Renal damage is present by at least three months of age, with proteinuria, glomerular mesangial matrix expansion and increased number of glomeruli with hypertrophy. However, GFR is still normal at this age. Hypertension and hyperlipidemia precede the overt renal damage and may both contribute to glomerulosclerosis and proteinuria (170).

Obese Zucker rats exhibit abnormal sympathoadrenal function and abnormal plasma catecholamines (170). It has been shown that obesity in the Zucker rat is in part reversible upon adrenalectomy. Readministration of glucocorticoids restores the metabolic defects which lead to obesity (177). Therefore there may exist steroid responsive elements in the sequences controlling the expression of *fa* which are more or less regulated as a result of the fatty mutation.

Obese Zucker rats have increased number and size of adipocytes. Fatty acid synthesis is highest in young fatty rats and gradually declines until there are no differences between obese and lean siblings. There is increased activity of adipocyte malic enzyme, acetyl-CoA carboxylase, 6 phosphogluconate dehydrogenase and fatty acid desaturase when obese animals are not food restricted. The increases in these enzymes levels or activities accompany an increased rate of lipogenesis (177).

The homozygote-wild type and the heterozygote siblings of obese animals have been considered lean, thus the *fa* allele has been considered recessive (174). However, several investigators have shown that there are phenotypically intermediate lean animals which may be representative of animals carrying one copy of the *fa* allele (186). Establishment of a Brown Norway/Zucker cross allows identification of polymorphic genetic markers tightly linked to the *fa* mutation (<0.5 cM from *fa*) (187). Offspring of these crosses can be used from a very early age to determine the effects of carrying zero, one or two copies of the *fa* allele (186).

H. Dietary influences on obesity, insulin resistance and hypertension

It is well known that environmental activities such as eating and exercise play a crucial role in determining body weight, blood pressure, and insulin sensitivity despite an

organism's genetic background (129, 130, 134 - 136, 188 - 194). Numerous studies have looked at the relationship of carbohydrate or fat feeding and various vitamins and minerals on the risk factors of cardiovascular disease. The contents of several nutrients are exaggerated in urbanized diets and have received attention in the studies of obesity, insulin resistance or hypertension. For instance, calcium is typically reduced (194) while sodium and fat are in excess (21, 190, 194). Studying the relationship of these nutrients to disease in human beings is difficult due to the colinearity of nutrients, dietary form and inadequate assessment techniques (190).

Louis K. Dahl recognized that genetic, as well as environmental factors such as weight gain, contributed to variations in observed blood pressure responses to salt intake. (129). He discovered a rat model (Dahl salt sensitive or salt resistant) of salt-resistance/salt sensitivity and showed in cross-transplantation studies that salt sensitivity leading to hypertension followed the kidney. However, the contribution of sodium intake to the clustering of other risk factors of cardiovascular disease has been studied by few. As previously discussed, in humans and animals acute administration of insulin increases renal sodium reabsorption (69, 73). Conversely, there appears to be a relationship of sodium intake with insulin sensitivity. Donovan et al. reported significant decrements in insulin sensitivity in normal subjects on high sodium diets (191). One suspected mechanism underlying the decrease in insulin sensitivity with high sodium intake is a sodium-mediated increase in free fatty acids and resultant substrate competition with down regulation of glycolytic enzymes (191).

The role of dietary calcium in hypertension has also been studied intensively. In a recent review, metanalysis of several studies showed reduced dietary calcium intake is frequently associated with human and animal hypertension. In general it appears that

calcium supplementation results in a prophylactic effect on the development of hypertension in animals and humans, but has not yet been shown to have therapeutic effects on already established high blood pressure (190). The relationship among dietary calcium, calcemia and hypertension seems paradoxical. While dietary supplementation of calcium seems to lower blood pressure, both hypocalcemia and hypercalcemia have been observed in hypertensive states. Calcitrophic hormones are also altered in hypertensives with increases in parathyroid hormone and decreases in calcitriol present. Epidemiological data supports a positive relationship among serum calcium and blood pressure and negative relationship between calcium intake and blood pressure (19, 190).

Weight gain has been recognized as a contributor to essential hypertension in both animal models and humans (55, 56, 73). The association of increased blood pressure with aging is highly correlated with weight gain. Weight loss can reduce blood pressure independently of sodium intake (73, 129) Furthermore, weight gain is also highly associated with the development of insulin resistance and hyperinsulinemia, although insulin resistance occurs in many lean hypertensives as well (55).

The incidence of cardiovascular disease is highly associated with diets that are high in fats, particularly saturated fats (136). As previously discussed, high fat feeding, with or without weight gain, has been shown to induce or exaggerate hypertension in both normal and obese animal models (129, 136, 139). High levels of dietary fat also contribute to the development of obesity, atherosclerosis, ischemic heart disease and various cancers. Reduced fat diets alone and coupled with exercise have been shown to dramatically reduce the risk factors of Syndrome X in humans (192).

Studying the associations of high fat diets with cardiovascular risks in human populations can be highly problematic. For instance, vegetarians have frequently been

compared to omnivores, as they tend to eat less fat, particularly saturated fats. Seventh Day Adventists, who are predominantly lacto-ovo-vegetarians, have a very low incidence of morbidity and mortality from cardiovascular diseases. However, Seventh Day Adventists beliefs cause them to abstain from many other potentially harmful substances such as alcohol (195). Rouse et al. got around this problem by comparing Adventist vegetarians to Mormon omnivores whose religious habits also eliminated alcohol, caffeine and other confounding foods and beverages. Adventists had significantly lower blood pressures, total fat intake, saturated fat intake and cholesterol than the Mormons (196). In general most studies indicate that decreasing dietary fat in humans results in a decrease in blood pressure (197).

Fewer studies are available which study the relationship of dietary fat with other risk factors of Syndrome X. In the San Luis Valley Diabetes Study, subjects which exhibited impaired glucose tolerance were asked to report 24-hour recall of their diet at baseline and one to three years after. The people who went on to develop NIDDM had the highest dietary fat intake, followed by those who remained glucose intolerant. The group which reverted to normal glucose tolerance had the lowest fat intake of all. An average 40g/day increase in dietary fat was associated with 3.4 fold risk of developing NIDDM (193). In one of the few dietary fat intervention studies in humans, Barnard et al. studied NIDDM, insulin-resistant and normal subjects for three weeks on a low-fat (10%; polyunsaturated /saturated fat ratio = 1.24) and treadmill exercise regimen. All groups exhibited significant decreases in cardiovascular risk factors, and the NIDDM group had the greatest changes. Insulin, blood pressure, triglycerides and BMI were reduced; thus it is difficult to tell whether these positive effects were due to weight loss, decreased dietary fat, increased activity level or a combination of all three (192).

In reviewing studies of the effects of high fat diet on cardiovascular risk factors, the quality of fat in the diets must be scrutinized (198). In a recent review, Iacono and Dougherty reported results from 18 studies with varying increases in the polyunsaturated fat linoleic acid. Twelve studies showed significant decreases in blood pressure with linoleic acid addition or vegetarian diet in normotensive individuals, while 4 showed lowered blood pressures in hypertensive individuals (198). Linoleic acid an omega-6 (18:2) fatty acid, found in seed oils, is the precursor of arachidonic acid which is the precursor for the 2-series prostaglandins. Formation of different prostaglandins is enzyme and tissue specific. With increased percentage of linoleic acid of total fat intake, increased prostacyclin is produced in the kidney, and salt and water excretion increases leading to lowered blood pressures. In addition, prostacyclin acts as a vasodilator, and may thereby decrease total peripheral resistance. However, increased arachidonic acid also increases thromboxane A_2 (a vasoconstrictor) production.

Saturated fat intake has been found to be the most important predictor of plasma cholesterol. Pronczuk et al. have recently described the variations in gerbil plasma cholesterol caused by different dietary fat compositions to be similar to that found in cebus monkeys and humans (199). The most important dietary fat determinant of increased cholesterolemia is myristic acid intake (14:0). Consistent with evidence discussed previously, dietary 18:2 decreased cholesterolemia. It was concluded that dietary 14:0 and 18:2 are the fatty acids which modulate plasma cholesterol during low cholesterol intakes. Therefore diets high in 14:0 may lead to increased cardiovascular risk, while diets high in 18:2 may reduce cardiovascular risk (199)

As discussed before, it is difficult to tease apart the genetic and environmental factors that contribute to cardiovascular disease, but epidemiological, intervention and

controlled animal studies have increased our understanding of both. The physiological responses of various animal species to high fat feeding were discussed previously in the section of this dissertation addressing experimental models of obesity, insulin resistance and hypertension.

I. Diet and gene interactions in obesity, insulin resistance and hypertension

Genetics and diet have been shown to interact in obesity and hypertension in humans (155). Individuals with familial dyslipidemia hypertension, who are genetically susceptible to coronary heart disease, experience earlier onset of hypertension and dyslipidemia with high fat diets (200). However there are few human studies which clearly assess the contributions of genetic and environmental factors (155, 193, 198).

As already established, the obese Zucker rat is a genetic model of syndrome X (169 - 185). While the double mutant is the genetic extreme there still appears to be some environmental modulation of the phenotype (201 - 208). Hypertensive Zucker obese rats exhibit an increased pressor effect to a relatively high salt diet (4g/100g), they do not show a decreased pressor effect from a relatively sodium-restricted (0.26g/100g) diet (203). In addition, lean Zucker rats seem relatively resistant to high fat diets, while obese Zucker rats appear sensitive to high fat feeding (202). However, obese Zucker rats fed an energy restricted diet, nonetheless, exhibit an obese body composition (201). Bray et al. showed that weight gain in both lean and obese Zucker rats was accelerated by high fat feeding. In addition, adrenalectomy attenuated dietary fat induced weight gain in lean and obese animals. Increased lipoprotein lipase activity observed in lean animals was not observed in obese animals fed high fat. Hence, genotype, high fat and adrenalectomy were found to interact in the development of obesity in Zucker rats (207).

Molecular studies indicate that development of lipogenesis, hepatic fatty acid synthesis and enzymatic activities are higher in obese Zucker rats compared to lean *Fa/?* rats on any diet. High fat feeding resulted in higher proportions of large-type adipocytes in both phenotypes (206). Penicaud et al. found that expression of fatty acid synthase, acetyl-CoA carboxylase and Glut4 were all increased in obese versus lean rats fed high fat diets. In contrast, high fat feeding in some studies of obese animals has been shown to reduce insulin-responsive glucose transporter protein (Glut 4) and glucose transport in skeletal muscle and adipose tissue (135). While liver fatty acid synthase expression and activity appear to be suppressed with high fat feeding in normal rats (140), this suppression does not occur in genetically obese rats (209). In addition, adipsin, a serine protease which may play a role in food intake regulation and is secreted by adipocytes is suppressed in *ob/ob* and *db/db* mice, Zucker obese rats but not in JCR:LA*cp* obese rats (another rat model of genetic obesity) and may be normalized with high fat feeding (142). The results of these and other studies clearly support a gene-diet interaction in the Zucker obese rat. Consequently, further studies employing the use of genotyping methods (187) may allow a more sensitive analysis of these interactions in animals which carry one mutant allele (186).

In conclusion, there are associations among obesity, insulin resistance and hypertension in both humans and animals. Hypertension in insulin resistant states may be due to some combination of hyperinsulinemia, increased sympathetic activity, renal sodium reabsorption or defective cation transport in vascular tissues. The clustering of these risk factors present in Syndrome X are clearly influenced by both genetics and environment. While the study of gene-environment interactions in humans is difficult,

animal models, such as Zucker rats, afford the unique opportunity to assess the contribution of both, in a more controlled environment.

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PART 3

EXPERIMENTAL INVESTIGATIONS OF RELATIONSHIPS AMONG OBESITY, INSULIN RESISTANCE AND HYPERTENSION

I. RECOVERY TO BASELINE INTRACELLULAR CALCIUM IS IMPAIRED IN FRESHLY ISOLATED VASCULAR SMOOTH MUSCLE CELLS FROM STREPTOZOTOCIN DIABETIC RATS[1]

A. Abstract

We have recently reported that insulin accelerates the rate of intracellular Ca^{2+} ($[Ca^{2+}]_i$) recovery (i.e. return to baseline) following agonist stimulation in cultured vascular smooth muscle cells (VSMC). Accordingly, we predicted that impaired cellular responses to insulin, whether due to insulinopenia or insulin resistance, would result in a delay in $[Ca^{2+}]_i$ recovery following agonist stimulation, and that such an impairment may contribute to exaggerated vascular resistance and reactivity in diabetic states. Consequently, we have evaluated the effects of insulinopenia on the ability of VSMCs to recover from angiotensin II (AII)-induced Ca^{2+} loads. Male Sprague-Dawley rats were injected with either vehicle or streptozotocin (STZ; 65 mg/kg I.V.) to induce insulinopenia, and sacrificed two weeks later for evaluation. Thoracic aortae were immediately removed, cleaned, denuded of endothelium, and enzymatically digested to release VSMCs. Freshly dispersed cells were then washed, counted, loaded with 10 μM Fura-2-AM, and Ca^{2+} responses to and rate of recovery from AII (200 nM) were fluorometrically studied in stirred suspensions (10^6 cells/mL). VSMCs from the STZ ($n = 5$) and control ($n = 8$) groups exhibited similar baseline $[Ca^{2+}]_i$ levels as well as

[1]This manuscript has been submitted for publication in similar form with co-author Michael B. Zemel.

comparable peak responses to AII. However, the STZ VSMCs exhibited a marked delay in the rate of $[Ca^{2+}]_i$ recovery to baseline following AII stimulation (2.4±1.2 nM/min vs. 8.4±1.2 nM/min in VSMCs from STZ and control rats, respectively; $p < 0.03$). These data support the concept that inability of insulin to stimulate $[Ca^{2+}]_i$ recovery in insulinopenia may contribute to exaggerated vasoconstrictor responses in diabetic states.

B. Introduction

Individuals with both insulinopenia and insulin resistance exhibit increased risk of high blood pressure, although the pathogenesis of hypertension in these diabetic states differs (1). Overt hypertension in Type I diabetes generally develops after the onset of renal disease; however, there is evidence suggesting that exaggerated vascular smooth muscle reactivity may precede renal dysfunction (1). Reports of hypertension in animal models of insulinopenia have varied with animal age and with methods of induction of insulinopenia and for blood pressure measurement (2-4). It appears that directly recorded, resting, intra-arterial blood pressure is similar in streptozotocin-induced diabetic rats and controls. In contrast, STZ diabetic animals restrained for tail-cuff blood pressure measurement exhibit higher blood pressures than controls (2). Increased vascular sensitivity to catecholamines is observed in chemically-induced diabetic rats as early as 5 weeks after β cell destruction (5). Impairment of acetylcholine-induced relaxation in streptozotocin-treated rats has also been reported (5).

The increase in blood pressure in insulin resistance has been attributed to high circulating levels of insulin and resultant increases in both sympathetic tone and renal sodium retention (1). However, we have recently reported that hypertension in insulin resistance states is maintained under ganglionic blockade and is, therefore, independent

of sympathetic neural support (6). In addition, we and others have recently shown insulin to attenuate intracellular calcium and vasoconstrictor responses to pressor agonists in both animal models of hypertension and humans (7 - 14). Chronic insulin infusion in dogs (13, 14) and in normal humans results in decreased peripheral vascular resistance and blood pressure (8, 9). Accordingly, we have proposed that a vascular smooth muscle manifestation of insulin resistance, rather than hyperinsulinemia, may be responsible for the increased peripheral vascular resistance characteristic of insulin resistant states. This would suggest that hypertension and exaggerated pressor responses in insulinopenic states may result from loss of insulin-induced vasodilation.

Previous studies in this laboratory have shown that cultured vascular smooth muscle cells incubated with insulin exhibit a more rapid recovery from agonist-induced calcium loads than control cells (10). Further, freshly isolated vascular smooth muscle cells from insulin resistant Zucker obese rats exhibited significantly slower intracellular calcium recovery rates than those from the normal Zucker lean rats (15). Accordingly, the objective of the present study was to determine whether freshly isolated cells from experimentally-induced insulinopenic rats exhibited impairments of vascular smooth muscle calcium regulation as compared with control rats.

C. Materials and Methods

Male Sprague Dawley rats were fed nonpurified diet (Agway Prolab Rat Mouse Hamster 3000, Syracuse, NY) and given water ad libitum in a controlled environment (21°C) with a 12 h light:dark cycle. This study was approved by the University of Tennessee Institutional Animal Care and Use Committee.

At five weeks of age 16 rats were injected in the tail vein with 65 mg/kg streptozotocin (n = 8) or citrate buffer (n = 8) vehicle solutions in 0.1 - 0.2 ml volume, pH = 4.5. Streptozotocin is a nitrosourea which results in degranulation and selectively destroys the β cells of the pancreas. Injection of 40 - 65 mg/kg results in marked hypoinsulinemia (1) and hyperglycemia in most animals (1 - 5).

After two weeks and an overnight fast, rats were anesthetized with sodium pentobarbital (50 mg/kg), weighed and venous blood glucose was analyzed (Accu-ChekIII, Boehringer Mannheim Diagnostics, Indianapolis, IN). Vehicle-injected rats (n = 8) and streptozotocin-injected rats (only animals with fasting blood glucose greater than 300 mg/dL, n = 6) were sacrificed by aortic transection.

Thoracic aortae were cleaned in HEPES buffer (HBSS), pH 7.4, containing 138 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 4.0 mM $NaHCO_3$, 25 mM. dextrose, 6 mM glutamine, 20 mM HEPES, and 0.5% bovine serum albumin. After removing the endothelium with gentle rubbing, the vascular smooth muscle cells were released by enzymatic digestion in HBSS with 20 U/mL elastase, and 260 U/mL collagenase. After washing, VSMCs were loaded with Fura-2-AM (acetylmethoxyester, Calbiochem, San Diego, CA) dissolved in DMSO for a final concentration of 10 μM and incubated at 37°C with gentle shaking. After 20 minutes for loading and de-esterification, Fura-2 loaded cells were washed and placed in stirred suspension. Intracellular calcium was measured fluorometrically (Hitachi F-2000, Naperville, IL - Appendix A) with dual excitation (340 nm and 380 nm) and single emission (510 nm).

Angiotensin II (200 nM) was added after a stable baseline calcium was achieved, Digitonin (100 mM) and Tris-EGTA (100 mM) were added approximately 300 and 400 seconds after angiotensin II addition to obtain maximum and minimum calcium values,

respectively. Concentrations were calculated using a K_D for Fura-2 of 224 in the equation of Grynkiewicz et al. (Appendix A) (16). The rate of recovery from peak to baseline was calculated by superimposing tracings on graph paper, drawing a best fit line and determining the slope of that line.

Viable cells, as visualized with trypan blue exclusion and counted on a hemocytometer, were studied at a density of 10^6 cells/mL. One streptozotocin determination was not included due to experimenter error (the stirring mechanism was turned off). Differences between insulinopenic and control groups were assessed using the unpaired Student's t-test assuming equal variances.

D. Results

Means ± standard errors (SEM) are reported in Table 1. Initial and experiment day body weights were comparable between the two groups although the mean weight change in control was higher than STZ (91 g and 65 g, respectively) animals. Plasma glucose was 124 ± 3 mg/dL in the control rats. In the STZ group, only those animals with plasma glucose >300 mg/dL were studied. A mean plasma glucose for the diabetic rats cannot be reported, as readings above 400 are beyond the fidelity of the glucose meter. Figure 1 depicts a representative tracing of $[Ca^{2+}]_i$ response to angiotensin II in stirred VSMC suspension.

There were no differences in basal cell calcium or peak responses to AII stimulation between vascular smooth muscle cells from streptozotocin and vehicle-injected controls. However, there was a marked difference in the rate of recovery from calcium loads. Cells from insulinopenic rats were able to clear the transient calcium

Table 1. Gross physiological measurements and vascular smooth muscle cellular responses to angiotensin II from vehicle- and streptozotocin-injected male Sprague Dawley rats.

	VEHICLE	STZ
Initial weight (g)	129 ± 9	142 ± 13
Experimental weight (g)	221 ± 9	206 ± 9
Blood glucose[1] (mg/dL)	124 ± 8	> 300
Baseline $[Ca^{2+}]_i$ (nM)	266.2 ± 11.4	260.1 ± 19.0
Peak $[Ca^{2+}]_i$ (nM)	317.6 ± 10.6	301.5 ± 19.3
Slope of return to baseline	0.14 ± 0.02	0.04 ± 0.02*
Recovery rate[2] (nM/min)	8.4 ± 1.2	2.4 ± 1.2*

Data are expressed as mean ± SEM

[1]Glucose measurements over 400 are beyond the fidelity of the meter

[2]Recovery = rate of return to baseline $[Ca^{2+}]_i$ levels following agonist stimulation.

* $p < 0.03$.

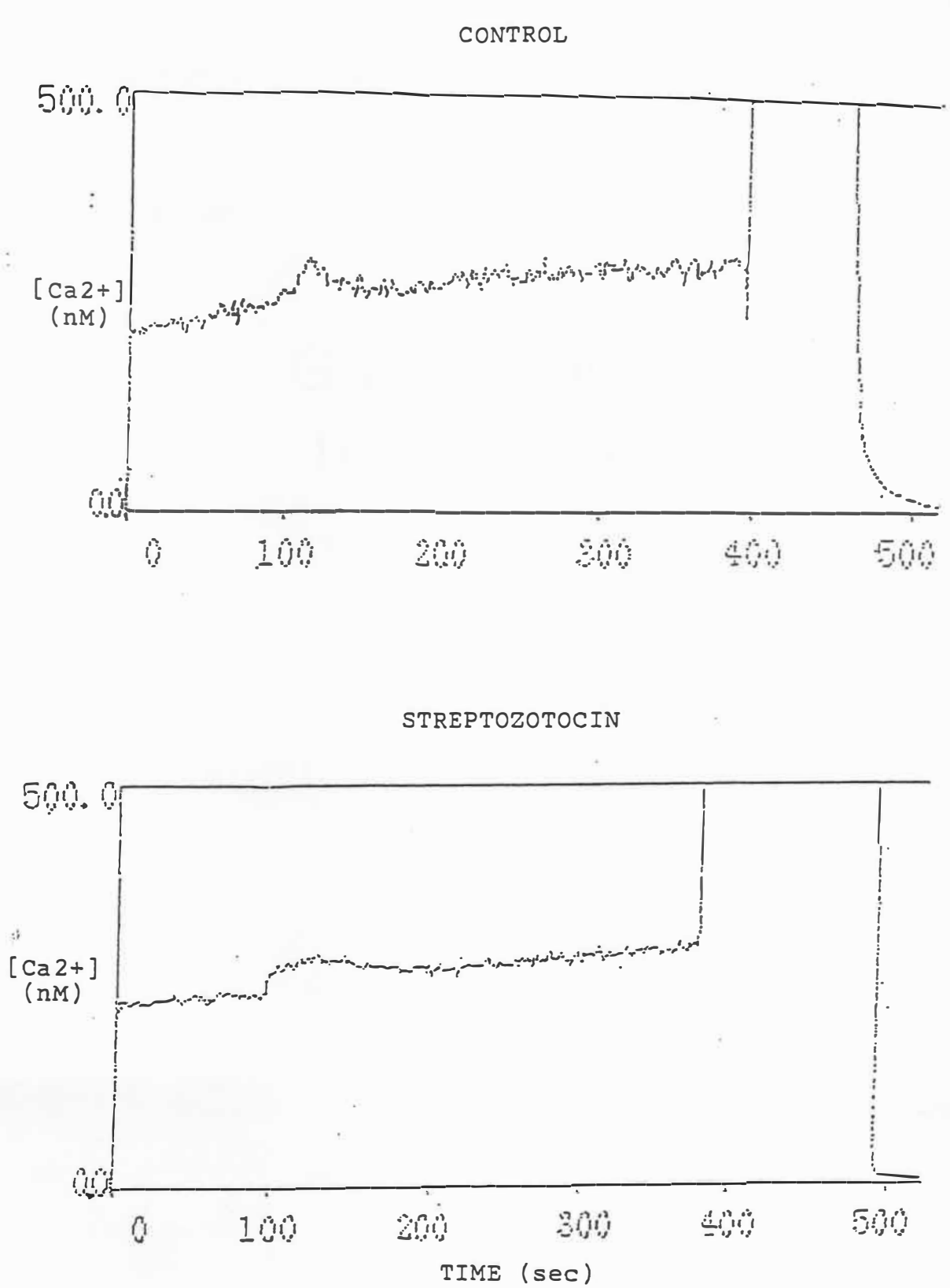

Figure 1 Representative tracings of angiotensin II stimulated $[Ca^{2+}]_i$ responses in aortic smooth muscle cells in stirred suspension. Angiotensin II was added at +100 seconds and digitonin and Tris-EGTA were added at +300 and +400 seconds, respectively.

increase at less than half the rate observed in cells from vehicle-injected controls ($p<0.03$, Table 1).

E. Discussion

Data from this study demonstrate there is an impairment in the rate of intracellular calcium recovery to baseline in isolated VSMC from insulinopenic rats compared with VSMC from vehicle-injected rats. These results are consistent with the recent report that insulin accelerates the rate of intracellular calcium recovery to baseline following agonist stimulation of cultured vascular smooth muscle cells (10). Moreover, isolated VSMC from insulin resistant obese Zucker rats exhibit similar impairments in calcium metabolism as those from STZ rats (15). The fact that this impairment is observed in VSMC from insulin deficient animals suggests that reduced VSMC responsiveness to insulin may contribute to increased intracellular Ca^{2+} and consequently, to increased vascular resistance in Type I diabetics.

It is not clear whether this impairment results from reduction of plasmalemmal calcium ATPase expression and/or activity, sarcoplasmic reticulum calcium ATPase expression and/or activity, or some other manifestation of insulin deficiency. Recent data from this laboratory has shown that insulin increases the transcription of both calcium pumps in cultured vascular smooth muscle (11). In addition, there is reduced transcription of both pumps in cells from Zucker obese (insulin resistant) compared to Zucker lean (insulin sensitive) rats (11). Erythrocyte membrane Ca^{2+}-ATPase pump activity has been shown to be decreased both in animal models of diabetes and in Type I and Type II diabetic subjects (17 - 20). Moreover, decreased $^{45}Ca^{2+}$ efflux has been demonstrated in aortae from insulin resistant and insulinopenic rats (21, 22).

The relative contributions of baseline or peak $[Ca^{2+}]_i$ response to agonists versus rate of $[Ca^{2+}]_i$ recovery to the net vasoconstrictor response of a given agonist in vivo is not clear. Although force generation by intact blood vessels generally correlates with steady state $[Ca^{2+}]_i$ levels, vascular smooth muscle is repeatedly subjected in vivo to multiple pressor stimuli. Accordingly, the true steady state $[Ca^{2+}]_i$ condition may be less relevant than the ability of the cell to mount a rapid recovery to each agonist-induced Ca^{2+} load.

We acknowledge that studying VSMCs in stirred suspension may introduce artifacts, for example, activation of stretch-dependent channels. Indeed, basal $[Ca^{2+}]$i in the present study was approximately double that observed in cultured cells (10). However, we have previously found comparable effects of insulin on VSMCs studies in suspension (10) vs. coverslips (23). Moreover, study of cells in suspension is unavoidable if comparisons of environmental manipulations (eg. STZ) are to be studied in freshly isolated VSMCs, as such non-genetic differences are unlikely to be maintained in culture (15).

It has been suggested that the increase in glucose transport observed in exercising muscle may be, in part, due to the same increased cytoplasmic calcium concentrations which allow contraction (24). Further, increased calcium in concentrations too low to bring about contraction has been shown to stimulate glucose transport in skeletal muscle (24). Adequate glycemic control using supplemental insulin has been shown to restore normal Ca^{2+} pump activity and systolic blood pressure in streptozotocin-treated rats, suggesting a contributing role of glucose in cellular calcium regulation in diabetic states (4, 7). Therefore, the independent pathways whereby insulin or exercise increase glucose transport may result from similar alterations in cytoplasmic calcium. Further, insulin

sensitivity and vascular tone would appear to be tightly linked, with an impairment in either resulting in increased cytosolic calcium and consequently, a corresponding impairment in the other.

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II. IMPAIRED RECOVERY OF VASCULAR SMOOTH MUSCLE INTRACELLULAR CALCIUM FOLLOWING AGONIST STIMULATION IN INSULIN RESISTANT (ZUCKER OBESE) RATS[1]

A. Abstract

We have previously shown insulin to attenuate vasoconstrictor responses to pressor agonists and to accelerate VSMC Ca^{2+}-ATPase mediated Ca^{2+} efflux and vascular relaxation. We have now sought to determine if VSMC from insulin resistant Zucker Obese (ZO) rats manifest exaggerated $[Ca^{2+}]_i$ responses to pressor agonists and impaired $[Ca^{2+}]_i$ recovery (rate of $[Ca^{2+}]_i$ return to baseline) compared to their lean controls (ZL). Thoracic aortae from ZO and ZL were enzymatically digested to release VSMC (n = 16 animals/group and 8 determinations using 2 aorta each/group). Freshly dispersed cells were washed, counted, loaded with Fura-2-AM, and $[Ca^{2+}]_i$ responses to and rate of recovery from angiotensin II (AII; 200 nM) and arginine vasopressin (AVP; 10 μM) were studied fluorometrically in stirred suspension (10^6 cells/mL). Peak $[Ca^{2+}]_i$ responses to AVP were not significantly different in ZO vs. ZL, while responses to AII were higher in ZL ($[Ca^{2+}]_i$ 180±7 vs. 160±4% of baseline in ZL and ZO, $p < 0.02$). Since we have recently shown insulin to increase AII-releasable Ca^{2+} stores in sarcoplasmic reticulum (unpublished data), this increase in peak $[Ca^{2+}]_i$ response to AII in ZL may reflect relative VSMC insulin resistance in ZO. Despite their increased peak AII

[1]This manuscript has been published in similar form with co-author Michael B. Zemel in: American Journal of Hypertension 1993;6:500-504.

response, ZL exhibited a more rapid recovery from both the AII-stimulated load (recovery rate = 66.1±8.9 vs. 42.1±9.0 nM/min in ZL and ZO, $p<0.02$) and the AVP-stimulated $[Ca^{2+}]_i$ load (22.2±2.3 vs. 18.4±4.6 nM/min). Consequently, we propose that the exaggerated vasoconstrictor responses associated with insulin resistance may result, in part, from failure of insulin to stimulate VSMC Ca^{2+} pump activity and thereby accelerate recovery from $[Ca^{2+}]_i$ loads.

B. Introduction

The role of hyperinsulinemia versus that of impaired insulin action in the pathogenesis of hypertension in insulin resistance is a topic of increasing controversy. Hypertension in insulin resistant states has been attributed to selective insulin resistance (1), in which skeletal muscle and adipose tissue exhibit resistance, while renal and sympathetic nervous system responses to insulin are preserved. Accordingly, the hyperinsulinemia resulting from this selective insulin resistance is thought to result in increased sympathetic neural output and renal sodium retention and thereby increase blood pressure (2). However, a growing body of evidence suggests an alternative; that insulin exerts vasodilatory effects and that vascular smooth muscle manifestation of insulin resistance results in a partial loss of this insulin-mediated vasodilation.

Consistent with this latter concept, Hall et al. (3, 4) have reported that chronic insulin infusion in normal dogs results in decreased blood pressure and peripheral vascular resistance. Similarly, insulin infusion results in vasodilation and decreased peripheral vascular resistance in normal humans (5 - 8). Indeed, Anderson, et al. (6, 7) reported that insulin infusion during euglycemic clamp studies of both normal and hypertensive individuals resulted in decreases in peripheral vascular resistance despite

increases in muscle sympathetic nerve activity. Thus, although insulin does appear to stimulate increased sympathetic neural output, it does not appear to directly exert hypertensive effects, but instead exerts hypotensive action. Further, we have recently reported that the hypertension observed in an animal model of insulin resistance, the Zucker obese rat, can not be attributed to increased sympathetic neural output, as blood pressure differences between Zucker obese rats and lean controls were maintained under ganglionic blockade (9).

Insulin appears to exert several direct effects on vascular smooth muscle which may explain its vasodilatory actions. We have found insulin to attenuate in vitro vasoconstrictor responses to pressor agonists (10), and that this effect is blunted in vascular smooth muscle from Zucker obese rats compared to their lean controls (11). Moreover, we have found insulin to accelerate the rate of both vascular smooth muscle plasmalemmal Ca^{2+}-ATPase-mediated Ca^{2+} efflux and vascular relaxation (12, 13).

Recently, we reported that insulin accelerates the rate of intracellular Ca^{2+} ($[Ca^{2+}]_i$) recovery following stimulation of cultured vascular smooth muscle cells with angiotensin II or arginine vasopressin (14). The peak $[Ca^{2+}]_i$ responses to angiotensin II (but not arginine vasopressin) were increased in insulin-treated cells (14). We attribute the latter effect to insulin stimulation of sarcoplasmic reticulum Ca^{2+}-ATPase expression (18), resulting in increased sarcoplasmic reticulum Ca^{2+} stores to be released following angiotensin II stimulation. Nonetheless, the acceleration of Ca^{2+} recovery time resulted in a reduction in the area under the intracellular Ca^{2+} response curve. Accordingly, in the present study we sought to determine whether vascular smooth muscle cells isolated from insulin resistant (Zucker obese) hypertensive rats manifest an impairment in the ability to recover from agonist-induced intracellular Ca^{2+} loads.

C. Materials and Methods

Zucker lean and obese rats were obtained from Charles River Inc. (Wilmington, MA). The fluorescent dye Fura-2-acetylmethoxyester (Fura-2-AM) was purchased from Calbiochem (San Diego, CA) and dissolved in DMSO. Collagenase, elastase, angiotensin II (AII), arginine vasopressin (AVP) and digitonin were obtained from Sigma Chemicals (St. Louis, MO). Digitonin was prepared in distilled water and collagenase and elastase were mixed in a HEPES-buffered salt solution (HBSS) containing 138 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 4 mM $NaHCO_3$, 25 mM D-glucose, 6 mM glutamine, 20 mM HEPES and 0.5% bovine serum albumin, pH 7.4. AII and AVP were prepared in serum-free HBSS.

Male Zucker lean (n = 16) and obese (n = 16) rats were anesthetized with sodium pentobarbital (50 mg/kg) and thoracic aortas were excised. Two aortas were pooled for each replicated determination. Blood and debris were removed by rinsing with cold HBSS. The intact aortas were then digested with collagenase (260 U/mL) and elastase (20 U/mL) for 15 minutes at 37°C. The vessels were then opened longitudinally, the endothelium was removed by gentle rubbing, and the vessels were cut into fine pieces. The minced aortas were further digested in the enzymes with occasional disruption by shaking to release the smooth muscle cells. Dispersed cells were then filtered, washed with HBSS and counted. Viable cells determined by trypan blue exclusion and counted with a hemocytometer were used at an approximate density of 10^6 cells/mL.

Cell suspensions were immediately chilled on ice for at least ten minutes prior to Fura-2-AM addition for $[Ca^{2+}]_i$ measurement. Fura-2 loaded cells were incubated in the dark for 20 minutes at 37°C with shaking, washed with HBSS and resuspended immediately prior to $[Ca^{2+}]_i$ determination. Intracellular calcium was determined using

dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry (Hitachi, F-2000, Naperville, IL) essentially as previously described (15, 16) (Appendix A). However, freshly dispersed cells were studied in stirred suspension rather than cultured cells being studied in monolayer.

After a stable baseline was established, agonists (200 nM AII or 10 μM AVP) were added. Three minutes were then allowed for the cells to recover fully from the agonist-induced $[Ca^{2+}]_i$ load prior to addition of digitonin (25 μM) and Tris/EGTA (100 mM/100 mM, pH 8.7) to determine maximal and minimal calcium fluorescence ratios, respectively. The concentration of intracellular calcium was calculated by the computer internal to the fluorometer from data points collected every 0.5 seconds using the equation of Grynkiewicz et al. (Appendix A) (17).

Recovery of intracellular calcium loads from AII and AVP-induced stimulation was determined from the rate of recovery to baseline after reaching maximal agonist-induced $[Ca^{2+}]_i$ levels. All data were analyzed for differences between lean and obese rats using the unpaired Student's t-test assuming equal variances.

D. Results

Typical tracings illustrating characteristic $[Ca^{2+}]_i$ responses to AII in cells from lean and obese rats are shown in Figure 2, and the data are summarized in Table 2. Peak $[Ca^{2+}]_i$ responses to AII were greater in the lean than the obese rats ($p<0.02$). However, the rates of recovery from AII-induced $[Ca^{2+}]_i$ loads were decreased by approximately 36% in cells from obese compared with cells from lean animals. Despite the reduced peak $[Ca^{2+}]_i$ response to AII in cells from the obese animals, the prolonged recovery rate resulted in an increase in the area under the $[Ca^{2+}]_i$ response

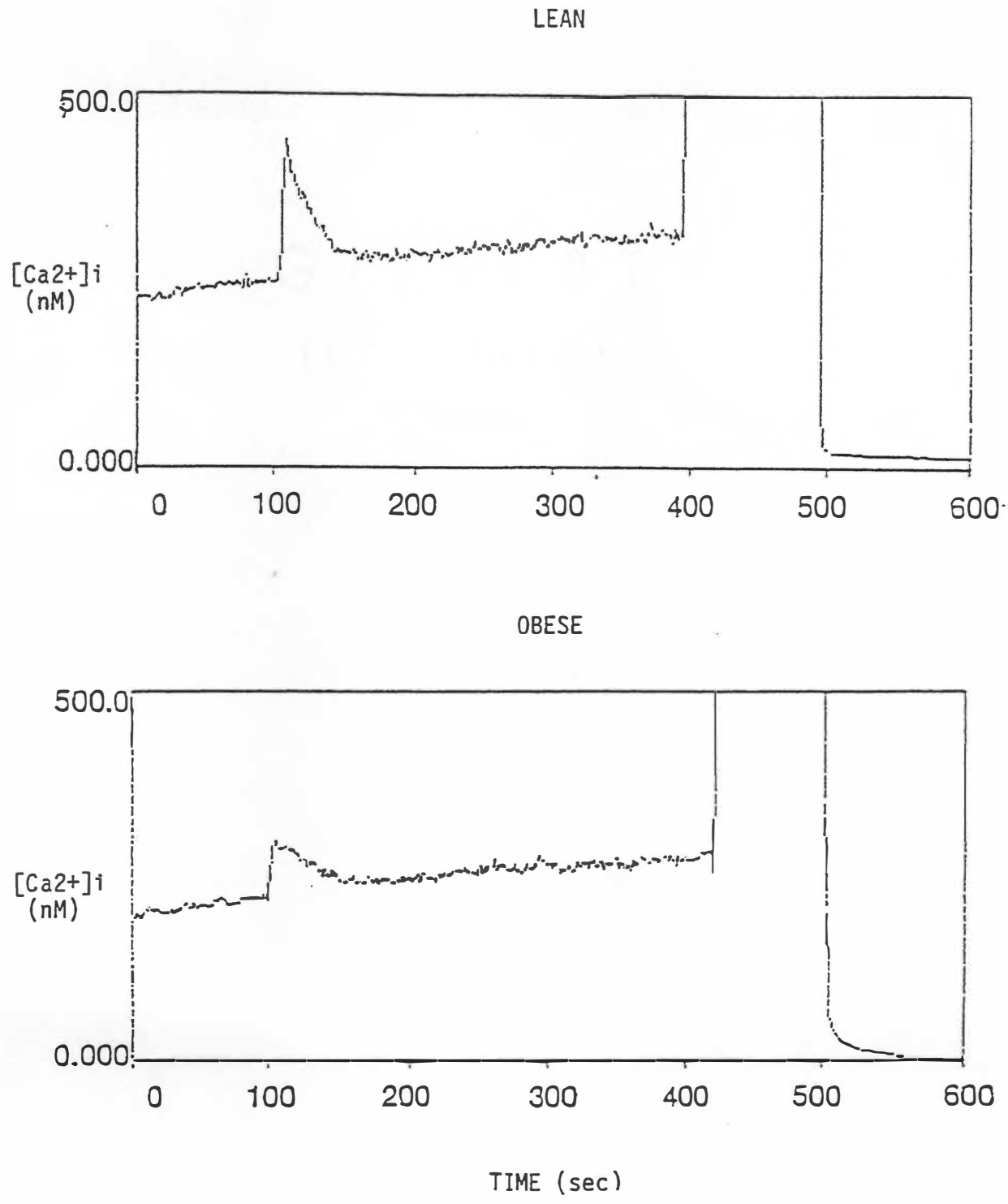

Figure 2. Characteristic tracings of the intracellular Ca^{2+} response to angiotensin II in vascular smooth muscle cells isolated from Zucker lean (upper panel) and obese (lower panel) rats. Angiotensin II was added at approximately +100 seconds followed by digitonin and Tris-EGTA at +400 and +500 seconds, respectively.

Table 2. Aortic smooth muscle cell responses to angiotensin II (AII) and arginine vasopressin (AVP) in Zucker lean and obese rats.

	Lean[1]	Obese[1]
Baseline $[Ca^{2+}]_i$ (nM)	230 ± 15	237 ± 15
Response to AII (%)[2]	180 ± 7	160 ± 4*
Response to AVP (%)[2]	151 ± 5	144 ± 5
Recovery from AII (nM/min)[3]	66.1 ± 8.9	42.1 ± 9.2*
Recovery from AVP (nM/min)[3]	21.6 ± 2.3	18.4 ± 4.6*
Area under AII Response curve (nM•sec)	17,116 ± 392	23,450 ± 228*
Area under the AVP Response curve (nM•sec)	28,890 ± 649	42,694 ± 872*

[1]Data are expressed as mean ± S.D.

[2]Response (%) = Peak/Baseline x 100

[3]Recovery = rate of return to baseline $[Ca^{2+}]_i$ levels following agonist stimulation.

*$p < 0.02$

curve. A similar decrease in the rate of $[Ca^{2+}]_i$ recovery and an increase in the area under the $[Ca^{2+}]_i$ response curve in the obese animals was observed when the cells were stimulated with AVP. Baseline $[Ca^{2+}]_i$ was not significantly different between the two groups (Table 2).

E. Discussion

Data from this study indicate that vascular smooth muscle cells from insulin resistant Zucker obese rats exhibit a decreased rate of return to baseline (i.e. recovery) from agonist-induced $[Ca^{2+}]_i$ loads. This phenomenon appears to be a vascular smooth muscle cell manifestation of insulin resistance, as we have recently reported that insulin accelerates the rate of $[Ca^{2+}]_i$ recovery in cultured rat vascular smooth muscle cells following stimulation with either AII or AVP (14). Interestingly, insulin also increased peak $[Ca^{2+}]_i$ responses to AII in the cultured cells (14), consistent with our present observation that this peak response was greater in vascular smooth muscle cells isolated from insulin sensitive (Zucker lean) rats than those isolated from insulin resistant (Zucker obese) rats.

Baseline $[Ca^{2+}]_i$ levels in the present study were somewhat higher than those reported by other investigators (14, 15). It is possible that the difference may result from studying cells which may have been traumatized by the enzymatic dispersion process used. However, this is unavoidable if freshly obtained vascular smooth muscle cells are to be studied in suspension. As an alternative, cells from lean and obese rats may be studied fluorometrically as monolayers following serial passage in culture. However, it is not clear whether true differences found in vivo would persist during

passage in cell culture, and it may be considered more likely that such differences would be evident in freshly obtained cells.

The relative contributions of peak $[Ca^{2+}]_i$ response to agonists versus rate of $[Ca^{2+}]_i$ recovery to the net vasoconstrictor response of a given agonist in vivo is not clear. Although force generation by intact blood vessels generally correlates with steady state $[Ca^{2+}]_i$ levels, vascular smooth muscle is repeatedly subjected in vivo to multiple pressor stimuli. Accordingly, the true steady state $[Ca^{2+}]_i$ condition may be less relevant than the ability of the cell to mount a rapid recovery to each agonist-induced Ca^{2+} load. In cultured cells, the integrated $[Ca^{2+}]_i$ response to AII (i.e. area under the $[Ca^{2+}]_i$ response curve) was decreased in insulin-treated cells despite the increase in peak response (14). Similarly, in the present study cells from the more insulin sensitive (lean) rats exhibited a reduced area under the AII $[Ca^{2+}]_i$ response curve compared to these from the obese rats. Cells from Zucker obese rats exhibited both a lower peak and slower $[Ca^{2+}]_i$ recovery rate, and these animals also exhibit hypertension and exaggerated in vivo and in vitro pressor responsiveness. Thus, it may be considered likely that the slower recovery rate and corresponding increase in net $[Ca^{2+}]_i$ exposure (area under the curve) is of greater importance than peak $[Ca^{2+}]_i$ responses in determining vascular tone in those animals.

Insulin has also been shown to decrease peak $[Ca^{2+}]_i$ responses to AVP (14, 15) in cultured rat aortic smooth muscle cells by reducing Ca^{2+} influx through receptor-operated channels (15) and decreasing voltage-mediated Ca^{2+} influx (15). However, we found no difference in the peak $[Ca^{2+}]_i$ response to AVP in cells from the insulin resistant obese rats versus those from the lean rats. Thus, insulin regulation of AVP receptor-operated vascular smooth muscle Ca^{2+} channels appears to be preserved in this

model of insulin resistance. In contrast, the rate of $[Ca^{2+}]_i$ recovery following stimulation with AVP was blunted in cells from the obese rats suggesting that insulin stimulation of $[Ca^{2+}]_i$ recovery is compromised in smooth muscle cells from insulin-resistant rats.

We have recently reported that insulin incubation stimulates the expression of plasmalemma Ca^{2+}-ATPase in cultured rat vascular smooth muscle cells (18) and, consequently, accelerates Ca^{2+}-ATPase-mediated Ca^{2+} efflux (12) and vascular relaxation (13). Thus, it is likely that insulin-induced stimulation of $[Ca^{2+}]_i$ recovery and corresponding decreases in the area under the $[Ca^{2+}]_i$ response curves are mediated by the plasmalemmal Ca^{2+}-ATPase. Accordingly, the decrease in $[Ca^{2+}]_i$ recovery rate in the obese rat in the present study probably reflects an impairment in insulin stimulation of Ca^{2+}-ATPase expression and activity. Indeed, we have previously found reduced aortic $^{45}Ca^{2+}$ efflux in Zucker obese rats (19) and a comparable decrease in erythrocyte Ca^{2+}-ATPase activity in both Zucker obese rats (20) and type II diabetics (21).

We have also recently found insulin to stimulate the expression of sarcoplasmic reticulum Ca^{2+}-ATPase in cultured rat vascular smooth muscle cells (18). If the increased expression results in increased sarcoplasmic reticulum Ca^{2+}-ATPase activity, an increase in sarcoplasmic reticulum Ca^{2+} storage would be expected. This may explain the increase in peak $[Ca^{2+}]_i$ responses to AII in insulin treated cells (14), as well as the diminished $[Ca^{2+}]_i$ response to AII observed in cells from the obese animals.

In summary, data from the present study provide a cellular rationale for recent observations of insulin-induced vasodilation in humans (6, 7) as well as insulin-mediated acceleration of vascular relaxation (13) and attenuation of <u>in vitro</u> vasoconstriction (10, 11). We propose that the impaired rate of $[Ca^{2+}]_i$ recovery in Zucker obese rats results from a vascular smooth muscle cell manifestation of insulin resistance. The resistance

appears to result in an impairment in the ability of insulin to stimulate both plasmalemma and sarcoplasmic reticulum Ca^{2+}-ATPase. The blunted peak $[Ca^{2+}]_i$ response to AII in Zucker obese rat vascular smooth muscle cells may also result from insulin resistance and a consequent failure of insulin to stimulate sarcoplasmic reticulum Ca^{2+}-ATPase, thereby resulting in a diminished AII-releasable Ca^{2+} store. Finally, we propose that the exaggerated vasoconstrictor responses characteristic of insulin resistance may result, in part, from failure of insulin to stimulate one or both Ca^{2+}-ATPases and thereby accelerate recovery from $[Ca^{2+}]_i$ loads.

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III. VARIATION OF BLOOD PRESSURES IN LEAN ZUCKER RATS FED LOW OR HIGH FAT DIETS.[1]

A. Abstract

The purpose of this study was to determine whether lean (*Fa/?*) Zucker rats which exhibit greater metabolic efficiency in response to a high fat (48%) diet also exhibit hypertension. Twenty-nine lean (*Fa/?*) and eleven obese (*fa/fa*) rats were fed low (12% energy) or high (48% energy) fat diets. Food intake and weight change were recorded weekly to obtain an index of energy efficiency. After six weeks, direct intra-arterial blood pressure was measured in conscious, unrestrained animals. Arterial blood was collected for analyses of plasma insulin and glucose. There was no effect of high fat diet on blood pressure in obese rats, although obese animals on either diet had significantly higher blood pressure than lean siblings ($p<0.05$). A positive correlation between blood pressure and energy efficiency ratio was present only in lean rats fed the high fat diet ($r=0.69$, $p<0.01$). This relationship was not present when all rats or lean rats on a low fat diet. Moreover, lean rats fed the high fat diet with blood pressures above the group median also exhibited significantly higher insulin ($p<0.02$), glucose ($p<0.01$) and energy efficiency ratios ($p<0.01$) than those with lower blood pressures. Thus, it appears there were two groups of lean animals present, one of which was phenotypically more sensitive to high fat feeding than the other. This suggests the possibility of a gene dosage effect of the *fa* allele in the Zucker rat.

[1]This manuscript has been submitted for publication in similar form with coauthors William J. Banz and Michael B. Zemel.

B. Introduction

Obesity, glucose intolerance, hypertriglyceridemia and hypertension are a clustering of metabolic and physical aberrations called "Syndrome X" which is closely associated with cardiovascular disease (1). Hyperinsulinemia has been implicated as the condition that links this "deadly quartet", although a cause-and-effect relationship has yet to be established (1). Dietary, exercise and pharmacological interventions have been intensely studied in an attempt to modify environmental factors contributing to this syndrome (2, 3). However, genetics may play a pivotal role in determining cardiovascular risk and the response to environmental interventions.

The obese Zucker rat (*fa/fa*) has been shown to be a single gene defect animal model of insulin resistance. Frank obesity is present in obese Zucker rats by age 3 to 5 wk when compared to lean (*Fa/Fa*) siblings (4). Hyperinsulinemia, hypertriglyceridemia and glucose intolerance are present by age 11 to 13 wk (5). While some investigators have found hyperglycemia in obese Zucker rats, others have not (5, 6). Nonetheless, skeletal muscle glucose transport is lower in obese animals (7 - 9). Obese Zucker rats are also hyperphagic to a described "breakpoint" which may be reached with the maximal possible size of each individual animal. The magnitude and duration of hyperphagia is influenced by dietary components; animals on a high fat diet reach this breakpoint sooner (10).

The obese Zucker rat has been extensively studied as a model of hypertension. Blood pressure studies of obese versus lean Zucker rats are conflicting; however, with direct arterial measurements in conscious, unrestrained animals it has been consistently demonstrated that obese Zucker rats are hypertensive by six weeks of age (11 - 16). In addition, vascular tissue from obese animals exhibits increased in vitro vascular

reactivity (17) and exaggerated intracellular calcium responses to vasoactive agonists (18).

Genetics and environment have been shown to interact in obesity and hypertension in humans. Subjects who are genetically susceptible to coronary heart disease experience earlier onset of hypertension and dyslipidemia with high fat diets (19). High fat feeding has also been shown to induce or exaggerate hypertension in both normal and obese animal models (20 - 22). Obese Zucker rats fed an energy restricted diet, nonetheless, exhibit an obese body composition (23). While lean Zucker rats seem relatively resistant to high fat diets, obese Zucker rats appear sensitive to high fat feeding (24). The purpose of the present study was to determine whether lean animals which exhibit greater metabolic efficiency and reduced insulin sensitivity in response to high fat diet also exhibit hypertension.

C. Materials and Methods

Animals. Lean Zucker rats (*Fa/?*) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and bred to produce lean (*Fa/?*) and obese (*fa/fa*) rats. Females were housed in plastic cages with wood shavings through mating and pregnancy until the pups were weaned at 21 days. Pups were sexed and the males were placed individually in wire hanging cages. All animals were fed nonpurified diet (Agway Prolab Rat Mouse Hamster 3000, Syracuse, NY) unless otherwise stated and lived in a controlled environment (21°C) with a 12 h light:dark cycle. This study was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Diet. At age 10 wk, 29 lean (*Fa/?*) and 11 obese (*fa/fa*) male Zucker rats were randomly assigned to a low (12% energy) or high (48% energy) fat diet (Table 3). The 12% fat diet was based on the AIN-*76*™ purified diet for rats and mice. Corn oil replaced corn starch in the high fat diet, and protein and essential micronutrient content were adjusted to be equivalent on an energy basis. Animals were allowed free access to their assigned diet and water. Weekly food intake and body weight were recorded for the duration of the six week study. Energy efficiency was calculated as weight gain (g) divided by energy intake (kcal) over the 6 wk feeding period.

Outcome measures. After 42 - 52 days of diet treatment animals were anesthetized with sodium pentobarbital (50 mg/kg) and a femoral arterial catheter (PE-10) was implanted, exteriorized at the nape of the neck and stored in a tissue culture cap. Forty eight hours post-surgery blood pressure was measured directly with a low volume transducer and Digimed blood pressure analyzer (Micromed Inc. Lexington, KY) in the conscious unrestrained state. The animals were then anesthetized and exsanguinated by cardiac puncture. Plasma glucose and insulin concentrations were determined using the standard glucose oxidase (Sigma, St. Louis, MO) and radioimmunoassay (INCSTAR, Stillwater, MN) methods, respectively (Appendix B).

Statistics. Two-way analysis of variance (ANOVA) of strain (lean vs. obese Zucker rats) and diet (high vs. low fat), multiple and simple regressions were performed to test for all possible relationships of the independent variables. A p value of less than 0.05 was considered statistically significant. When two-way ANOVA interactions were significant,

Table 3. Components of low (12% energy) and high (48% energy) fat diets

Component	12% fat diet[1]	48% fat diet[1]
Cornstarch[2]	55.0 (g/100g)	24.7 (g/100g)
Corn oil[2]	5.0	25.5
Casein	20.0	24.8
Cellulose	5.0	6.2
Sucrose	10.0	12.4
AIN Mineral mix	3.5	4.3
AIN Vitamin mix	1.0	1.2
D-l-methionine	0.3	0.5
Choline bitartrate	0.2	0.3
Energy	3.86 (kcal/g)	4.80 (kcal/g)

[1]12% and 48% of energy derived from fat

[2]Corn oil was substituted for cornstarch in the high fat diet.

the Least Significant Difference test (LSD) was utilized to distinguish differences in group means.

Based on our initial statistical analysis, we examined two populations of lean animals fed high fat diets: those below and those above the median mean arterial blood pressure. We then performed Student's t-tests comparing the two groups to see if there were statistically significant differences in insulin, glucose and energy efficiency ratio.

D. Results

As expected, obese Zucker rats had significantly higher mean arterial blood pressures than lean siblings, regardless of diet (138±2 versus 149±5 mmHg, $p < 0.02$). Simple regression relationships of all lean animals revealed a significant correlation between mean arterial blood pressure and blood glucose ($r = 0.48$, $p < 0.05$), while multiple regression demonstrated significant relationships of mean arterial blood pressure with plasma glucose ($p = 0.006$) and energy efficiency ratio ($p = 0.04$). In the lean animals fed the high fat diet, mean arterial blood pressure was significantly correlated with weight change ($r = 0.62$, $p < 0.01$), blood glucose ($r = 0.60$, $p < 0.02$) and energy efficiency ratio ($r = 0.69$, $p < 0.01$) and insulin had a significant correlation with energy efficiency ratio ($r = 0.62$, $p < 0.01$) in lean animals on a high fat diet. Moreover, multiple regression analysis within this group demonstrated that variation in energy efficiency ratio, weight change and insulin accounted for 89% of the variation in mean arterial pressure ($p < 0.000001$). As obese animals were included for reference purposes only, these correlations can not be evaluated due to the small number of animals. Among lean animals on the low fat diet these relationships were not evident. The mean values for independent variables are presented in Table 4.

Table 4. Physiological measurements of lean and obese Zucker rats fed low (12% energy) or high (48% energy) fat diets.

	Lean 12%	Lean 48%	Obese 12%	Obese 48%
Sample size	14	15	5	6
Initial weight (g)	339.7 ± 7.5	348.2 ± 8.1	463.0 ± 10.2[a]	457.5 ± 16.8[a]
Weight change (g)	95.5 ± 1.9	134.9 ± 6.5[b]	127.0 ± 15.6	184.5 ± 8.4[b]
Insulin (ng/mL)	23.4 ± 2.7	23.5 ± 4.2	93.9 ± 9.7[a]	104.5 ± 9.3[a,b]
Glucose (mg/dL)	201.8 ± 12.0	200.9 ± 13.4	329.1 ± 44.2	313.0 ± 25.4
G/I ratio[1]	12.2 ± 3.0	12.7 ± 2.5	3.7 ± 0.7[a]	3.2 ± 0.4[a]
MAP[2] (mmHg)	140.4 ± 2.8	134.9 ± 3.9	149.3 ± 9.0[a]	148.6 ± 6.6[a]
CEI[3] (kcal)	3337 ± 95	3492 ± 81	3974 ± 106	4018 ± 70
EER[4]	0.029 ± 0.002	0.038 ± 0.002[b]	0.032 ± 0.002	0.046 ± 0.002[b]

[a]significant phenotype effect $p < 0.05$.

[b]significant diet effect $p < 0.05$.

[1]G/I ratio is the ratio of plasma glucose (mg/dL) to plasma insulin (ng/mL).

[2]The sample sizes for MAP measurements were 12, 12, 4 and 3, as ordered above.

[3]CEI = Cumulative energy intake over six weeks.

[4]EER = Energy efficiency ratio (weight gain (g)/CEI (kcal))

The distribution of blood pressures in lean animals fed the high fat diet suggested the presence of two populations of animals (Figures 3 and 4). Accordingly, we separated these animals into two groups based on median mean arterial blood pressure (132 mmHg). Those animals with mean arterial blood pressures above the median also exhibited increases in energy efficiency ratio ($p < 0.01$) and plasma levels of insulin ($p < 0.02$) and glucose ($p < 0.01$) compared to those with lower blood pressures (Table 5).

E. Discussion

In the present study, feeding lean animals a high fat diet unmasked heterogeneity in mean arterial blood pressure, plasma insulin and glucose, and energy efficiency. Interestingly, lean animals fed a high fat diet with the highest mean arterial blood pressures also had higher circulating insulin and glucose as well as a higher energy efficiency ratio. The ability of a high fat diet to raise mean arterial blood pressure was related to the ability of a high fat diet to exert a diabetogenic effect and possibly promote fat storage. Thus, there appeared to be two populations of lean animals, suggestive of a possible *fa* gene dosage effect that may be environmentally alterable in heterozygous animals.

The obese Zucker rat (*fa/fa*) is a genetic extreme of obesity and insulin resistance. The phenotype is relatively resistant to environmental manipulations which would otherwise reduce these pathologies when compared to lean (*Fa/?*) animals (23). In addition, obese animals are relatively sensitive to environmental manipulations which would exacerbate obesity and insulin resistance (24). Therefore, studies of environmental effects on modulation of phenotypical *fa* gene expression have proved difficult in obese animals as they are already at the phenotypical extreme.

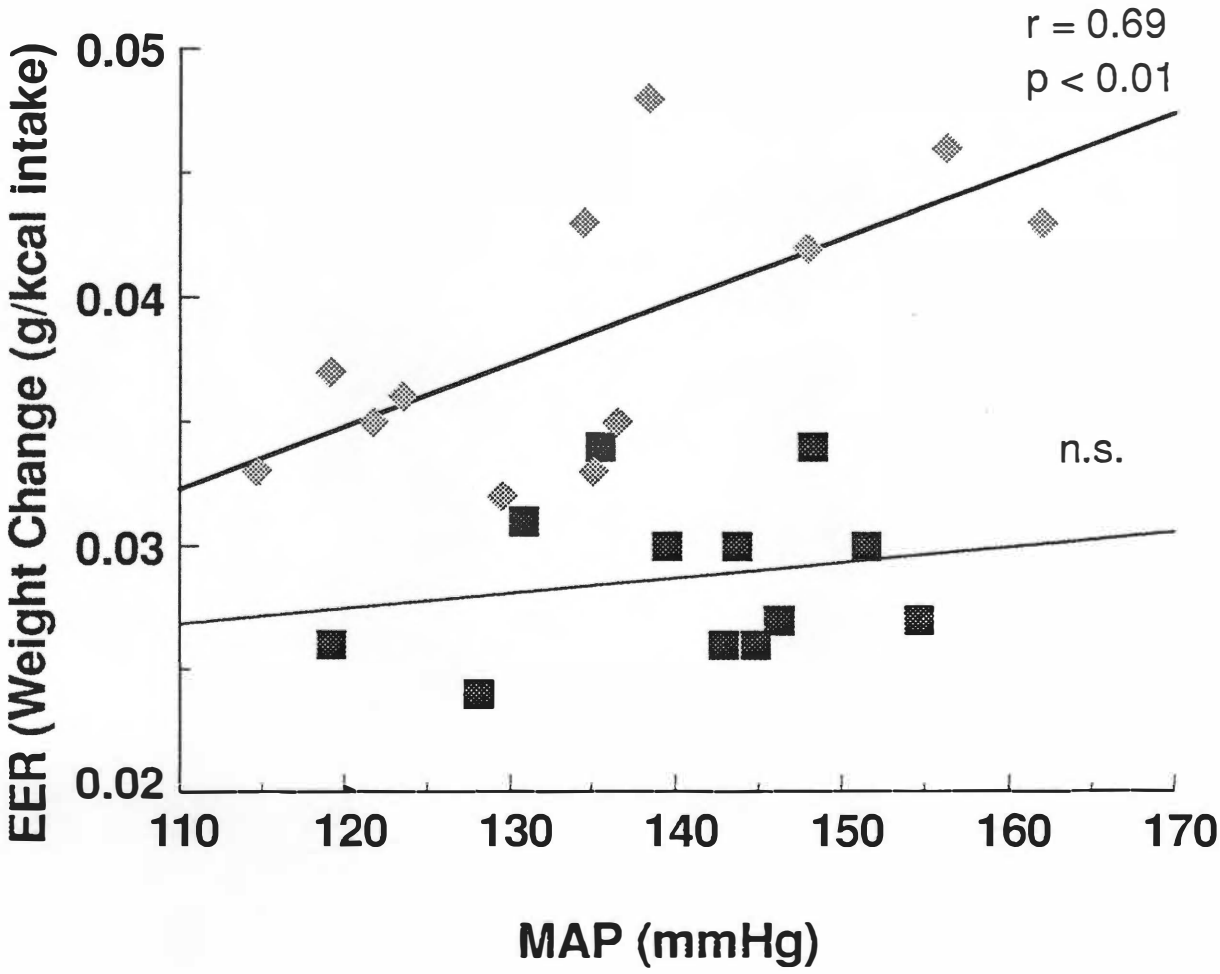

Figure 3. Regressions of mean arterial pressure (MAP) and energy efficiency ratio (EER) in all lean (*Fa/?*) Zucker rats, fed a low (12% - squares) or high (48% - diamonds) fat diet. The mixed regression line was not significant.

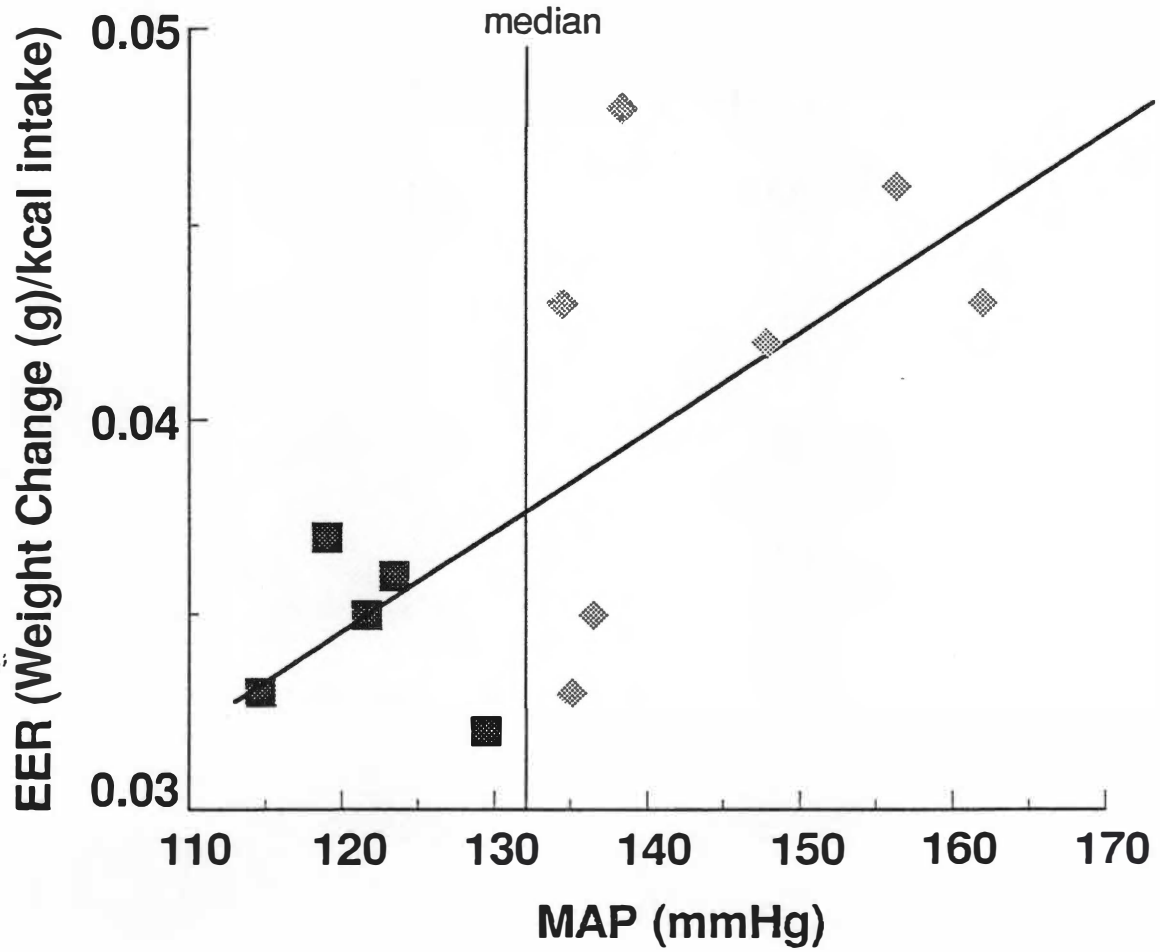

Figure 4. Regression of mean arterial pressure (MAP) and energy efficiency ratio (EER) in lean (*Fa/?*) Zucker rats fed a high (48%) fat diet ($r = 0.69$, $p < 0.01$). Animals with mean arterial blood pressures below and above the median 132 mmHg are shown in squares and diamonds, respectively.

Table 5. Physiological measurements of lean Zucker rats fed high fat diets grouped around the median mean arterial pressure of 132 mmHg.

	Below median MAP	Above median MAP
Sample size	5	7
Initial weight (g)	354.8 ± 12.7	344.4 ± 15.5
Weight change (g)	120.2 ± 5.1	147.1 ± 10.3[a]
Insulin (ng/mL)	14.8 ± 4.3	32.7 ± 5.9[a]
Glucose (mg/dL)	165.7 ± 10.1	212.8 ± 12.8[a]
G/I ratio	13.3 ± 1.9	7.7 ± 1.4[a]
CEI (kcal)	3433 ± 127	3564 ± 121
EER	0.035 ± 0.001	0.041 ± 0.002[a]
MAP (mmHg)	122 ± 3	144 ± 4[a]

[a]significant difference between groups, $p < 0.02$.

CEI = Cumulative energy intake, EER = energy efficiency ratio, and MAP = mean arterial blood pressure, G/I ratio = glucose (mg/dL) to insulin (ng/mL) ratio.

As expected, obese animals in the present study exhibited higher energy efficiency, blood pressure, plasma glucose and insulin and greater weight gain than lean siblings that were not significantly affected by high fat feeding.

The effect of a high fat diet on the development of hypertension is related, in part, to insulin resistance (20 - 22, 25). Weight gain resulting from increased energy efficiency on a high fat diet, despite similar caloric intake, may also result in hypertension (21, 26). In addition, insulin resistance, enhanced by dietary fat, may lead directly to increased blood pressure at the vascular smooth muscle level, as vascular reactivity and cell calcium metabolism is aberrant in this tissue from insulin resistant animals (14, 18). Moreover, compensatory hyperinsulinemia, secondary to insulin resistance, may result in increased sympathetic activity and renal sodium retention and further contribute to hypertension (3).

Until recently it has been impossible to distinguish between genotypes of lean vs. obese Zucker rats until later in development, when phenotypic markers (rectal temperature, body weight and fat pad size) are discernible. In addition, distinguishing between lean animals who carry zero or one copy of the affected gene has depended on the known breeding history of parent animals. Therefore, little research has been done to show gene dosage effects of the altered allele (*fa*), but a few studies have shown lean heterozygote (*Fa/fa*) animals to be phenotypically intermediate on several physical and metabolic parameters (27 - 31). At 17 days, inguinal fat pad weight and inguinal fat pad weight / body weight ratio, as well as inguinal fat pad cell size and number, of lean Zucker heterozygotes (*Fa/fa*) were intermediate between obese (*fa/fa*) and homozygote lean (*Fa/Fa*) Zucker pups (30). Lipid metabolic enzymes such as lipoprotein lipase and 6-phosphogluconate dehydrogenase were also intermediately produced in adipose and

liver tissue. Insulin binding in brain and liver is reduced in both lean heterozygote (*Fa/fa*) and obese (*fa/fa*) animals when compared to lean homozygote (*Fa/Fa*) animals (27, 29). Dynamic pancreas release of insulin is increased in lean heterozygote (*Fa/fa*) and obese (*fa/fa*) rats compared to homozygote lean (*Fa/Fa*) Zucker rats (Blonz et al. 1985). Also intermediate values in brown adipose tissue thermogenesis have been shown in lean Zucker heterozygotes (31).

With the recent discovery of a restriction fragment length polymorphism recognized by a human genomic subclone sequence (vc85) in a Brown Norway (BN)/Zucker cross, it is now possible to distinguish obese (*fa/fa*) animals at an early age and to identify lean homozygotes (*BN/BN*) and heterozygote (*BN/fa*) offspring (32). Utilizing this methodology, Truett et. al has shown that there are significant differences in body and inguinal fat pads weights at 7 and 14 days of age in the three genotypes for *fa* in Brown Norway/Zucker offspring (33). It has also been demonstrated that the heterozygote effect on body weight increases in magnitude by the fourth week of life around the same time the double mutant exhibits a marked increase in body weight (34). Therefore it seems there is a developmental progression in the expression of the *fa* allele with animals carrying one copy exhibiting phenotypical intermediacy early in life which becomes less obvious as animals carrying two copies of *fa* become the phenotypical extreme (33, 34). Moreover, we have recently confirmed heterozygote effects of *fa* at a later age and exacerbation of *fa* effects with high dietary fat using this model (35).

The experiments presented here may be useful in validating Brown Norway/Zucker offspring carrying *fa* as sufficient models of obesity, insulin resistance and hypertension with appropriate controls (*BN/BN*). The effects of a different background of genes, contributed by Brown Norway, on the traditional Zucker phenotype

can now be compared. These data are only suggestive of gene dosage as it is not yet possible to genotype Zucker rats without first crossing them with a genetically dissimilar strain. Consequently, studies to confirm that *fa* gene dosage alters the severity and developmental progress of obesity, insulin resistance and hypertension in Brown Norway/Zucker offspring carrying the *fa* gene are presently in progress.

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IV. DIETARY AND GENDER-RELATED EFFECTS OF HETEROZYGOSITY OF THE RAT FATTY (*fa*) ALLELE.

A. Abstract

Zucker rats carrying one copy of the obesity gene *fa* may exhibit intermediate phenotypes between lean (*Fa/Fa*) and homozygous mutants (*fa/fa*). Offspring of later generations of a BN/Crl X Crl:Zuc:*fa* F1 intercross that segregates for an RFLP tightly associated with the *fa* gene were genotyped and placed on a 12% or 48% energy as fat diet for seven weeks. Approximately 72 hours after femoral arterial catheter implantation, blood pressures were measured in the conscious, unrestrained state. Heterozygous males on high fat diet had higher mean arterial pressures than homozygous littermates ($p<0.03$). Although there were no differences in energy utilization between genotypes, females were less efficient than males ($p<0.0001$). Moreover, males fed high fat diet were more efficient than those consuming low fat diet ($p<0.03$), an effect of diet not seen in females. Perirenal fat pads, expressed either as absolute weight or as percentage of body weight were greater in males vs. females ($p<0.05$). Perirenal and epididymal fat pads were greater in rats with one vs. zero copy of the *fa* allele ($p<0.008$), as well as rats fed 48% vs. 12% fat diets ($p<0.0001$). Females exhibited significantly lower plasma glucose, insulin and total cholesterol than males. In addition, high fat diet resulted in increased plasma glucose ($p<0.03$) in females and insulin in males ($p<0.05$). Lastly, high fat resulted in decreased concentration of adipose and soleus Ca^{2+}-ATPase, while one copy of *fa* had this effect on soleus alone. The results of the present study not only show heterozygote effects of the *fa* allele, but suggest that these effects may be modulated by both gender-related factors and dietary manipulations.

B. Introduction

There are mutant genes in the human population which are responsible for the development of obesity and hypertension (1 - 3). If considered recessive, those with two copies of the mutant allele would be rare and grossly obese or hypertensive, while others "carry" only one copy and would appear lean or borderline hypertensive, respectively. However, if the specific gene, protein product and function have not yet been identified it may not be evident if one copy of the mutant allele affects the normal phenotype. Depending on the results of the mutation, such as no protein product, unregulated protein product or mutant competitive protein product, there may be environmental factors which exacerbate or attenuate deleterious effects of heterozygosity (1).

Genetics and environment have been shown to interact in obesity and hypertension in humans (2 - 4). Individuals, with familial dyslipidemic hypertension (FDH) who are genetically susceptible to coronary heart disease experience earlier onset of hypertension and dyslipidemia with high fat diets (2). Further, high fat feeding has been shown to induce or exaggerate insulin resistance and hypertension in both normal and obese animal models (5 - 11). The composition of dietary fat has been found to be an important factor in the development of cardiac risk factors (4, 12). Saturated fats, particularly, myristate (14:0), have been found to be the most atherogenic fats, while polyunsaturated fats, such as linoleate (18:2), may even be protective (12). In general, it is suggested that dietary intake of saturated fats increases cardiovascular risks more than intake of polyunsaturated fats, however over consumption of either is deleterious (4).

The obese Zucker rat (*fa/fa*) is a genetic extreme of obesity and also exhibits insulin resistance and elevated blood pressures (13). This phenotype is relatively resistant to environmental manipulations which would otherwise reduce these pathologies

when compared to lean animals (*Fa/?*) (7, 8). In addition, obese animals (*fa/fa*) are relatively sensitive to environmental manipulations which would exacerbate obesity and insulin resistance (7). Therefore, studies of environmental effects on modulation of phenotypical *fa* gene expression have proved difficult in obese animals as they are already at the phenotypical extreme. Obese Zucker rats (*fa/fa*) fed an energy restricted diet, nonetheless, exhibit an obese body composition (8). While lean Zucker rats (*Fa/?*) seem relatively resistant to high fat diets, obese Zucker rats (*fa/fa*) appear sensitive to high fat feeding (7).

The fatty mutation in Zucker rats has been considered a recessive mutation (14, 15). However, recent evidence comparing siblings carrying zero, one or two copies of the *fa* allele show that it is not a purely recessive trait as previously thought (16 - 20). Until recently it has been impossible to distinguish between genotypes of lean (*Fa/?*) versus obese (*fa/fa*) Zucker rats until later in development, when phenotypic markers (rectal temperature, body weight and fat pad size) are discernible (21). In addition, distinguishing between lean animals with zero or one copy of the affected gene has depended on the known breeding history of parent animals. Therefore, little research has been done to show gene dosage effects of the altered allele (*fa*), but a few studies have shown lean heterozygote animals (*Fa/fa*) to be phenotypically intermediate on several physical and metabolic parameters (16 - 20, 22).

Intermediate values reflecting brown adipose tissue thermogenesis have been shown in lean Zucker heterozygotes (*Fa/fa*) (20). In addition, insulin was increased in heterozygote lean (*Fa/fa*) and obese (*fa/fa*) compared to homozygote lean (*Fa/Fa*) Zucker rats (17). Inguinal fat pad weight and fat cell size and number in lean Zucker heterozygotes (*Fa/fa*) were also intermediate between obese (*fa/fa*) and homozygote

lean (*Fa/Fa*) Zucker pups by three weeks of age (19). Furthermore, lipid metabolic enzymes, such as lipoprotein lipase and 6-phosphogluconate dehydrogenase, were also intermediately produced in adipose and liver tissue. Finally, insulin binding in brain and liver is reduced in both lean heterozygote (*Fa/fa*) and obese (*fa/fa*) when compared to lean homozygote (*Fa/Fa*) animals (16, 18).

Recent discovery of a restriction fragment length polymorphism (RFLP) recognized by a human genomic subclone sequence (vc85) which segregates with *fa* in a Brown Norway (BN)/Zucker cross, allows identification of obese animals (*fa/fa*) at an early age. Furthermore, lean homozygote (*BN/BN*) and heterozygote (*BN/fa*) offspring can now be distinguished, but only in subsequent generations of this cross (21). Consequently, we sought to demonstrate that: a.) effects of one copy of *fa* are present later in life than previously reported, b.) there are interactions between copy number of the *fa* allele with dietary fat and c.) heterozygous (*BN/fa*) Brown Norway/Zucker offspring may be used as models of obesity, insulin resistance and hypertension (Syndrome X) that are susceptible to environmental manipulation.

C. Materials and Methods

Animals. Offspring of subsequent generations of a BN/Crl X Crl:Zuc-*fa* F1 intercross were supplied by Pennington Biomedical Research Center (Table 6). Pups were placed individually in wire hanging cages. All animals were fed semipurified diet (Agway Prolab Rat Mouse Hamster 3000, Syracuse, NY) upon arrival and lived in a controlled

Table 6. Distribution of BN/Z-*fa* offspring by sex, genotype, diet and litter.

Diet	Sex	Copies *fa*	N	Litters represented
12% Fat diet	Female	0	4	1,2,2,4
		1	6	3,4,5,5,6,6
	Male	0	8	1,1,2,2,3,3,4,6
		1	5	3,5,5,6,6
48% Fat diet	Female	0	4	1,1,2,2
		1	5	1,3,4,5,6
	Male	0	8	1,1,2,2,3,3,3,5
		1	5	1,4,5,6,6
TOTAL			45	1 - 6

environment (21°C) with a 12 hour light:dark cycle. This study was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Genotype assignment. Offspring were genotyped at Pennington Biomedical Research Center. High molecular weight DNA was prepared by phenol-chloroform (Autoextract Kit, Isogene Biotechnology, Overland Park, KS) extraction from tail segments. DNA was digested with the restriction endonuclease *Taq-I* (Boehringer Mannheim, Indianapolis, IN) and electrophoresed through 1% LE agarose gel (Sigma, St. Louis, MO) in 40 mM tris(hydroxymethyl)aminomethane acetate and 1 mM EDTA, transferred to nylon membranes (Hybond N+, Amersham, UK) according to manufacturer's instructions. Blots were hybridized with a 2.1 kb subclone vc85 (American Type Culture Collection 61067), labelled with ^{32}P-alpha-dATP random priming kit (DECAprime, Ambion, Austin, TX) in 10% dextran sulfate, 1M NaCl, 1% sodium dodecyl sulfate and 10 ug/ml sheared salmon sperm DNA at 65°C for 16-24 hours.

Diet. Male and female rats (5 wks) were randomly assigned to a low (12% energy) or high (48% energy) fat diet (Table 7). Weekly food intake and body weight were recorded for the duration of the seven week study. Coconut oil (Table 8) replaced corn starch in the high fat diet, and protein and essential micronutrient content was adjusted to be equivalent on an energy basis. Animals were allowed free access to their assigned diet and water. Energy efficiency was calculated as weight gain (g) divided by energy intake (kcal) throughout the feeding period.

Table 7: Components of low (12% energy) and high (48% energy) fat diets

Component	12% fat diet[1]	48% fat diet[1]
Sucrose	29.9 (g/100g)	37.2 (g/100g)
Cornstarch[2]	35.1	0
Coconut oil[2]	0	19.4
Casein	20.0	24.8
Corn oil	5.0	6.2
Cellulose	5.0	6.2
AIN Mineral mix	3.5	4.3
AIN Vitamin mix	1.0	1.2
D-l-methionine	0.3	0.4
Choline bitartrate	0.2	0.3
Energy	3.86 (kcal/g)	4.80 (kcal/g)

[1]12% and 48% of energy derived from fat

[2]Coconut oil was substituted for cornstarch in the high fat diet.

Table 8: Fatty acid composition of the coconut oil used in the diet.

Fatty acid	Percentage
< 12:0	5.45
12:0	48.37
14:0	20.94
16:0	11.27
18.0	6.66
18:1	5.64
18:2	1.36
20:0	0.14
TOTAL	99.83

Surgery and blood pressure measurement. After approximately 7 weeks of diet treatment each animal was anesthetized with sodium pentobarbital (PB - 50 mg/kg) and a femoral arterial catheter (PE 10) was implanted, exteriorized at the nape of the neck and stored in a tissue culture cap. Approximately 72 hours post-surgery, blood pressure was measured directly via a low volume transducer and Digi-med monitor (Micromed Inc. Lexington, KY) in the conscious, unrestrained state.

Plasma collection and assays. The animals were then anesthetized (50 mg/kg PB) and exsanguinated by cardiac puncture of the left ventricle. Plasma glucose, cholesterol, triglyceride, and insulin concentrations were determined using the standard glucose oxidase, cholesterol, and triglyceride kits (Sigma, St. Louis, MO) and radioimmunoassay (INCSTAR, Stillwater, MN) methods, respectively (Appendix B).

Western blot analysis of Ca^{2+}-ATPase. Aorta, soleus and adipose tissue samples were washed and minced in phosphate-buffered saline and submerged in equal volumes of suspension buffer and 2X gel loading buffer. Samples were then heated at 100°C for 10 minutes, sonicated and centrifuged. The supernatant was removed and a modified Lowry procedure (Appendix B) was performed to determine protein concentrations. 200 µg of protein samples were loaded and electrophoresed through a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were then electroblotted for 1 hour using transfer buffer containing Tris, glycine, methanol and SDS. After blocking, blots were incubated in blocking solution containing a 1:1000 dilution of the mouse-anti-rat monoclonal antibody to the plasmalemmal Ca^{2+}-ATPase (Affinity Bioreagents, Neshanic Station, NJ) in ascites fluid. After washing 2 times in tris-buffered saline containing

Tween 20 (TBST), the blots were incubated in 5 μCi ^{125}I-labelled goat-anti-mouse IgG (ICN, Irvine, CA). The blots were then washed 3 times with TBST, dried briefly, and exposed to film (NEN Dupont, Wilmington, DE) for 7-10 days.

Statistical analysis. Analysis of data compiled in this study had to account for highly unbalanced groups. Data were analyzed employing the MIXED Procedure of SAS (23). Each of the dependent variables (energy efficiency ratio (EER), plasma insulin, plasma glucose, fat pads weights, mean arterial pressure, heart rate, plasma triglycerides, plasma cholesterol, glucose to insulin ratio and Ca^{2+}-ATPase concentrations) were regressed on the independent variables diet, sex, genotype, litter, diet and sex, diet and genotype, genotype and sex, and diet by sex by genotype. Diet, sex, genotype and interactions were considered fixed factors, while litter was considered a random factor. Thus the effect of diet, sex and genotype are theoretically applied to all possible litters of BN/Z offspring. F statistics for analysis of variance of the fixed factors were computed using Type III sum of squares for unbalanced data.

D. Results

Weight gain over the seven weeks on diet was greater in males than females. In addition, males of either genotype on high fat diet gained significantly more weight than males on low fat diet. Weight gain in males carrying one copy of *fa* was greater than males carrying zero copy of *fa* only on the high fat diet (Figure 5).

Male animals on high fat diets were more energy efficient (weight change/energy intake) than those on low fat diets. In addition, males were more energy efficient than

females on either diet (Table 9). There was no apparent effect of genotype on energy efficiency.

Perirenal fat pads, expressed either as absolute weight or percentage of body weight were greater in animals fed high vs. low fat and in males vs. females on high fat diet (Table 9). Similar effects of diet were seen with epididymal fat pads expressed as percentage of body weights (Table 10). Moreover, a diet and genotype interaction was observed revealing that males with one copy of *fa* had a significantly greater increase in perirenal and epididymal fat pad weights expressed as percentage body weight on high fat diet than males with zero copy of *fa* (Table 10).

Plasma cholesterol was significantly higher in male than in female animals on high fat diet (Table 9). A diet and sex interaction revealed that males responded to high fat feeding with increased plasma cholesterol while females did not. Plasma triglycerides were not significantly affected by gender, diet or genotype (Table 9 and 10).

Plasma glucose was increased in males compared to females and in females with high fat feeding (Table 9). Plasma insulin was significantly higher in males fed high fat diets vs. males fed low fat and females on high fat diet.

Mean arterial blood pressure was significantly different in males carrying one versus zero copy of the *fa* allele only on high fat diets when paired by litter and analyzed by paired t-test ($p<0.03$). There were no significant differences in heart rate by gender, genotype, diet or any interaction.

Overall, high fat feeding decreased Ca^{2+}-ATPase protein concentration in soleus and perirenal fat ($p<0.03$) but not in aorta. In addition, one copy of *fa* resulted in reduced Ca^{2+}-ATPase protein in the soleus (Figure 6).

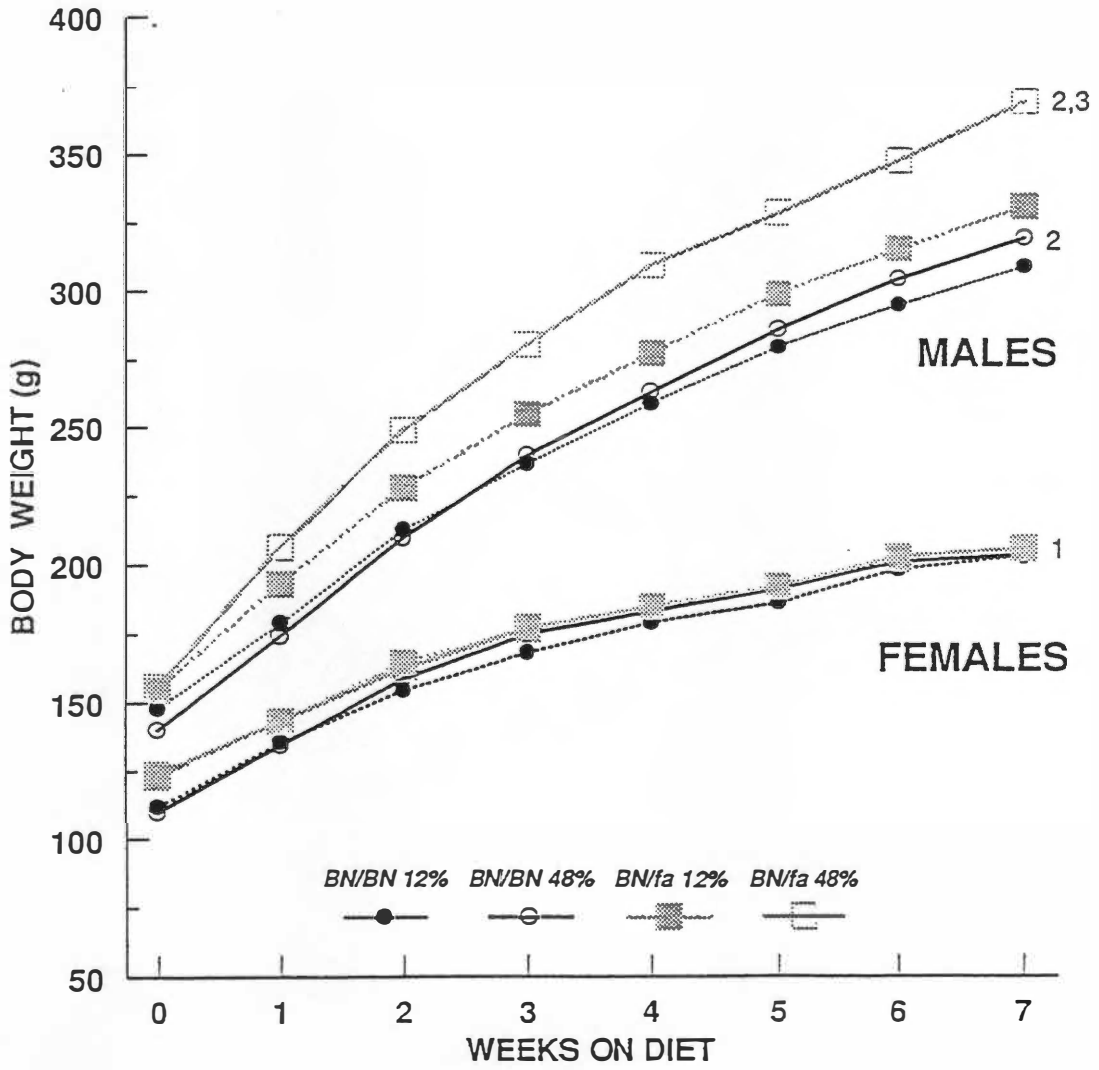

Figure 5 Mean growth curves of Brown Norway/Zucker male and female offspring on low (12% energy) and high (48% energy) fat diets. The weight gain over 7 wks was compared. [1]indicates significant difference from males, [2]indicates significant difference from 12% fat diet and [3]indicates significant difference from *BN/BN*, all at $p<0.05$.

Table 9. Physiological measurements of Brown Norway/Zucker offspring fed low (12% energy) or high (48% energy) fat diets.

GENDER	FEMALE		MALE	
DIET	12%	48%	12%	48%
N	10	9	13	13
EER	0.030±0.00[a]	0.030±0.002[a]	0.046±0.002[b]	0.053±0.002[c]
PFP % BW	0.90±0.14[a]	1.33±0.15[b]	0.91±0.14[a]	1.77±0.13[c]
TC (mg/dL)	79.4±4.1[a]	75.5±4.3[a]	86.8±4.0[a]	98.8±4.0[b]
TG (mg/dL)	46.4±6.0[a]	48.4±5.8[a]	50.7±5.6[a]	53.7±5.5[a]
Glucose (mg/dL)	143.9±6.1[a]	161.9±6.3[b]	158.8±5.8[a,b]	168.0±5.5[b]
Insulin (ng/mL)	2.43±1.01[a]	2.43±0.98[a]	4.01±1.11[a]	6.70±0.96[c]
MAP (mmHg)[1]	126±4[a]	128±5[a]	126±4[a]	131±4[a]
HR	365±18[a]	355±21[a]	366±18[a]	359±18[a]

Values with the same letter (a, b, or c) are not significantly different at $p<0.05$.

[1]The sample sizes for mean arterial pressure were 8, 6, 10, and 8 as ordered above.

KEY: N = sample size, EER = energy efficiency ratio (weight change (g)/energy intake (kcal)) over 7 weeks, PFP % BW = perirenal fat pads expressed as percent body weight TC = total cholesterol, TG = triglyceride, MAP = mean arterial blood pressure, HR = heart rate

Table 10. Physiological measurements of male Brown Norway/Zucker offspring fed low (12% energy) or high (48% energy) fat diets.

DIET	12%		48%	
GENOTYPE	*BN/BN*	*BN/fa*	*BN/BN*	*BN/fa*
N	8	8	5	5
EER	0.047 ± 0.002^{a}	0.046 ± 0.003^{a}	0.053 ± 0.003^{b}	0.053 ± 0.003^{b}
PFP % BW	0.73 ± 0.16^{a}	$1.11\pm0.22^{a,b}$	1.41 ± 0.16^{b}	2.12 ± 0.19^{c}
EFP % BW	1.17 ± 0.13^{a}	1.34 ± 0.17^{a}	1.66 ± 0.13^{b}	2.30 ± 0.15^{c}
TC (mg/dL)	81.8 ± 4.5^{a}	$91.7\pm6.3^{a,b}$	98.9 ± 4.6^{b}	98.8 ± 6.3^{b}
TG (mg/dL)	49.0 ± 6.5^{a}	52.3 ± 7.7^{a}	51.5 ± 6.3^{a}	55.8 ± 7.7^{a}
Glucose (mg/dL)	149.4 ± 6.7^{a}	$168.3\pm9.5^{a,b}$	$164.3\pm7.2^{a,b}$	171.8 ± 8.5^{b}
Insulin (ng/mL)	$5.10\pm1.19^{a,b}$	2.93 ± 1.74^{a}	$5.65\pm1.19^{a,b}$	7.73 ± 1.27^{b}
MAP (mmHg)[1]	127 ± 5^{a}	125 ± 6^{a}	128 ± 5^{a}	134 ± 6^{a}
HR	375 ± 19^{a}	355 ± 25^{a}	366 ± 21^{a}	352 ± 25^{a}

Values with the same letter (a, b or c) are not significantly different at $p<0.05$.

[1]Sample sizes for mean arterial pressure were 8, 6, 10, and 8 as ordered above.

KEY: N = sample size, EER = energy efficiency ratio (weight change (g)/energy intake (kcal)) over 7 weeks, PFP % BW = perirenal fat pads expressed as percent body weight, EFP % BW = epididymal fat pads expressed as percent body weight, TC = total cholesterol, TG = triglyceride, MAP = mean arterial blood pressure, HR = heart rate

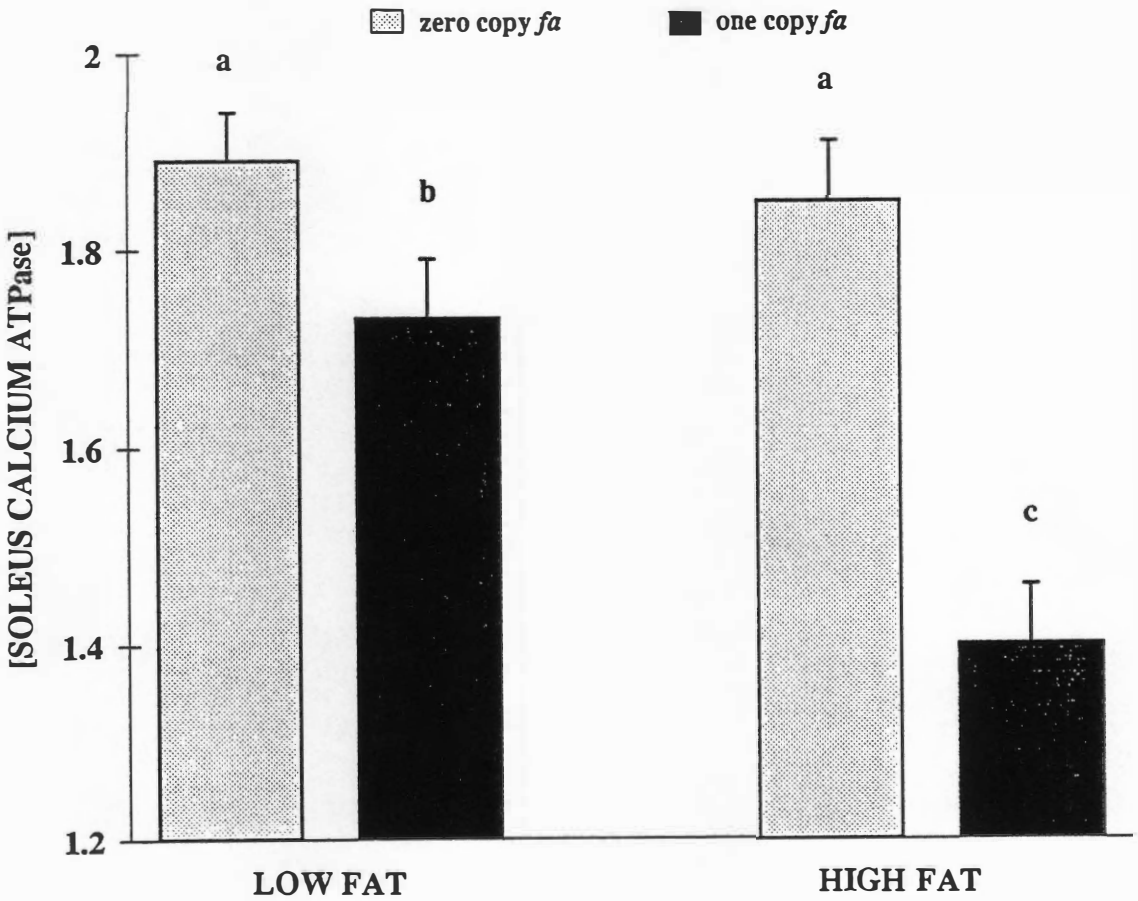

Figure 6. Densitometric evaluation of soleus Ca^{2+}-ATPase concentration in Brown Norway/Zucker offspring on low (12% energy) or high (48% energy) fat diets. Bars with the same letters (a,b or c) are not significantly different at $p<0.05$.

A diet by genotype interaction was observed wherein the effect of high fat reduction of Ca^{2+}-ATPase was significant only in animals with one copy of *fa* ($p<0.001$).

E. Discussion

The results of the present study clearly demonstrate effects of gender, high fat diet, genotype and interactions of these on physiological variables related to obesity, insulin resistance and hypertension (Syndrome X). We have thus begun to characterize a potentially useful model for the study of gene-environment interactions in the development of cardiovascular risk factors.

Effects of high fat feeding emerged, regardless of gender or genotype from this study. Energy efficiency was increased with high fat feeding in males but not females. Furthermore, animals on the high fat diet exhibited greater energy efficiency with increased fat storage in perirenal and epididymal fat pads. In addition, fasting plasma insulin was significantly higher in males on high fat diet. Thus it appears that development of insulin resistance and obesity observed with high fat feeding in several other studies may also occur in Brown Norway/Zucker offspring. However, in contrast to previous studies of both rats and dogs, high fat feeding alone did not result in hypertension (5, 6). This discrepency may be due to differing experimental design with respect to the duration of the diets, total fat content and composition of fat in the diets (6).

Interestingly, high fat diet also resulted in reduction of soleus and perirenal fat plasmamembrane Ca^{2+}-ATPase concentrations. Our laboratory has previously found relationships between insulin and intracellular calcium regulation in vascular smooth muscle (VSM) (24, 25). In cultured VSM cells, insulin results in increased expression of

plasma membrane and sarcoplasmic reticulum calcium pumps (24). Moreover, insulin increases the recovery to baseline calcium after agonist-induced calcium increases (25). This latter effect of insulin is impaired in aortic cells from insulin resistant obese Zucker rat (26).

Furthermore, others have found increases in intracellular calcium enhance insulin-mediated glucose transport in skeletal muscle (27) and adipose tissue (28). However, when intracellular calcium is increased beyond an optimal range, insulin-stimulated glucose transport is inhibited (28). Accordingly, the observed decreases in Ca^{2+}-ATPase may result in reduced insulin sensitivity secondary to increases in intracellular calcium. Conversely, it is also possible that diet-induced insulin resistance may result in diminished ability of insulin to stimulate Ca^{2+}-ATPase expression. Consequently, further studies are clearly needed to elucidate the mechanism by which high fat leads to reduction of this protein in skeletal muscle and adipose tissues.

There were several effects of gender which remained significant across diets and genotypes. Surprisingly, female rats in this study exhibited a marked decrease in energy efficiency, or ability to convert caloric intake into body mass, when compared to male rats. Consistently, females also had lower perirenal fat pad weights expressed as percent body weight. In addition, females had significantly lower plasma insulin and total cholesterol than males. These results suggest that female rats are more insulin sensitive than males, at least early in development, and may be resistant to metabolic impairments resulting in increased risk factors for syndrome X. Consistant with this concept is the observation of android obesity in syndrome X, where deposition of visceral fat is associated with increased levels of circulating androgens in both male and female humans (29, 30).

The interactions of diet and gender in this study support further investigation of this animal model for the study of obesity and insulin resistance. A closer look at energy efficiency revealed that while females were less efficient than males on either diet, males consuming high fat were more efficient than males consuming low fat diets. Likewise, while females were more insulin sensitive than males regardless of diet, males consuming high fat had significantly higher plasma insulins than the males on low fat diets. Moreover, while plasma cholesterol was actually somewhat lower in females fed high fat compared to females fed low fat diets, it was significantly higher in males fed high fat compared to males fed low fat diets. Clearly there is a gender and diet interaction in this model which allows female rats to remain relatively insensitive to high fat feeding, while increased cardiovascular risk factors (weight gain, increased cholesterolemia and increased insulinemia) mimicking syndrome X begin to be present in males fed high fat.

Unlike gender and diet, genotype alone had only significant effects of increasing both epidiymal and perirenal fat pad weights and reducing Ca^{2+}-ATPase concentration in the soleus. However, these data demonstrate effects of carrying one copy of the *fa* allele later in development than previously reported (16 - 20, 22) and may be indicative of a predisposition to increased adiposity and calcium dysregulation. Truett et al. studied siblings carrying zero, one, or two copies of the *fa* allele at 7 and 14 days of age (22). Analysis of gross physical measures revealed a linear effect of *fa* copy number on body weight and inguinal fat pads weights at 7 days of age, thus indicating a codominant effect of *fa* in early development. Quadratic effects of *fa* copy number began to appear later in life, indicating a shift towards recessivity.

In the present study, it was observed that *fa* may indeed act recessively in the groups that were maintained on normal diets. However, in male animals on high fat

diets there were significant phenotypical differences attributable to the copy number of *fa*. High fat diet increased the epididymal fat pads weights of animals carrying one copy of *fa*, while there was no difference between low fat fed and high fat fed animals carrying zero copy of the *fa* allele. In addition, heterozygosity of *fa* in combination with high fat consumption in males resulted in the greatest energy efficiency and the highest fasting plasma insulin. Furthermore, males with one copy of *fa*, on the high fat diet exhibited higher blood pressures (analyzed by paired t-test) than high fat fed male littermates with zero copy of *fa*. Therefore, the copy number of *fa*, continues to affect the phenotype of 14 week old male animals under specific (high fat) dietary conditions. Thus, the deleterious effects of high fat feeding on cardiovascular risk factors such as weight gain, hyperinsulinemia, insulin resistance and hypertension are further enhanced by male gender in animals carrying one copy of the *fa* allele.

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PART 4

CONCLUSIONS

I. IMPACT OF THIS RESEARCH AND FUTURE DIRECTIONS

Studies presented here support accumulating evidence from several laboratories that insulin is intricately involved in regulation of calcium metabolism. Conversely, recent evidence also predicts a role of calcium in insulin regulation. This reciprocal relationship is very likely altered in insulin resistant states and in conditions where calcium-handling abnormalities are primary. Further studies should include elucidation of the metabolic targets (such as Ca^{2+}ATPase and Glut4) which are affected by both insulin and calcium and defining the intermediates (such as PP1 and calmodulin) involved.

The final study reported here clearly demonstrated gene (*fa*) and environment (diet) interactions which have previously been ignored in a historically valuable animal model of Syndrome X. Due to the difficulty of controlling the environment of human subjects, animal models are at times the only solution to isolating gross physiological effects of the environment. The BN/Z model appears to be useful for studying Syndrome X, especially in light of the observed gender effects and gene-environment interactions.

The research presented here adds to a very large pool of knowledge regarding the relationships among obesity, insulin resistance and hypertension. Despite the wealth of information describing cardiovascular risk, we are still searching for cause and effect in this disease process. Moreover, with each new finding, there are yet more unanswered questions. This is why the work presented within this document is so important. Examination of molecular and cellular mechanisms, systems integration, and whole animal interactions with environment is crucial considering the prevalence of cardiovascular disease.

With the recent cloning of the *ob* gene (which codes for a yet-undefined humoral factor that appears to affect satiety and fat storage) and the association of *ob* with *db* (the mouse homologue for *fa*) as a putative receptor, the causative nature of many genetic obesities will, in the next ten or so years, be defined. This will also be of value for understanding cases of Syndrome X which are not solely genetically determined. However, it is doubtful that simple cures (such as those aimed at regulation or return of protein products) will allow us to control all of the associated symptoms of Syndrome X. Understanding the effects of insulin resistance on individual tissues (through transgenic studies) and the development of Syndrome X in the aging population should help us strive towards preventative goals, regardless of genetic predisposition. The genetic revolution must be tempered by the understanding that genetic influence never rules out environmental effects, as organisms do not exist without both.

For future research, I will continue to investigate the roles of genetics and environment in obesity and cardiovascular disease. Specifically, in light of our recent findings, I will begin by further investigating the mechanisms responsible for the gender differences we have observed. I would like to examine development profiles in both intact female and male animals compared with castrated animals of both genders to elucidate the role of steroid hormones (androgens, estrogen and cortisol regulation of gene expression) in Syndrome X (See literature review for references).

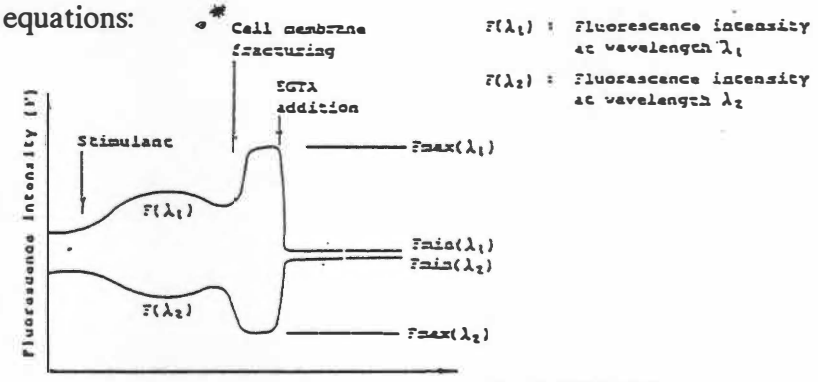
APPENDICES

APPENDIX A

Fluorometry theory and general procedures adapted from the Hitachi F-2000 Fluorometer (Naperville, IL) owners manual:

When a molecule (Fura-2-Ca^{2+}) is excited by light (excitation wavelengths = 340 and 380 nm) and electrons move to an excited state, optical energy is emitted (emission wavelengths = 510 nm) called FLUORESCENCE (emission is longer than excitation due to vibrational energy) as the electrons move back towards the ground state.

Fluorescence intensity at both excitation wavelengths is measured and the concentration of calcium is computed by the computer internal to the fluorometer using the following equations:



Z_1 : $F(\lambda_1)$ of sample alone (auto-fluorescence)

Z_2 : $F(\lambda_2)$ of sample alone (auto-fluorescence)

K : Constant (dissociation constant in concentration calculation. Fura 2 - 224 nM)

$$Rmin = \frac{Fmin(\lambda_1) - Z_1}{Fmin(\lambda_2) - Z_2} \qquad Rmax = \frac{Fmax(\lambda_1) - Z_1}{Fmax(\lambda_2) - Z_2}$$

$$R = \frac{F(\lambda_1) - Z_1}{F(\lambda_2) - Z_2}$$

$$\text{Concentration } C = K \cdot \frac{R - Rmin}{Rmax - R} \cdot \frac{Fmin(\lambda_2)}{Fmax(\lambda_2)}$$

*Digitonin permeabilizes the cell membrane and EGTA chelates calcium away from Fura-2.

There are four important components to be analyzed from each tracing shown in the text: Baseline, peak response, rate of recovery to baseline and the area under the response curve.

APPENDIX B

Sigma Enzymatic Glucose Determination (Procedure No. 510, St. Louis, MO):

This procedure is based on two enzymatic reactions. In the first, glucose, water and oxygen react in the presence of the enzyme **glucose oxidase** to form gluconic acid and hydrogen peroxide. In the second the hydrogen peroxide reacts with o-dianisidine (colorless) in the presence of the enzyme **peroxidase** to form oxidized o-dianisidine (brown).

Twenty-five microliters of standard (100 mg/dL) or plasma were added to 0.5 mL of water, followed by 5 mL of the enzyme-color reagent solution (containing glucose oxidase, horseradish peroxidase and o-dianisidine) and mixed. The tubes were incubated at 37°C for 45 m at room temperature. Absorbances of the standard and samples were read in reference to a blank (water and enzyme-color reagent) in a Beckman spectrophotometer (DU30, Fullerton, CA) at 475 nm. The sample values were then calculated utilizing the following formula:

$$\text{Plasma Glucose [mg/dL]} = A_{test} / A_{standard} \times 100$$

Triglycerides and cholesterol were studied using Sigma kits with similar design, although different reagents, with a colorimetric shift detected by spectrophotometry and compared to standard.

Incstar Insulin ^{125}I Radioimmunoassay (RIA) Kit (06130, Stillwater, MN) and rat standard 25 ng/mL:

The concept behind RIA is relatively simple. Binding sites (B) for a hormone (in this case, insulin) are provided by an antiserum establishing [B_{total}]. They are exposed to a low concentration of labelled hormone [^{125}I-insulin] and to the same unlabelled

hormone (insulin) to be measured. Thus the concentration of ^{125}I-insulin is a function of the dilution of ^{125}I-insulin by unlabelled insulin. The concentration of bound radioactive tracer [^{125}I-insulin•B] in consideration of the total radioactivity in the absence of hormone ^{125}I-insulin•B_0]. Results are obtained by comparing sample outcomes to a standard curve utilizing various concentrations of unlabelled insulin standards.

Two hundred microliters of plasma or standard, 100 µL antiserum containing first antibody and 100 µL ^{125}I-insulin were combined and incubated for 20 hours at 4° C. A second antibody precipitating complex was then added (0.5 mL) and the tubes were spun for 20 minutes at 760 x g, following a 25 minute incubation at room temperature. The supernatant was removed and the orifice of the tubes was blotted. The samples were then counted on a Packard Cobra-II gamma counter (Meriden, CT) for 10 minutes each. Sample concentrations of insulin were computed by the counter in comparison with the standard curve after consideration of total counts (100µL ^{125}I-insulin only) and non-specific binding (200 µL 0 standard, 100 µL ^{125}I-insulin and 0.5 mL precipitating complex).

Modified Lowry Procedure from: Lowry et al, Journal of Biological Chemistry 1951;193:265:

Protein treated with alkaline copper sulfate forms a copper-protein chelate which allows copper to act as a catalyst for protein reduction of phosphotungstic acid and phosphomolybdic acid (Folin reagent) to form a chromophore detectable in the visible spectrum. Tyrosine and tryptophan residues are oxidized resulting in the reduction of the Folin reagent and color formation.

Dilutions of 0.01% Bovine Serum Albumin (BSA) in 0.9% saline were utilized to create a standard curve of 0 (blank), 10, 20, 40, 80 and 100 μg/μL. Samples were diluted 1000 fold in 0.9% saline. One mL of diluted standards or samples were mixed and incubated with 3 mL of Lowry reagent (containing 2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate and 1.0% SDS mixed 100:1 with 4.0% $CuSO_4 \bullet 5H_20$) for 15 minutes at room temperature. Three hundred μL of Folin-Ciocalteu reagent premixed 1:1 with deionized H_2O was added, tubes were mixed and further incubated for 45 minutes at room temperature. Absorbances were read at 660 nm on a Beckman spectrophotometer (DU30, Fullerton, CA) against the blank and protein concentration was determined using the following formula:

$$\text{Concentration} = \text{Absorbance of sample}/K$$

K = constant = average (absorbance of standards/concentration of standards)

VITA

Margaret Ann Maher (Abel) was born September 21, 1965 in Dearborn, Michigan the last of six children. She attended Walnut Lake Elementary School, Bloomfield Hills, MI, after which her family moved to Texas. She graduated from A&M Consolidated High School, College Station, TX in May, 1983. In September of that year she attended Texas A&M University where she earned a bachelors degree in Psychology in May of 1989. During her undergraduate years she worked for the Texas A&M University Medical School Physiology Department and decided to pursue an advanced degree in Physiology. She began her doctoral work in January, 1990 and defended her dissertation in April, 1995. She will join the faculty, as Assistant Professor, at The University of Wisconsin, La Crosse in June of 1995.