VASCULAR ACTIONS OF INSULIN IN CARDIOMETABOLIC DISEASE: EFFECTS OF METFORMIN, PHYSICAL ACTIVITY, AND INTRINSIC AEROBIC FITNESS

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by

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I dedicate this dissertation to my husband, Dylan Keith Gray,
and our sons Eoin Alexander Gray and Jack Elliott Gray.
Thank you for your unconditional love, support, kindness, and patience.

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ABBREVIATIONS:

ACh - acetylcholine

ANOVA – analysis of variance

AUC – area under the curve

DEXA – dual energy x-ray absorptiometry

DR – diet restriction

EDHF – endothelium derived hyperpolarizing factor

eNOS - endothelial nitric oxide synthase

ET-1 – endothelin-1

FFA – free fatty acid

G2A-Red – second order arterioles perfusing the red portion of the gastrocnemius

G2A-White – second order arterioles perfusing the white portion of the gastrocnemius

GFA – gastrocnemius feed artery

HbA1c – glycosylated hemoglobin A1c

HDL – high density lipoprotein

HOMA-IR – homeostasis model assessment of insulin resistance

i.p. - intraperitoneal

IPGTT – intraperitoneal glucose tolerance test

LETO: Long Evans Tokushima Otsuka

LDL – low density lipoprotein

L-NAME - L-NG-Nitroarginine methyl ester

MAPK – mitogen activated protein kinase

MET – metformin

min - minute

MOPS - 3-(N-morpholino) propanesulfonic acid

NEFA – nonesterified fatty acids

NO – nitric oxide

OLETF – Otsuka Long Evans Tokushima Fatty rat

PE – phenylephrine

PI3K - phosphoinositide 3-kinase

PSS – physiological saline solution

PSSA – physiological saline solution albumin

PVDF - polyvinylidene fluoride

RUN – voluntary wheel running

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SED – sedentary

SEM – standard error of the mean

SFA – soleus feed artery

SNP – sodium nitroprusside

TG – triglycerides

T2DM –Type 2 diabetes mellitus

LIST OF PUBLICATIONS

This dissertation is based on the following manuscripts:

1.) **Crissey JM,** Padilla J, Jenkins NT, Martin JS, Scott Rector R, Thyfault JP, and Laughlin MH. Metformin does not enhance insulin-stimulated vasodilation in skeletal muscle resistance arteries of the OLETF rat. **Accepted in** *Microcirculation* 2013.

2.) **Crissey JM,** Jenkins NT, Duncan KA, Thorne PK, Bayless, DS, Vieira-Potter VJ, Rector RS, Thyfault JP, Laughlin MH, and Padilla J. Adipose tissue and vascular phenotypic modulation by voluntary physical activity and dietary restriction in obese insulin resistant OLETF rats. **Submitted to** the *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* on November 1, 2013.

3.) **Crissey JM,** Padilla J, Vieira-Potter VJ, Thyfault JP, and Laughlin MH. Divergent roles of nitric oxide in insulin-stimulated aortic vasorelaxation of low and high intrinsic aerobic capacity rats. **Manuscript in Preparation.**

VASCULAR ACTIONS OF INSULIN IN CARDIOMETABOLIC DISEASE: EFFECTS OF METFORMIN, PHYSICAL ACTIVITY, AND INTRINSIC AEROBIC FITNESS

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ABSTRACT

Insulin has important vascular and metabolic actions. The vascular effects of insulin typically account for as much as 40-50% of insulin-stimulated glucose disposal. However, the vascular reactivity to insulin is impaired in obesity and type 2 diabetes, in part due to an imbalance in endothelium-derived nitric oxide (NO) and endothelin-1 (ET-1), limiting the perfusion and delivery of glucose and insulin to target tissues. Consequently, aberrations in the vascular actions of insulin contribute to reduced glycemic control and insulin sensitivity. The studies described in this dissertation were designed to test hypotheses that focus on mechanisms and treatments of impaired vascular reactivity to insulin using rodent models of insulin resistance, obesity, and type 2 diabetes. We investigated the efficacy of metformin (Chapter 2), or daily physical activity (Chapter 3) treatments to improve the vasomotor response to insulin in conduit and skeletal muscle resistance arteries of obese, insulin resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Additionally we evaluated the influence of inherited aerobic fitness (Chapter 4), independent of physical activity levels, on the vascular reactivity to insulin in a large conduit artery of rats selectively bred for low or high aerobic running capacity. We present evidence that obesity-related impairments in micro- and macro-vasomotor reactivity to insulin is largely independent of changes in body

composition and adiposity. Specifically, metformin does not enhance the microvascular reactivity to insulin, despite reductions in body weight, food consumption, and improvements in glycemic control in OLETF rats. In contrast, daily physical activity in OLETF rats, significantly improved insulin-stimulated vasorelaxation, an effect that was in part mediated by reduced insulin-stimulated ET-1 aortic vasocontraction. Our data also indicate that low intrinsic aerobic fitness is paradoxically associated with greater insulin-mediated vasorelaxation and an exclusive dependence on nitric oxide in aortic vasomotor function. Collectively, our results shed new light on mechanisms underlying the influence of intrinsic aerobic fitness on the vasoreactivity to insulin, and provided strong evidence of physical activity's powerful insulin-sensitizing effects on the vasculature with obesity.

CHAPTER 1

INTRODUCTION

Obesity, Type 2 Diabetes, and Cardiovascular Disease

Obesity, type 2 diabetes and associated cardiovascular diseases continue to be leading causes of morbidity and mortality in the United States and around the world. Over the past few decades the prevalence of obesity and obesity-related diseases, including type 2 diabetes has risen dramatically. The obesity epidemic has turned into a global problem and largely accounts for the marked increase in the prevalence of type 2 diabetes worldwide (WHO report 2004). In the United States alone the prevalence of: overweight adults have increased from 47% to 66%, obese adults have increased from 15% to 32%, and type 2 diabetics have increased from 5% to 9% from 1976-2004 (66, 67). In 2007, nearly 23.6 million Americans had type 2 diabetes, and an additional 57 million had pre-diabetes (29). In 2010 more than one-third of all Americans were obese (135), and by 2050 as many as one in every three Americans are projected to be diagnosed with type 2 diabetes (29). Global projections estimate that type 2 diabetes will rise from 285 million individuals worldwide in 2010 to over 439 million in 2030 (187). Clearly, the prevalence of obesity and type 2 diabetes are rapidly increasing and represent major health challenges.

Obesity and type 2 diabetes are both major risk factors for the development of cardiovascular disease (33, 57, 159). It has long been known that obese diabetic individuals have increased morbidity and mortality from cardiovascular causes (60). Within the past decade, cardiovascular disease has become the leading cause of death in insulin-resistant individuals (98, 99), and the risk of cardiovascular disease is at least two to four times higher

in adults with diabetes compared to those without diabetes (167). Cardiovascular disease affects approximately 60% of the adult population over the age of 65 and represents the number one cause of death in the United States (33). Although the association between obesity, type 2 diabetes and cardiovascular disease are well recognized, the underlying relationships among these cardiometabolic diseases are poorly understood.

Accumulating evidence indicates that insulin resistance, a distinguishing patho-physiological hallmark of obesity and type 2 diabetes; and endothelial dysfunction, a well-established early marker of cardiovascular risk factors (90), together play key roles in the development and etiology of cardiometabolic disease. While insulin resistance is a hallmark attribute of metabolic disorders including obesity and type 2 diabetes, it is also a prominent component of hypertension, coronary artery disease, and atherosclerosis, all of which are characterized by endothelial dysfunction (153). The vast majority of diabetic and prediabetic individuals, have been shown to have endothelial dysfunction and exhibit insulin resistance (44). It is well established that obesity and type 2 diabetes, characterized by moderate-to-severe insulin resistance and endothelial dysfunction are major risk factors for the development of cardiovascular disease (57, 126). Thus, the metabolic derangements associated with insulin resistance are often accompanied by cardiovascular complications, including endothelial dysfunction in cardiometabolic disease.

Endothelial Dysfunction and Insulin Resistance

The vascular endothelium is as a key regulatory site of cardiovascular control. The endothelium is a single layer of cells that line all the vessels in the body, including conduit vessels, resistance vessels, precapillary arterioles and capillaries (180). Vascular endothelial

function is fundamental for the health of the vessel wall and vasomotor control in both conduit and resistance vessels. In contrast, a diseased endothelium contributes to the early pathophysiology of cardiovascular disease, and predicts future cardiovascular events (102, 184). Endothelial dysfunction represents one of the earliest abnormalities in the development of atherosclerosis, and contributes to the progression of later stage cardiovascular disease (186). In this context impaired endothelial function has emerged as a significant risk factor for cardiovascular disease (69, 163, 164), and the preservation or restoration of a healthy endothelium appears to be protective against the development of cardiovascular disease (62, 64, 65).

Evidence also indicates that the vascular and associated metabolic actions of insulin are fundamental to cardiovascular health. Classically insulin resistance is characterized by a blunted ability of peripheral tissues to regulate glucose homeostasis in response to insulin. The metabolic effects of insulin, including suppression of hepatic glucose production and stimulation of skeletal muscle glucose uptake are impaired in obesity and type 2 diabetes (46). In addition to the classic metabolic actions of insulin promoting glucose disposal, insulin has important hemodynamic actions in vascular tissue, including capillary recruitment, peripheral vasodilation, and increased regional blood flow (8, 39, 125, 183). Insulin stimulated vasodilation enhances skeletal muscle perfusion, augments nutritive flow, and increases delivery of insulin and glucose to skeletal muscle. In healthy individuals insulin-mediated increases in skeletal muscle blood flow account for as much as 40-50% of insulin-stimulated glucose disposal (8, 95, 132). However abnormalities in the vascular reactivity to insulin in obesity and type 2 diabetes contribute to decreased glucose disposal by limiting perfusion, and delivery of glucose and insulin to skeletal muscle (37, 38, 97, 185).

Insulin resistance is best defined as a cluster of metabolic and vascular abnormalities in insulin action associated with the development of metabolic syndrome and cardiovascular disease (169). Insulin resistance is associated with endothelial dysfunction; however, the mechanistic relationship between these aberrations and the role of vascular endothelial insulin signaling is incompletely understood.

Vascular Insulin Signaling

Insulin stimulates vascular endothelial cells to produce proteins that influence susceptibility to the development of atherosclerosis (151). Current conjecture is that insulin exerts important vascular actions in the endothelium including nitric oxide (NO) production, via the phosphatidylinositol 3-kinase (PI3K) pathway (Figure 1), leading to vasodilation of large and small vessels, and recruitment of muscle capillaries to increase nutritive flow, augmenting glucose uptake in skeletal muscle (39, 183). The opposite vascular action of insulin promotes vasoconstriction by activation of the mitogen-activated protein kinase (MAPK) pathway stimulating the production and release of endothelin-1 (ET-1) (Figure 1) (125, 126). It is theorized that in obesity and type 2 diabetes an imbalance between these insulin-signaling pathways promotes a dysfunctional, pro-inflammatory, and pro-atherogenic endothelial phenotype (54, 71) contributing to the development of atherosclerosis and cardiovascular disease. Selective impairments of the PI3K pathway with

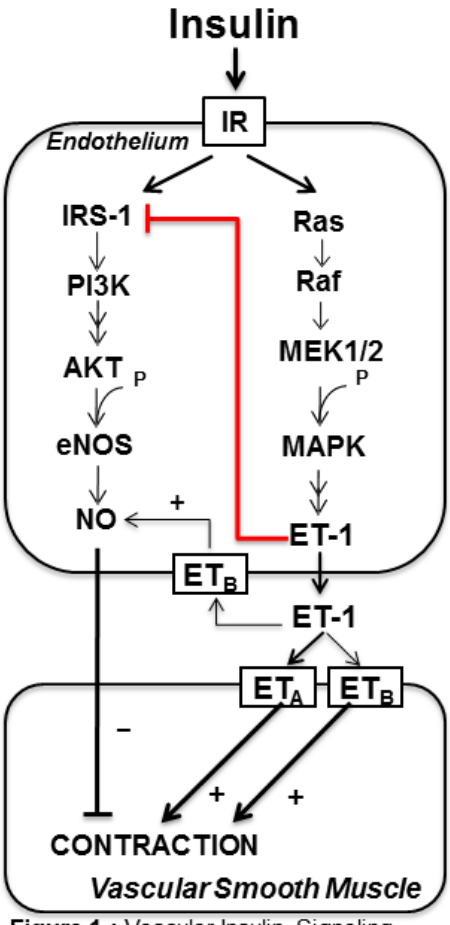

Figure 1 : Vascular Insulin Signaling

insulin resistance, reduces the bioavailability and vasodilatory effects of NO (27, 71). Concurrent overstimulation of the MAPK pathway increases the production and bioactivity of the potent vasoconstrictor, pro-inflammatory, and pro-atherogenic peptide ET-1 (19, 54, 71). Overproduction of ET-1 enhances insulin-mediated vasoconstriction and interferes with NO-mediated vasodilation (84). Abnormalities in vascular insulin signaling are central to the development of metabolic and vascular complications in cardiometabolic disease.

Our laboratory and others have shown that the vascular reactivity to insulin is impaired in obese type 2 diabetic humans and animals (96, 97, 110, 120, 122, 185). Aberrations in endothelial insulin signaling reduces insulin-stimulated NO-mediated vasodilation, and enhances insulin-mediated ET-1 vasoconstriction, contributes to impairments in insulin-stimulated blood flow and insulin-mediated glucose uptake by target tissues (7, 37, 41, 96, 97, 112, 125, 126). Insulin also has important direct actions on the vascular endothelium beyond regulating blood flow and capillary recruitment. As discussed above, insulin signaling stimulates the endothelial production of pro-atherogenic ET-1, involved in the initiation and progression of atherosclerosis (151). Obesity increases the risk of atherosclerotic disease and is associated with elevated ET-1 expression in the vascular endothelium in humans (166). Additionally, conditional knockout of the insulin receptor in endothelial cells causes a 2-to 3-fold increase in the atherosclerotic lesion size in the aorta of apolipoprotein E–null mice, and endothelial dysfunction in the carotid artery (152). Thus, a loss of endothelial insulin signaling is associated with endothelial dysfunction in large conduit arteries and accelerated atherogenesis. Accumulating evidence indicates that impairments in metabolic and vascular actions of insulin (along the arterial tree) are

interrelated and play key roles in insulin resistance and endothelial dysfunction with obesity, type 2 diabetes and cardiovascular disease.

Sex Differences in Insulin Action

Overall evidence indicates that females have greater hepatic (9) and skeletal muscle (134, 144) but lower cardiac (134) insulin sensitivity compared to males. Additionally females exhibit reduced susceptibility to fatty acid induced peripheral insulin resistance (58). However, not all studies are in agreement, some studies report lower (10), comparable (58) or greater (24, 144) insulin sensitivity in females compared to males. One possible explanation for these contradictory findings is that physical activity levels, and aerobic fitness, are key determinants of insulin action. Although, Basu et al. reported lower insulin-mediated glucose uptake in women compared to men, VO2max was also significantly lower in the women (10). In another study, in which males and females had similar aerobic fitness, insulin sensitivity (as assessed by a hyperinsulinemic euglycemic clamp) was 41% greater in females compared to males (134). In this study sex and maximum aerobic capacity accounted for 68% of the variability in skeletal muscle glucose uptake, and women were more sensitive to insulin that equally fit men due to enhanced skeletal muscle insulin sensitivity. Clearly both aerobic fitness and sex influence peripheral insulin action. However, it remains to be determined to what extent sex differences in skeletal muscle insulin sensitivity are associated with differences in the vascular reactivity to insulin involved in the pathogenesis of cardiometabolic disease.

In summary characterizing the mechanisms responsible for impairments in vascular reactivity to insulin associated with metabolic complications induced by insulin resistance in

obesity and type 2 diabetes is important to better understand the interrelationship between endothelial dysfunction and insulin resistance in the initiation and progression of cardiovascular disease. A greater understanding of the etiology of endothelial dysfunction and insulin resistance in the vasculature is important for uncovering insulin-sensitizing approaches to ameliorate cardiovascular abnormalities associated with metabolic disease. Therefore the central purpose of this dissertation was to gain insights into the mechanisms of altered vascular reactivity to insulin in rodent models of insulin resistance, obesity, and type 2 diabetes. In the following studies we investigated the effects of: (i) metformin, (ii) daily physical activity, and (ii) intrinsic aerobic fitness on the vasomotor reactivity to insulin in conduit and skeletal muscle resistance arteries with obesity and insulin resistance.

SPECIFIC AIMS

STUDY 1:

Metformin has been reported to have vasculoprotective effects (77, 88, 91, 113) linked to reduced cardiovascular events (142). Many of metformin's therapeutic effects are thought to be due in part to its ability to stimulate AMP-activated protein kinase (AMPK) in a variety of tissues (28, 192). In the vasculature AMPK has been shown to phosphorylate eNOS in both rat and human endothelial cells (36, 124), leading to enhanced production of NO and vasodilation. While metformin has been shown to reverse vascular dysfunction in conduit, large arteries, and the mesenteric circulation (79, 88, 113, 114); its ability to ameliorate or reverse vascular dysfunction in the skeletal muscle resistance vasculature was unknown. Furthermore, it was unclear whether metformin's insulin sensitizing effects were due to direct effects on the vascular reactivity to insulin.

SPECIFIC AIM 1: *In Study 1 (Chapter 2) we tested the hypothesis that chronic metformin treatment enhances insulin-mediated vasodilation in skeletal muscle resistance arteries of obese, insulin resistant rats.*

At 20 weeks of age healthy Long-Evans Tokushima Otsuka (LETO) and obese, insulin resistant pre-diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats were randomly assigned to untreated or metformin (MET) treated groups forming four groups: 1) LETO (n=15), 2) LETO + MET (n=14), 3) OLETF (n=15), and 4) OLETF + MET (n=14). This study was designed to (i) investigate the use of metformin as treatment against impaired vascular reactivity to insulin in obesity, and (ii) elucidate possible heterogeneity of metformin's impact on insulin-induced vasoreactivity throughout the skeletal muscle

vasculature. At 32 weeks of age (following 12 weeks of metformin treatment) gastrocnemius feed arteries, soleus feed arteries, 2nd order arterioles from the red portion of the gastrocnemius, and 2nd order arterioles from the white portion of the gastrocnemius were isolated to determine: 1) the vasoactive response to insulin alone, and 2) insulin-induced vasodilation in the presence of an endothelin-1 (ET-1) receptor blocker. We also determined the overall role of metabolic health in the vasomotor response to insulin with metformin by glucose tolerance tests and HbA1c measures.

STUDY 2:

Physical activity and exercise can have a profound influence on either maintaining or improving vascular function in obesity and type 2 diabetes (50, 62), and are associated with improvements in cardiovascular mortality and morbidity (18, 63-65, 85, 111). Impairments in endothelial insulin signaling, observed in cardiometabolic disease, is associated with the development of atherosclerosis in large conduit arteries (152). Physical activity has been shown to improve the vasomotor response to insulin in diseased animals and humans (47, 62, 86, 110, 120, 122). However, the ability of physical activity to prevent impairments in the vascular reactivity to insulin in conduit arteries (linked to atherosclerotic risk) with obesity, independent of changes in body composition, was not known.

SPECIFIC AIM 2: *In Study 2 (Chapter 3) we tested the hypothesis that daily physical activity protects against impairments in vascular insulin signaling and insulin-stimulated aortic vasorelaxation with obesity and insulin resistance.*

We randomized sedentary Otsuka Long Evans Tokushima Fatty (OLETF) rats, rats that are hyperphagic, physically inactive, and develop insulin resistance by 12 weeks of age,

to one of three groups: 1) continued sedentary (SED; n=10); 2) voluntary wheel running + *ad libitum* fed, (RUN; n=10); or 3) sedentary + diet restriction (fed 70% of *ad libitum* fed SED animals) to lower adiposity to levels in RUN rats (DR; n=10); for 8 weeks (12-20 weeks of age). This study was designed to investigate the use of physical activity as preventive treatment against the progression of vascular insulin resistance. At 20 weeks thoracic aorta rings were isolated to determine: 1) endothelial and vascular smooth muscle function; 2) insulin-stimulated aortic vasorelaxation; and 3) insulin-mediated ET-1 aortic vasocontraction. We also determined the effects of physical activity on insulin signaling [eNOS, and phospho-eNOS (Ser1177), ERK1/2, and phospho-p44/42 ERK1/2 (Thr202/Tyr204)] in abdominal aorta segments by Western blotting.

STUDY 3:

Increased physical activity levels and exercise training often lead to improvements in aerobic fitness, which is an independent and strong predictor of cardiovascular disease and overall mortality (15, 35, 128, 143). Although obesity is associated with cardiovascular risk and mortality, studies that fail to account for the influence of underlying aerobic fitness distort the relationship between obesity and mortality (118, 119). Moreover, the role of enhanced aerobic fitness, which occurs concomitant with increased physical activity, to modulate the vascular reactivity to insulin was unknown.

SPECIFIC AIM 3: *In Study 3 (Chapter 2) we tested the hypothesis that intrinsic low aerobic capacity is associated with vascular dysfunction and impaired vascular reactivity to insulin in the aorta, independent of physical activity levels.*

For this study we utilized mature adult female rats selectively bred for low (LCR) or high (HCR) running capacity. At 44 weeks of age the thoracic aorta was isolated from low fit LCR (n=21) and high fit HCR (n=17) rats to determine: 1) endothelial and vascular smooth muscle function, 2) role of nitric oxide (NO) and endothelin-1 (ET-1) in aortic endothelial vasorelaxation, 3) insulin-stimulated aortic vasorelaxation, 4) insulin-stimulated aortic vasorelaxation in the presence of ET-1 inhibition, and 5) insulin-stimulated aortic vasorelaxation in the presence of an eNOS inhibitor. We also performed glucose tolerance tests in the animals to determine the relationship between the vasoreactivity to insulin and post-prandial glycemic control.

CHAPTER 2

STUDY ONE:

METFORMIN DOES NOT ENHANCE INSULIN-STIMULATED VASODILATION IN SKELETAL MUSCLE RESISTANCE ARTERIES OF THE OLETF RAT

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Running Title: Metformin, resistance arteries, and insulin

Key Words: metformin, type 2 diabetes, insulin resistance, microvascular disease

ABSTRACT

OBJECTIVE: To test the hypothesis that chronic metformin treatment enhances insulin-induced vasodilation in skeletal muscle resistance arteries and arterioles. **METHODS:** We assessed the effect of metformin treatment (from 20-32 wks of age) of obese Otsuka Long Evans Tokushima Fatty (OLETF) rats and lean Long Evans Tokushima Otsuka (LETO) rats (300 mg/kg) on insulin-stimulated vasodilation in isolated skeletal muscle feed arteries and arterioles. **RESULTS:** Metformin treatment significantly lowered food intake, body weight, percent body fat, and glycosylated hemoglobin (HbA1c) in OLETF rats. Metformin resulted in a ~30% reduction in insulin-induced vasodilation of soleus feed arteries (SFA) from OLETF rats. Inhibition of endothelin-1 (ET-1) signaling with tezosentan produced 20% dilation and eliminated the difference between metformin-treated and untreated OLETF rats in insulin-induced dilation of SFA. In contrast to the SFA, metformin did not alter insulin-stimulated vasodilation in gastrocnemius feed arteries (GFA), or 2nd order arterioles in the red (G2A-R) or white (G2A-W) portions of the gastrocnemius muscle of OLETF rats. Metformin had no effects on vasomotor responses of arteries from LETO. **CONCLUSIONS:** Although metformin exerts favorable effects on body composition and HbA1c, it does not enhance the vasodilatory responses to insulin in the skeletal muscle feed arteries or arterioles of the obese OLETF rat.

INTRODUCTION

Insulin resistance is characterized by a blunted ability of peripheral tissues to regulate glucose homeostasis in response to insulin. The metabolic effects of insulin, including suppression of hepatic glucose production and stimulation of skeletal muscle glucose uptake are impaired in type 2 diabetes mellitus (T2DM) (46). In addition to insulin's metabolic effects, insulin also stimulates dilation of skeletal muscle resistance arteries. Current evidence indicates that abnormalities in the vascular reactivity (vasodilation) to insulin in T2DM contribute to decreased glucose disposal by limiting perfusion, and consequently, delivery of glucose and insulin to skeletal muscle (38, 97, 185). Therefore, it is becoming clear that impairments in both the vascular and metabolic actions of insulin are interrelated and play key roles in insulin resistance with obesity and T2DM.

Insulin's vascular actions are complex and are balanced by endothelial signaling pathways controlling the production of the vasodilator, nitric oxide (NO) (39, 183), and the vasoconstrictor endothelin-1 (ET-1) (125, 126). Insulin-stimulated vasodilation enhances skeletal muscle perfusion and increases delivery of insulin and glucose to skeletal muscle. In healthy individuals, insulin-mediated increases in skeletal muscle blood flow account for as much as 40-50% of total insulin-stimulated glucose uptake in skeletal muscle (8, 95, 132). Conversely, with obesity, insulin resistance and T2DM, there is a reduction of insulin-stimulated NO-mediated vasodilation, and enhanced insulin-induced ET-1-mediated vasoconstriction. This imbalance in the vascular actions of insulin is thought to contribute to impairments in insulin-stimulated glucose uptake by target tissues (7, 37, 41, 96, 97, 112, 125, 126). Indeed, our laboratory and others have shown that the vascular reactivity to insulin, and the resulting increase in skeletal muscle perfusion, is impaired in obese and

physically inactive T2DM humans and animals, ultimately attenuating nutritive blood flow and contributing to skeletal muscle insulin resistance (90, 97, 110, 120, 122, 185).

Most treatment and prevention therapies for T2DM involve the use of anti-hyperglycemic drugs targeting whole body insulin resistance; however the vascular effects of these drugs remain largely unknown. Metformin has long been used to treat T2DM and, based on current American Diabetes Association recommendations, is a first line treatment along with diet and exercise (1, 3). It is thought that metformin decreases blood glucose concentration by reducing basal hepatic glucose production and enhancing skeletal muscle insulin sensitivity (2, 142). In addition to being widely used in the treatment of T2DM, metformin is also increasingly prescribed as a preventative therapy in individuals at risk for the development of overt T2DM [8, 9]. In fact, results from the Diabetes Prevention Program show that treatment with metformin reduced the incidence of T2DM by 31% in non-diabetic impaired glucose tolerant individuals (92).

Several studies have reported that metformin reverses vascular dysfunction in conduit, large arteries and in the mesenteric circulation (79, 88, 113, 114); however, the ability of metformin to ameliorate or reverse vascular dysfunction in skeletal muscle resistance vasculature has not been well studied. Furthermore, while we have demonstrated that soleus muscle feed arteries are protected against insulin-resistance related vascular dysfunction, relative to gastrocnemius muscle feed arteries (14), it is unclear whether metformin impacts insulin-induced vasoreactivity uniformly throughout the skeletal muscle vasculature and whether these effects are modulated by obesity and insulin resistance. Thus, the purpose of this study was to test the hypothesis that chronic metformin treatment enhances vascular reactivity to insulin in feed arteries and 2nd order arterioles of the gastrocnemius (primarily

mixed/fast twitch) and in the feed artery of the soleus (primarily slow twitch) muscles of insulin resistant pre-diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats; and has no effects on these arteries and arterioles in healthy lean rats. The OLETF rat is a model of T2DM characterized by a mutated cholestykinin-1 receptor that results in hyperphagia and obesity (89). These rats exhibit early increases in serum insulin, have hyperglycemia and advanced insulin resistance by 12-20 weeks of age, and continue to progress to overt T2DM between 20-40 weeks (32, 89, 101).

METHODS

Animals

Four week old male obese OLETF (n = 29) and healthy lean Long Evans Tokushima Otsuka (LETO; n = 29) rats (Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan) were individually housed in cages maintained in temperature (21°C) and light controlled, 12:12-hr light-dark cycle (lights off at 1800h), animal quarters. All rats were provided *ad libitum* access to water and standard rodent chow (Formulab 5008, Purina Mills, St. Louis, MO) comprised of ~ 26% protein, 18% fat, and 56% carbohydrate. Body weights and food intakes were recorded on a weekly basis. The University of Missouri Institutional Animal Care and Use Committee approved all experimental protocols.

Experimental Design

At 20 weeks of age LETO and OLETF rats were randomly assigned to untreated or metformin treated groups forming 4 groups: 1) LETO (n=15), 2) LETO + metformin (LETO + MET, n=14), 3) OLETF (n=15), and 4) OLETF + MET (n=14). It should be noted that some data appear in recently-published reports from our laboratory addressing fundamentally

different research questions from that of the present study, i.e. the functional adaptation of the skeletal muscle microvasculature to exercise training (110) and the effects of training and metformin on adipose tissue inflammation (82). Metformin treatment was initiated at 20 weeks of age, as this is the age at which the OLETF rat is known to begin progressing from advanced insulin resistance to overt T2DM (32, 89, 101). We administered metformin (Bosche Scientific) at a dose of 300 mg/kg in the rats' drinking water for three months (20-32 weeks of age), based on previous rodent studies utilizing chronic metformin administration (25, 114). Rats were started on half a dose of metformin (150mg/kg/day) for one week for drug acclimatization, and then increased to 300mg/kg/day, with no noticeable side effects. We carefully monitored the water intake of the rats so that the maximum dose of metformin was not exceeded. The animals had *ad libitum* access to their water (with metformin) until anesthesia was given the morning of sacrifice. The rationale for adopting this approach of treating the animals with metformin until anesthesia is that we wanted to be consistent with what might occur in clinical settings (human patients are not normally instructed to avoid their medications prior to a fasting blood draw).

At 32 weeks of age we anesthetized the rats with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) in the morning following an overnight fast, and measured the animals' body weight and percent body fat (described below). The soleus and gastrocnemius muscles were then harvested for feed artery and arteriole isolation. Subsequently, we collected blood samples for analysis and euthanized the rats by exsanguinations.

Our rationale for this experimental design was that comparisons between LETO and OLETF rats would discriminate potential differences between the vascular reactivity to insulin due to obesity and diabetes. However, the primary focus of this study was to test the

hypothesis that metformin effectively treats advanced insulin resistance, prevents the progression to overt T2DM [analogous to the administration of metformin to pre-diabetic patients in the Diabetes Prevention Program (92)], and improves insulin-mediated vascular function. This was accomplished by comparing OLETF and OLETF+MET rats. To complement these objectives we also treated LETO animals with metformin to achieve a balanced experimental design and examine possible effects in a group of healthy control animals. Finally, we chose to study our animals at 32 weeks of age, as this is the age that obese OLETF rats exhibit pancreatic beta cell failure, advanced insulin resistance, and T2DM (32, 89, 101, 157). However, it is important to note that not all OLETF rats progress to frank T2DM by 32 weeks, thus herein we refer to our 32-week-old OLETF rats as obese and insulin resistant.

Body Composition and Blood Parameters

On the day of experiments we measured body mass of the rats and determined body composition by using a dual energy x-ray absorptiometry machine (Hologic QDR-1000) calibrated for rodents. Whole blood was collected for analysis of glycosylated hemoglobin (HbA1c) by the boronate-affinity HPLC method (Primus Diagnostics, Kansas City, MO) in the Diabetes Diagnostics Lab at the University of Missouri. Serum samples were prepared by centrifugation and stored at -80°C until analysis. A commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO) analyzed all serum chemistry parameters on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA) using commercially available assays according to the manufacturer's guidelines. Plasma insulin concentrations were determined using a commercially available, rat-specific ELISA (Alpco Diagnostics, Salem, NH). Samples were run in duplicate and manufacturer's controls and

calibrators were used according to assay instructions. The homeostasis model assessment-insulin resistance (HOMA-IR) was calculated according to the formula of Matthews et al. (115): [fasting glucose (mg/dl) • fasting insulin (μIU/mL)]/405). Intraperitoneal glucose tolerance tests (IPGTT) were performed at 31 weeks of age (n=6-8 per group), one-week prior to sacrifice, as previously described (158). Briefly, food was removed from the cages 12 hours before each received an intraperitoneal injection of dextrose (50% solution, 2 g/kg body weight). Venipuncture blood samples were collected from the lateral tail vein immediately before (0 minutes) dextrose administration and 15, 30, 45, 60, and 120 minutes after injection. Insulin sensitivity was estimated as the product of the area under the curve (AUC) for glucose using the trapezoidal method (173).

Isolation of Skeletal Muscle Feed Arteries and Arterioles

Gastrocnemius feed arteries (GFA), soleus feed arteries (SFA), 2nd order arterioles from the red portion of the gastrocnemius (G2A-Red), and 2nd order arterioles from the white portion of the gastrocnemius (G2A-White) were isolated, cannulated, and pressurized as previously described (14, 110, 190). During dissection, muscles were placed in ice-cold MOPS-buffered physiological saline solution (PSS) containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS (pH 7.4). Following isolation, vessels were cannulated on glass micropipettes, pressurized, and mounted in MOPS-PSS bath (37 °C) on an inverted microscope outfitted with a video camera for continuous measurement of intraluminal diameter with calibrated videocalipers, as previously described (4, 100). We adjusted the hydrostatic pressure reservoirs connected to the cannulated micropipettes, containing PSS plus albumin (1g/100 mL), to achieve approximate *in vivo* intraluminal pressures of 90 and 67 cmH_2O, for

GFA/SFA and G2A-Red/G2A-White vessels, respectively (4, 188). We treated the vessels with 80mM KCl to verify viability, washed them with PSS, and allowed the vessels to equilibrate for 1 hour at 37˚C. Following equilibration, arteries were pre-constricted with phenylephrine (PE; 10^{-5}-10^{-4} M) to achieve $\geq$30% tone for examination of vasomotor responses to insulin. We have found that skeletal muscle feed arteries and arterioles from OLETF rats do not uniformly develop spontaneous vascular tone, therefore after confirming vessel viability with KCl, we added PE to the vessel bath prior to the first insulin dose-response curve regardless of spontaneous tone. However, for subsequent curves, if sufficient tone ($\geq$ 30% of initial diameter) was present, no further PE was added.

We determined vasomotor responses to insulin in one SFA, GFA, G2A-Red, and G2A-White vessel per animal. The vasoreactivity to insulin was assessed at four concentrations (1, 10, 100, and 1000 μIU/mL) of bovine insulin (dissolved in 0.01 N HCl and diluted 1:10 with PSS containing 1% BSA). To evaluate the role of ET-1 in the vasomotor response to insulin, we incubated vessels with tezosentan (3 μM), a non-selective ET_A and ET_B receptor antagonist, for 20 minutes, and re-assessed the vasoreactivity to insulin (1-1000 μIU/mL). For all insulin curves, the four serial doses of insulin were given in 10-minute intervals, and intraluminal vessel diameters were recorded at the end of the 10 minutes, immediately prior to the introduction of the next dose of insulin. Between the insulin and insulin + tezosentan dose response curves, we washed the vessel baths with fresh PSS and allowed a re-equilibration period of 30 minutes. Following insulin curves, we determined maximal vessel diameter, with Ca^{2+}-free PSS containing 100μM sodium nitroprusside (SNP).

Drugs and Solutions

All drugs and solutions were obtained from Sigma (St. Louis, MO), except albumin (USB Corporation, Cleveland, OH). We prepared all PSS solutions and drugs prior to the study, froze them, and thawed aliquots each day of experiments. All drug solutions were prepared in PSS, except insulin, which was prepared in PSS with BSA (1g/100mL). We prepared SNP fresh daily and protected the serial dilutions from light.

Data Analysis and Statistics

Results are presented as mean ± SEM. Vasodilator responses are presented as percent possible (maximal) dilation, calculated as: $[((D_d - D_b)/(D_{max} - D_b))\bullet 100]$, where D_d is diameter after insulin treatment, D_b is baseline diameter, and D_{max} is maximal vessel diameter. To detect group differences in animal (Table 1) and vessel characteristics (Table 2) we used a one-way analysis of variance (ANOVA) with Fisher's Least Significant Differences (LSD) post-hoc analysis. We confirmed equality of variances for all animal and vessel characteristic variables. Vasomotor responses to insulin were examined using a mixed-design repeated-measures ANOVA, with group as the between-subjects factor and insulin as a repeated factor, followed by Fisher's LSD post-hoc analysis. To compare the vascular reactivity to insulin, and insulin in the presence of tezosentan, we performed a separate mixed-design repeated measures ANOVA, and evaluated main effects of metformin in OLETF and LETO groups, as well as interactions between group, insulin dose, and tezosentan effects. We used IBM SPSS Statistics 19 for Windows (Chicago, IL) for all statistical analyses, and established *a priori* statistical significance of $P < 0.05$.

RESULTS

Animal and Vessel Characteristics

Consistent with our previous reports (14, 32), our untreated OLETF rats had greater body weight, percent body fat, and food intake), and blood lipids, and were insulin resistant (fasting hyperglycemia, hyperinsulinemia, and elevated HbA1c) compared to healthy LETO animals (all $P < 0.05$, Table 2.1 and Figure 2.1). Metformin treatment significantly reduced food intake, body mass, and percent body fat in OLETF rats ($P < 0.05$, Table 2.1). Moreover, metformin significantly lowered HbA1c, an index of chronic glycemic control ($P < 0.001$, Figure 2.1). However, metformin treatment did not lower blood lipids, glucose, insulin, or insulin resistance, as assessed by both the HOMA-IR (115) and glucose responses to an IPGTT compared to untreated OLETF rats (Table 2.1). While metformin did not alter glucose AUC during the IPGTT, insulin AUC was significantly reduced in metformin treated OLETF animals. Although there were no statistically significant differences in plasma glucose, insulin, and HOMA-IR between groups, each of these parameters tended to be lower in metformin-treated OLETF rats (Table 2.1).

Maximal vessel diameters, pre- and post-tezosentan treatment diameters, percent dilation to tezosentan, and percent pre-constriction relative to maximal vessel diameter are presented in Table 2. Tezosentan (20 min incubation with 3μM) produced significant dilation in all resistance arteries within all groups, except for the G2A-Red and G2A-White arterioles of the LETO+MET group (Table 2.2). These results suggest the presence of basal ET-1 constrictor tone. The % dilation produced by tezosentan was significantly greater in the GFA and G2A-Red of OLETF rats than in the same arteries from LETO rats (Table 2.2). Additionally, the SFA of OLETF+MET tended to have a greater dilation to tezosentan compared to SFA of

OLETF (P = 0.09, Table 2.2). Between-group comparisons (within vessel) of the magnitude of pre-constriction indicated no significant differences among groups in SFA, GFA, and G2A-White. Also there were no differences among groups in percent pre-constriction prior to the insulin + tezosentan dose-response experiments, except that pre-constriction of G2A-Red was significantly lower in OLETF compared to LETO rats ($P < 0.05$). Within vessels there were no significant differences among groups in diameter prior to the insulin dose-response experiments. It is has been shown that metformin can blunt contraction to PE (105, 171). However in our experiments this does not appear to be an issue, reflected in the lack of change in percent pre-constriction between the LETO and LETO+MET groups (Table 2.2).

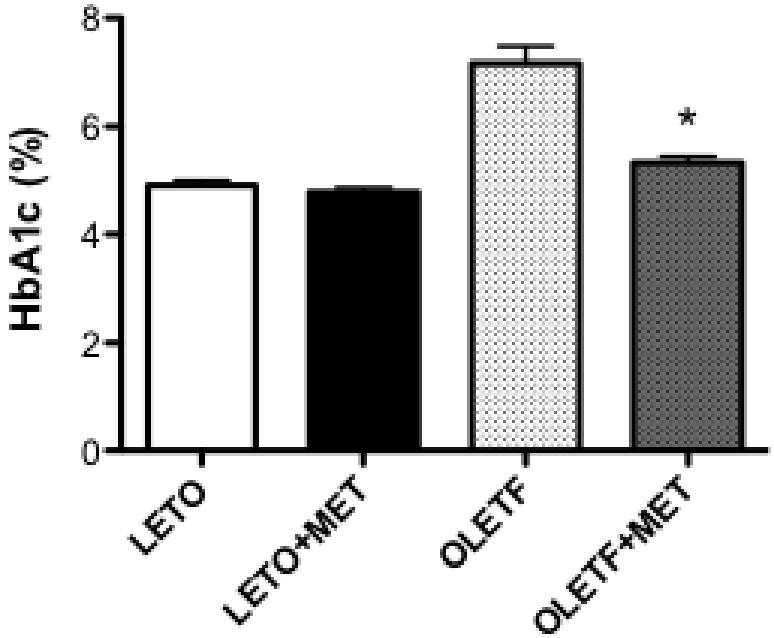

Figure 2.1 Glycosylated Hemoglobin (HbA1c, %) in 32-week old LETO (n=12), LETO + MET (n=11), OLETF (n=13), and OLETF + MET (n=15) rats. All data are expressed as means ± SEM. *Significantly different than OLETF rats ($P < 0.05$).

Table 2.1 Animal Characteristics				
Variable	**LETO**	**LETO + MET**	**OLETF**	**OLETF +MET**
Body Weight (g)	469 ± 12	445 ± 9	687 ± 11*	650 ± 7*#
Body Fat (%)	15.5 ± 0.6	13.5 ± 0.4	35.3 ± 1.1*	32.4 ± 1.0*#
Absolute Food Consumption ($g.wk^{-1}$)	152 ± 4	133 ± 4*	226 ± 4*	208 ± 4*#
Relative Food Consumption ($g.wk^{-1}\ bw^{-1}$)	0.317 ± 0.004	0.295 ± 0.004*	0.334 ± 0.004*	0.323 ± 0.004*#
Total Cholesterol ($mg.dL^{-1}$)	94 ± 3	93 ± 3	153 ± 8*	146 ± 8*
HDL-C ($mg.dL^{-1}$)	26.8 ± 0.6	26.4 ± 0.5	34.0 ± 1.7*	35.4 ± 1.8*
LDL-C ($mg.dL^{-1}$)	58.6 ± 2.8	58.9 ± 2.7	47.2 ± 5.9	43.5 ± 5.7*
Triglycerides ($mg.dL^{-1}$)	44.3 ± 3.3	39.9 ± 2.0	359.8 ± 40.5*	337.4 ± 46.1*
Glucose ($mg.dL^{-1}$)	152 ± 4	150 ± 6	300 ± 12*	283 ± 21*
Insulin ($mg.dL^{-1}$)	3.1 ± 0.4	2.6 ± 0.3	9.9 ± 2.1*	7.1 ± 1.0*
HOMA-IR (glucose*insulin/405)	1.2 ± 0.1	1.0 ± 0.1	7.5 ± 1.8*	5.0 ± 0.9*
Glucose AUC ($mg.dL^{-1}$*120 min)	38553 ± 2867	36508 ± 1798	64839 ± 2875*	59821 ± 5300*
Insulin AUC ($ng.mL^{-1}$*8 hr)	505 ± 148	356 ± 53*	728 ± 281*	497 ± 97

Data presented as mean ± SEM (n = 14–15/group). *Significantly different than LETO ($P < 0.05$), and #significantly different than OLETF ($P < 0.05$). HDL, high-density lipoprotein; LDL, low-density lipoprotein; and TG, triglycerides; HOMA-IR homeostasis model assessment- insulin resistance; and AUC, area under the intraperitoneal glucose tolerance curve.

Table 2.2 Vessel Characteristics

			Vessel Diameter (μm)			% Pre-constriction	
Vessel	**Group**	**N**	**Maximal**	**Before Tezosentan**	**After Tezosentan**	**% Dilation to tezosentan**	**Insulin**	**Insulin + tezosentan**
SFA	LETO	13	184 ± 11	99 ± 10	114 ± 13[a]	22.0 ± 6.8	52.0 ± 4.1	44.8 ± 3.0
	LETO + MET	13	177 ± 12	97 ± 11	115 ± 15[a]	19.8 ± 7.3	46.7 ± 3.4	47.2 ± 3.9
	OLETF	15	209 ± 6	125 ± 12	144 ± 13[a]	17.9 ± 5.5	50.2 ± 3.5	48.3 ± 3.7
	OLETF + MET	14	212 ± 7	97 ± 6	133 ± 8[a]	35.9 ± 9.3†	48.2 ± 2.4	50.1 ± 2.8
GFA	LETO	14	289 ± 12	217 ± 10	225 ± 8[a]	4.5 ± 1.4	41.4 ± 2.4	40.9 ± 3.7
	LETO + MET	14	287 ± 12	188 ± 10	202 ± 10[a]	8.2 ± 3.3	40.9 ± 2.4	43.1 ± 3.3
	OLETF	13	289 ± 12	221 ± 18	243 ± 14[a]	14.4 ± 6.1*	44.4 ± 3.0	41.5 ± 3.5
	OLETF + MET	13	305 ± 7	206 ± 8	225 ± 9[a]	10.8 ± 3.2	45.0 ± 2.2	39.9 ± 3.0
G2A-Red	LETO	12	191 ± 12	124 ± 10	153 ± 10[a]	23.5 ± 4.7	41. 9 ± 4.0	49. 9 ± 2.6
	LETO + MET	12	178 ± 8	129 ± 10	141 ± 8	13.3 ± 6.5	39.5 ± 2.7	48.2 ± 4.1
	OLETF	13	197 ± 10	154 ± 12	174 ± 12[a]	8.7 ± 2.1*	44.8 ± 3.4	38.9 ± 3.5*
	OLETF + MET	12	204 ± 6	152 ± 8	182 ± 6[a]	16.2 ± 4.0	39.4 ± 4.0	40.1 ± 4.0
G2A-White	LETO	12	178 ± 11	126 ± 11	142 ± 13[a]	10.3 ± 4.2	38.2 ± 3.0	45.3 ± 3.4
	LETO + MET	11	181 ± 16	151 ± 14	162 ± 13	8.85 ± 4.0	36.2 ± 4.4	42.4 ± 4.7
	OLETF	13	189 ± 12	149 ± 10	167 ± 9[a]	15.5 ± 8.0	33.4 ± 2.0	38.9 ± 3.7
	OLETF + MET	12	192 ± 12	169 ± 13	181 ± 13[a]	3.5 ± 1.0	33.6 ± 2.4	35.6 ± 3.6

Data presented are mean ± SEM. [a]$P \leq 0.05$ vs. before tezosentan. * $P \leq 0.05$ vs. LETO. †$P = 0.09$ vs. OLETF. SFA, soleus feed artery; GFA, gastrocnemius feed artery; G2A-Red, second order arterioles perfusing the red portion of the gastrocnemius; G2A-White, second order arterioles perfusing the white portion of the gastrocnemius; MET, metformin.

Effects of Metformin on Microvascular Reactivity to Insulin

Although insulin produced a dose-related dilation of SFA of healthy LETO rats, it only produced a significant dilation (16%) from baseline in the SFA of healthy LETO rats at the highest supra-physiological dose of 1000 μIU/mL insulin (Figure 2.2A). There were no differences in the vasoreactivity to insulin between LETO and LETO+MET animals. In contrast in SFA of OLETFs insulin produced significant vasodilation above baseline at all insulin doses, and greater vasodilation at each successive dose, indicating a progressive vasodilatory response to insulin. No statistically significant vasodilatory effects of insulin were observed in the GFA (Figure 2.3A and C), G2A-Red (Figure 2.4A and C), or G2A-White (Figure 2.5A and C) vessels in LETO or OLETF animals.

Metformin treatment did not significantly alter the vascular reactivity to insulin in SFA or gastrocnemius arteries of healthy LETO animals (Figures 2.2A, 2.3A, 2.4A, and 2.5A). Conversely, the vasodilatory response to insulin in SFA of OLETF rats treated with metformin was decreased at all insulin doses (Figure 2.2C). Metformin treatment of OLETF rats did not appear to affect insulin responses of GFA, G2A-Red, or G2A-White arteries of OLETF rats. (Figures 2.3C, 2.4C, and 2.5C).

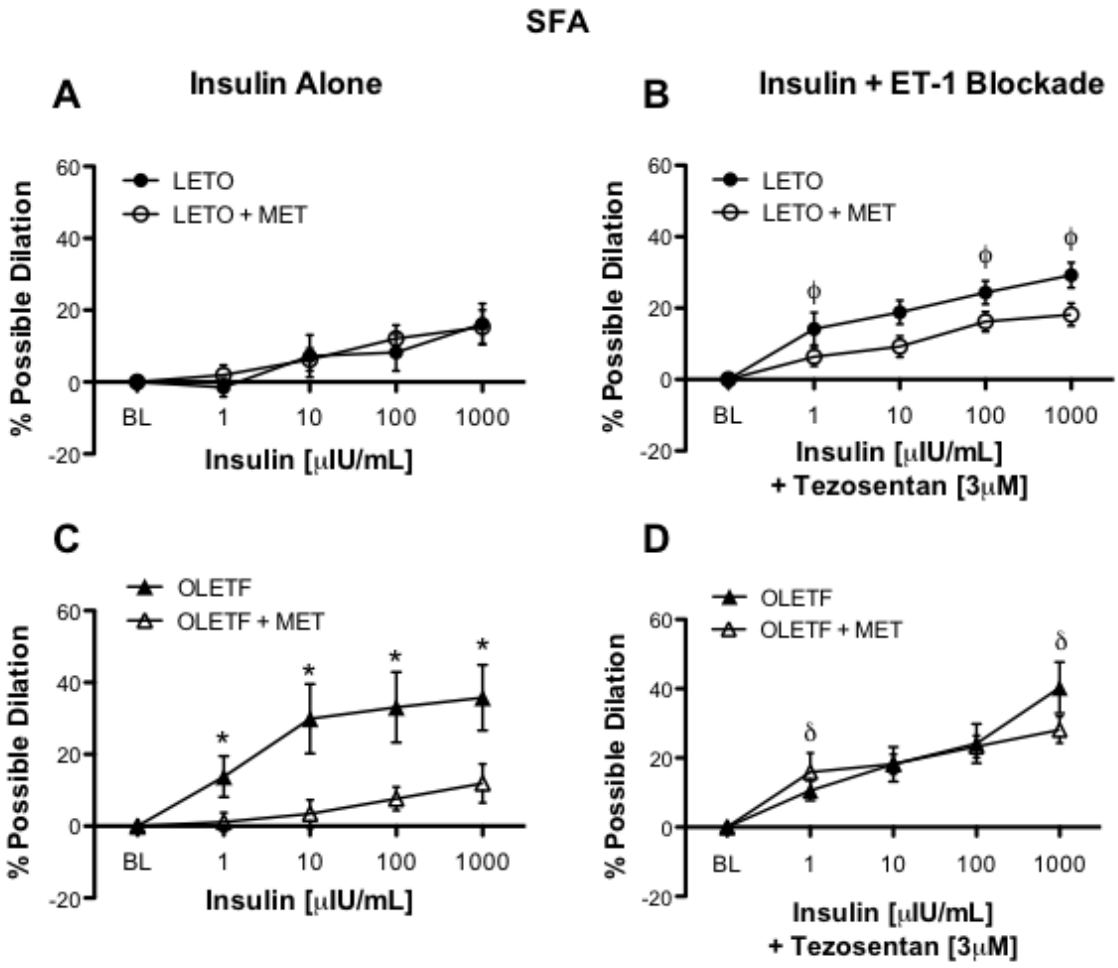

Figure 2.2 Insulin-induced vascular reactivity in SFA of LETO (A and B) and OLETF (C and D) rats. Left panels (A and C) present data from insulin dose-response curves performed under control conditions, and right panels (B and D) present data from insulin dose-response curves in the presence of 3 μM tezosentan, a non-specific inhibitor of Endothelin-1 (ET-1) receptors. *Significant difference between metformin treatment and untreated group. ϕSignificant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. δSignificant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μIU/mL, micro international units per milliliter. Note: Insulin produced a significant dilation from baseline in the SFA of LETO only at 1000 μIU/mL (2A), however insulin-induced dilation of the SFA was significantly greater than baseline at all insulin doses in OLETF (2B).

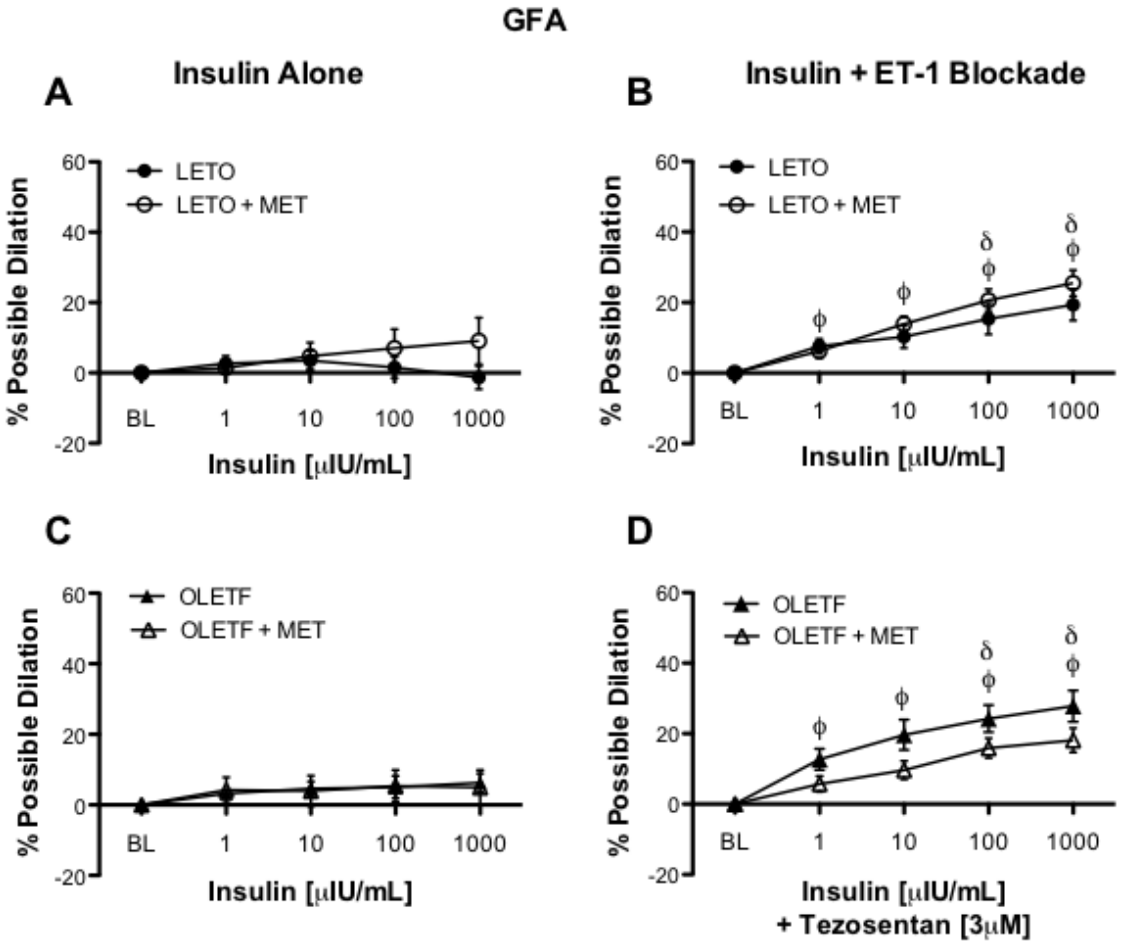

Figure 2.3 Insulin-induced vascular reactivity in GFA of LETO (A and B) and OLETF (C and D) rats. Left panels (A and C) present data from insulin dose-response curves performed under control conditions, and right panels (B and D) present data from insulin dose-response curves in the presence of 3 μM tezosentan, a non-specific inhibitor of Endothelin-1 (ET-1) receptors. ϕSignificant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. δSignificant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μIU/mL, micro international units per milliliter.

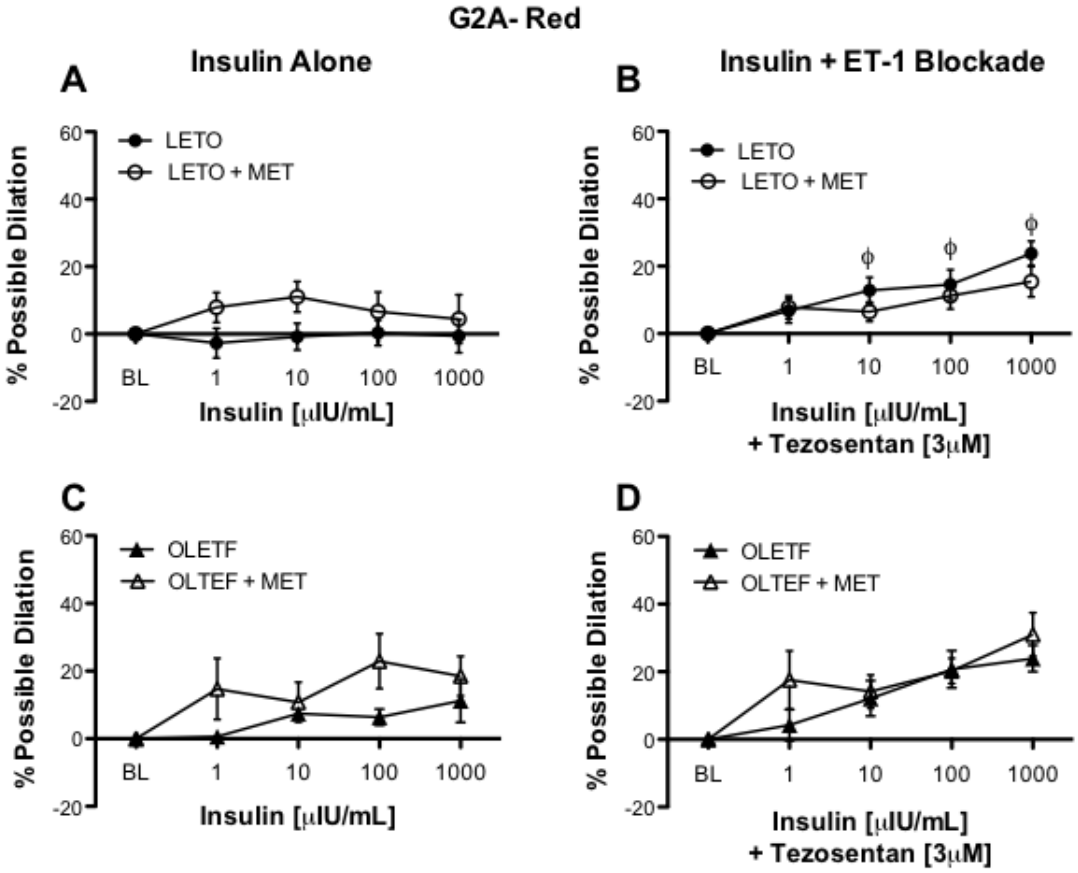

Figure 2.4 Insulin-induced vascular reactivity in G2A-Red of LETO (A and B) and OLETF (C and D) rats. Left panels (A and C) present data from insulin dose-response curves performed under control conditions, and right panels (B and D) present data from insulin dose-response curves in the presence of 3 μM tezosentan, a non-specific inhibitor of Endothelin-1 (ET-1) receptors. ϕSignificant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. δSignificant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μIU/mL, micro international units per milliliter.

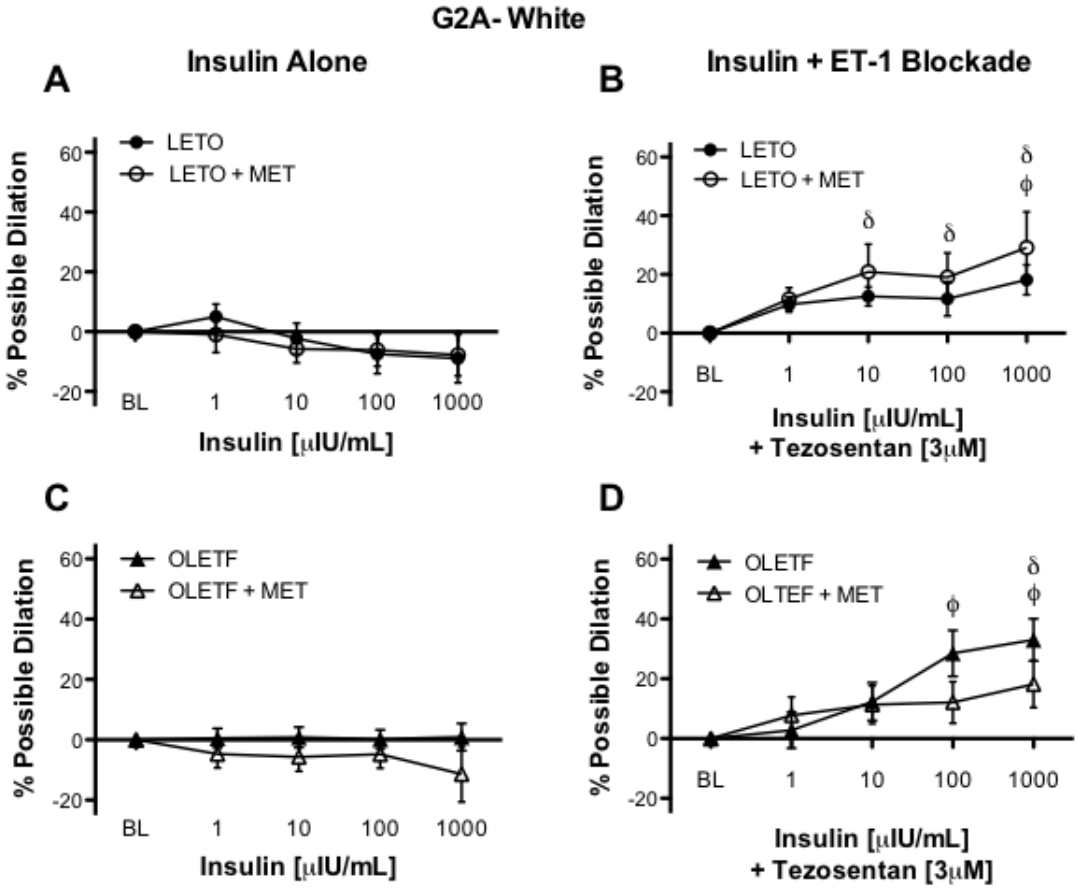

Figure 2.5 Insulin-induced vascular reactivity in G2A-White of LETO (A and B) and OLETF (C and D) rats. Left panels (A and C) present data from insulin dose-response curves performed under control conditions, and right panels (B and D) present data from insulin dose-response curves in the presence of 3 μM tezosentan, a non-specific inhibitor of Endothelin-1 (ET-1) receptors. ϕSignificant effect of insulin + ET-1 blockade compared to insulin alone in untreated animals. δSignificant effect of insulin + ET-1 blockade compared to insulin alone in metformin-treated animals. μIU/mL, micro international units per milliliter.

Role of Endothelin-1 in Vasomotor Response to Insulin

Tezosentan treatment dilated all arteries in all groups. In the presence of tezosentan, arteries/arterioles from LETO animals dilated in response to insulin to a greater extent compared to control conditions. Insulin-induced dilation of the SFA was increased at 1, 100, and 1000 μIU/mL concentrations of insulin (Figures 2.2A vs. 2B) after treatment with tezosentan. Tezosentan increased dilation in GFA at all insulin doses (Figures 2.3A vs. 2.3B); in the G2A-Red at 10, 100, and 1000 μIU/mL insulin (Figure 2.4A vs. 2.4B); and in the G2A-White at 1000 μIU/mL insulin (Figures 2.5A vs. 2.5B).

In OLETF rat SFAs, although the insulin-stimulated dose/response curve for vasodilation appeared depressed by tezosentan treatment, there was no statistically significant difference between the effects of insulin after ET-1 blockade in the SFA (Figures 2.2C vs. 2.2D). Similarly, in the G2A-Red (Figures 2.4C vs. 2.4D) the response after ET-1 blockade was not different than dilation to insulin alone. In the GFA, tezosentan increased insulin-induced dilation at all insulin, doses (Figures 2.3C vs. 2.3D). Tezonsentan increased insulin-induced dilation of G2A-White vessels at 100 and 1000 μIU/mL insulin (Figures 2.5C vs. 2.5D).

Although ET-1 receptor blockade did not alter insulin-induced vasodilation in the SFA (Figures 2.2A and 2.2B) or G2A-Red arteries (Figures 2.5A and 2.5B) of LETO rats, ET-receptor blockade increased insulin-induced vasodilation compared to insulin alone in the GFA of metformin-treated LETO at 100 and 1000 μIU/mL insulin (Figure 2.3A vs. 2.3B). Tezosentan also increased insulin-induced dilation in G2A-White of LETO + MET at 10, 100, and 1000 μIU/mL insulin, respectively (Figures 2.5A vs. 2.5B)

In OLETF-metformin treated animals, ET-1 blockade significantly increased vasodilation to insulin in all of the arteries except the G2A-Red (Figures 2.4C and 2.4D) [SFA at 1 and

1000 µIU/mL insulin (Figure 2.2C vs. 2.2D); GFA at 100 and 1000 µIU/mL insulin (Figures 2.3C vs. 2.3D); and G2A-White at 1000 µIU/mL insulin (Figures 2.5C vs. 2.5D)]. Blockade of ET-1 receptors by tezosentan abolished differences between insulin-induced vasodilation of SFAs from untreated and metformin-treated OLETF groups (Figures 2.2C and 2.2D), suggesting that insulin-induced ET-1 mediated vasoconstriction contributes to the reduced vasodilatory response to insulin in the SFA of metformin treated OLETF rats.

Role of Metabolic Health in Vasomotor Responses to Insulin with Metformin

We evaluated correlations between insulin-induced vasodilation and glycemic control outcomes to better understand the vascular response to insulin with metformin treatment. We only examined insulin-induced dilation in the SFA, as this was the only vessel that significantly dilated in response to insulin. Across all groups and insulin doses there was no significant correlation between HbA1c or HOMA-IR and insulin-induced dilation in the SFA, suggesting that insulin-induced vasodilation is not correlated with clinical parameters of insulin resistance in the OLETF rat.

DISCUSSION

Metformin is one of the most commonly used therapeutic drugs for pre-diabetic and diagnosed T2DM patients. Metformin not only reduces chronic hyperglycemia, but also has been reported to have vasculoprotective effects (77, 88, 91, 113) and is linked to reduced cardiovascular events (142). The primary purpose of this investigation was to test the hypothesis that chronic metformin treatment enhances vascular reactivity to insulin in resistance arteries of skeletal muscle of insulin resistant, pre-diabetic OLETF rats. The major

findings of our study are that (i) three months of metformin treatment in hyperphagic, obese, and insulin resistant OLETF rats did not enhance insulin-stimulated vasodilation in skeletal muscle resistance arteries, despite improvements in one chronic measure of glycemic control (HbA1c); and (ii) paradoxically, metformin reduced insulin-stimulated vasodilation in the OLETF SFA via increased insulin-induced activation of ET-1.

It is important to note that metformin significantly lowered food intake, body weight, adiposity, and HbA1c indicating that the dose and duration of metformin treatment used were sufficient to produce whole body metabolic effects characteristic of this common anti-hyperglycemic drug (1, 91, 178, 181, 182). The reduced adiposity induced by metformin likely contributed to the HbA1c improvements by enhancing regulation of hepatic glucose production. Importantly, however, metformin did not improve fasting glucose, glucose AUC during an IPGTT, or HOMA-IR, suggesting that skeletal muscle insulin sensitivity was likely not altered by metformin treatment in this animal model. Our speculation of unaltered insulin sensitivity is consistent with a study in humans showing unchanged insulin-stimulated muscle glucose uptake and whole body glucose uptake following treatment with metformin (70, 191). Since we did not directly assess insulin sensitivity, the trends for improvement with HOMA-IR and glucose AUC may underestimate metformin's real effect. Further, the apparent paradox of improved HbA1c despite unchanged glucose tolerance can be explained by differences between long and short-term indices of glycemic control. HbA1c most likely reflects the regulation of hepatic glucose output, insulin-stimulated glucose uptake, and insulin production from the pancreas over the course of days to weeks, whereas fasting glucose and IPGTT only reflect glycemic control at one particular point in time. Thus, our data suggest, but do not prove, that improvements in glycemic control occurred as a result of

reduced body weight and adiposity rather than from changes in glucose tolerance or insulin-induced vasodilation in skeletal muscle resistance arteries.

Metabolic and vascular studies in humans and animals utilizing metformin in T2DM have yielded conflicting results for both vascular function and changes in insulin sensitivity. In regard to metabolic outcomes, some studies report modest improvements in insulin-mediated glucose disposal (51, 150), some report no change (43, 45, 70), while others only report effects on fasting glucose clearance (5, 74, 131). Only a handful of studies have investigated vascular effects of metformin in humans and animals. Metformin is reported to increase basal and post oral glucose tolerance test forearm blood flows (79), and enhance insulin-stimulated muscle blood flow during exercise (70) in obese patients with T2DM. Also, metformin has been reported to improve acetylcholine (ACh) endothelium-dependent vasodilation in mesenteric arteries of diabetic rats (114), and improve ACh-stimulated forearm blood flows in insulin resistant T2DM humans (113). In the latter study metformin improved fasting insulin sensitivity (HOMA-IR) in T2DM patients, and interestingly, the degree of fasting insulin resistance following metformin treatment was the sole predictor of improved endothelial function. Given that the metformin-treated OLETF rats in the present study did not exhibit improvements in fasting glucose or insulin levels, it may therefore not be surprising that they also did not display improved vascular reactivity to insulin in skeletal muscle resistance arteries. Furthermore, our finding of reduced insulin-stimulated vasodilation in the SFA of metformin-treated OLETF rats is consistent with a study that reported no effect of metformin on endothelial dysfunction (ACh-stimulated forearm blood flow) in T2DM patients (131). Additionally, insulin-induced dilation in the SFA of OLETF rats revealed no significant correlation between HbA1c or HOMA-IR and insulin-induced

dilation in the SFA across all groups and insulin doses, suggesting that insulin-induced vasodilation is not correlated with clinical parameters of insulin resistance in the OLETF rat. Overall, these results suggest that the insulin-sensitizing actions of metformin primarily occur in the liver (e.g. reduced hepatic glucose production) and in skeletal muscle (e.g. enhanced insulin-stimulated glucose uptake) (2, 106, 142) with minimal effects on insulin-mediated vasodilation in skeletal muscle. The reported effects of metformin on muscle blood flow responses to insulin (70) do not appear to be mediated by changes in direct effects of insulin on skeletal muscle resistance arteries or arterioles.

Role of ET-1 in insulin-induced vasoregulation in SFA of OLETF rats.

ET-1 receptor blockade of SFAs from OLETF rats appeared to decrease insulin-induced dilation from 30 to 40 % possible dilation at doses of 10 and 100 μIU/ml to only 20% dilation. These results suggest that ET-1 released in response to these doses of insulin, in the absence of ET-1 receptor blockade, is signaling a net dilation. Our results do not allow determination of the mechanism for this effect but it is possible that endothelial ETB receptor effects are increased in the endothelium of OLETF rat SFA's resulting in enhanced insulin-induced dilation as shown in Figure 2C.

Our observation of a reduced vasodilatory response to insulin in the SFA of metformin-treated OLETF rats appears to be partially the result of changes in ET-1 signaling. Indeed, in the presence of ET-1 inhibition, insulin-stimulated vasodilation in the SFA of metformin-treated OLETF rats was increased compared to responses to insulin alone, so that the blunted insulin-induced vasodilation produced by metformin treatment in SFA of OLETF rats disappeared after blockade of the ET-1 receptors. Thus metformin treatment appeared to decrease insulin-induced dilation (Figure 2.2C) but blockade of ET-1 receptors seemed to

abolish the difference between insulin-induced dilation in SFA of untreated and metformin treated OLETF rats (Figure 2.2D). Furthermore, metformin tended to induce a greater dilatory response in the SFA during pre-treatment with the ET-1 inhibitor, tezosentan (prior to insulin stimulation), compared to untreated OLETF rats (Table 2.2), suggestive of a greater contribution of ET-1 induced constriction to basal tone in the SFA of metformin-treated OLETF rats. This enhanced dilation to ET-1 receptor block may contribute to the blunted dilation response to insulin (Figure 2.2C) in that it would decrease the available vasodilator reserve of these SFAs. Importantly, these effects of metformin on the SFA of OLETF rats were not observed in any of the skeletal muscle resistance arteries of healthy LETO animals treated with metformin (Figures 2.2-5). Collectively, our data suggest that while metformin exerts favorable effects on body composition and HbA1c, it did not improve insulin-induced vasodilation in skeletal muscle resistance arteries of obese, insulin-resistant OLETF rats. Although metformin treatment appeared to alter ET-1 signaling in SFA of OLETF rats, our results do not allow us to evaluate whether metformin increased insulin-induced ET-1 production by the endothelium or altered the relative roles of ETA and ETB receptor signaling in endothelium or vascular smooth muscle of these arteries.

Our finding that insulin did not induce vasodilation in skeletal muscle arteries and arterioles of healthy lean LETO rats (except at the highest supraphsyiological dose of insulin in the SFA, Figure 2.2A) is not unexpected, given published data from Eringa et al. reporting a lack of insulin induced dilation in skeletal muscle resistance arteries of lean Zucker rats(53). However, in the presence of ET-1 receptor blockade insulin induced 15-30% dilation in the SFA of LETO rats (Figure 2.2B). These findings are also consistent with the 20% dilation with ET-1 blockade in skeletal muscle resistance arteries of lean Zucker rats

(53).

Metformin has been shown to decrease physical fitness and increase lactate levels, which could translate to reduced ambulation. Although we did not directly assess physical fitness or physical activity in this study, we acknowledge that it is possible that metformin treated animals may have reduced ambulation, which could possibly explain reduced insulin-induced vasodilation of skeletal muscle resistance arteries. In future studies we plan to measure ambulatory cage activity and physical fitness in metformin treated animals.

In summary, our data demonstrate that 12 weeks of metformin treatment improved glycemic control, reduced adiposity and body weight, and did not improve insulin-stimulated vasodilation in skeletal muscle resistance arteries or arterioles of OLETF rats. The effect of metformin treatment on insulin-induced dilation in isolated SFA was not present in the presence of ET-1 receptor blockade. Metformin treatment did not alter insulin-induced vasodilation in gastrocnemius muscle feed arteries and arterioles of OLETF rats. Also, ET-1 receptor blockade had similar effects on insulin-induced dilation in gastrocnemius arteries of LETO and OLETF rats. Future work is needed to definitively determine the effects of metformin on *in vivo* skeletal muscle vascular responses to insulin and the role of these processes in the protection against the development of T2DM. Overall, this study provides evidence that while treatment of advanced insulin resistance in the obese OLETF rat with metformin exerts favorable effects on body composition and reverses impairments in HbA1c, it does not enhance the vasodilatory responses to insulin in isolated skeletal muscle resistance arteries.

PERSPECTIVES

Metformin is a widely-used anti-diabetic drug, however its effects on insulin-stimulated dilation of skeletal muscle resistance arteries were unknown. Our study demonstrates that metformin does not enhance insulin-stimulated vasodilation of skeletal muscle resistance arteries in the hyperphagic, obese OLETF rats (an established rodent model of obesity/T2DM). Thus, metformin's beneficial effects on body weight, food consumption, and glycemic control appear to be independent of changes in the microvascular actions of insulin.

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GRANTS

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CHAPTER 3

STUDY TWO:

VOLUNTARY WHEEL RUNNING PREVENTS IMPAIRMENTS IN AORTIC INSULIN-STIMULATED RELAXATION OF OBESE INSULIN RESISTANT OLETF RATS

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ABSTRACT

Impairments in the vascular actions of insulin play a critical role in the development of metabolic and vascular dysfunction in obesity. Physical activity enhances endothelial function and insulin-stimulated blood flow in obese insulin resistant humans and animals. The specific role of physical activity, independent of changes in body composition, on the vascular actions of insulin in large conduit arteries is unknown. **OBJECTIVE:** To test the hypothesis that daily voluntary wheel running in rats prone to obesity and insulin resistance protects against impairments in aortic insulin-stimulated vasorelaxation and vascular insulin signaling, independent of changes in adiposity. **METHODS:** Sedentary, hyperphagic, and obese Otsuka Long Evans Tokushima Fatty (OLETF) rats were randomly assigned to: (1) continued sedentary (SED, n=10), (2) voluntary wheel running (RUN, n=10), or (3) sedentary + diet restriction (DR, n=10; fed 70% of SED to lower adiposity to levels of RUN) for 8 weeks (12-20 weeks of age). At 20 weeks we assessed aortic vasoreactivity and determined aortic insulin signaling in all three groups. **RESULTS:** RUN and DR rats exhibited markedly lower adiposity (7.1±0.4 and 15.7±1.1 %body fat, respectively), relative to SED (27±1.2% body fat), as well as improved blood lipid profiles and systemic markers of insulin resistance. Endothelial dependent vasorelaxation was not different among groups. However, the impaired vascular reactivity to insulin in the aorta of SED rats was augmented in RUN but not DR rats. The improved insulin-stimulated relaxation in the aorta of RUN animals was in part mediated by a decrease in insulin-mediated ET-1 vasocontraction compared to SED. The ratio of peNOS/eNOS and pERK/ERK in response to insulin in the aorta was similar in RUN and SED, suggesting that aortic insulin signaling to nitric oxide and ET-1 was unaltered by physical activity. **CONCLUSIONS:** Collectively, we show that

physical activity-related improvements in insulin-stimulated vasorelaxation may not be contingent on changes in adiposity or vascular insulin signaling, in obese insulin resistant OLETF rats.

INTRODUCTION

Despite the well-known health benefits of regular physical activity, humans have become progressively more physically inactive and sedentary over the past several decades. Currently more than one-third of Americans are obese (135) and the underlying causes of this obesity epidemic appear to be largely related to a sedentary lifestyle and over-nutrition (21-23, 34, 172). Cumulative evidence indicates that lack of regular physical activity and obesity are important contributors to the development of metabolic syndrome, type 2 diabetes, and cardiovascular disease (20, 68). Cardiovascular diseases are the leading cause of morbidity and mortality in obese, insulin-resistant individuals (125).

Obesity is characterized by insulin resistance and endothelial dysfunction, both major risk factors for the development of cardiovascular disease (33, 57, 159). While endothelial dysfunction is well established as a classic marker of vascular disease, evidence indicates that insulin resistance is also a prominent component of hypertension, coronary artery disease, and atherosclerosis (153). In addition to the classic metabolic actions of insulin promoting glucose disposal, insulin has important vascular actions, including increased blood flow, vasodilation and capillary recruitment (125). Abnormalities in the vascular reactivity to insulin in obese and physically inactive type 2 diabetic humans and animals, contribute to reduced skeletal muscle glucose uptake by limiting perfusion, resulting in metabolic dysregulation in obesity and type 2 diabetes (90, 97, 110, 120, 121, 185). It is clear that

impairments in the vascular actions of insulin play a critical role in the development of metabolic and vascular dysfunction in obesity.

Increased physical activity improves the cardiovascular actions of insulin in diseased animals and humans. Evidence in humans shows that exercise training enhances endothelial function and insulin-stimulated blood flow in conduit vessels of insulin-resistant sedentary (62), and type 2 diabetic individuals (47, 86). Furthermore, we have previously demonstrated that daily voluntary wheel running effectively prevents, and reverse impairments in insulin-mediated vasodilation in the skeletal muscle microcirculation of obese insulin resistant Otsuka Long Evans Tokushima Fatty (OLETF) rats (120, 122). However, it is not clear if these effects are specifically driven by increased activity or if they are also related to reduced adiposity typically seen with physical activity. Thus, the specific role of physical activity, independent of changes in body composition on the vascular actions of insulin in conduit vessels is relatively unknown. A greater understanding of the influence of lifestyle modifications, including regular physical activity and diet modifications on vascular insulin resistance may lead to more effective strategies aimed at prevention and treatment of obesity-related metabolic and cardiovascular diseases.

Accordingly, the purpose of this study was to test the hypothesis that eight weeks of daily voluntary wheel running improves insulin-stimulated relaxation in the aorta and aortic vascular insulin signaling, independent of changes in adiposity, in obese insulin resistant OLETF rats. We designed this study to examine the efficacy of daily voluntary wheel running in preventing disease progression. The OLETF rat is characterized by a mutated cholestykinin-1 receptor that results in hyperphagia and obesity (89). OLETF rats exhibit early increases in serum insulin, have hyperglycemia and advanced insulin resistance by 12-

20 weeks of age, and continue to progress to overt type 2 diabetes between 20-40 weeks (32, 89, 101).

METHODS

Animals

Four week old male obese OLETF (n = 30) rats (Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan) were individually housed in cages maintained in temperature (21°C) and light controlled, 12:12-hr light-dark cycle (lights off at 1800h), animal quarters. All rats remained sedentary between four and twelve weeks of age and were provided *ad libitum* access to water and standard rodent chow (Formulab 5008, Purina Mills, St. Louis, MO) comprised of ~ 26% protein, 18% fat, and 56% carbohydrate. The University of Missouri Institutional Animal Care and Use Committee approved all experimental protocols.

Experimental Design

At 12 weeks, rats were randomized to one of three groups: 1) sedentary 12-20 weeks (SED; n=10, 2) sedentary + diet restriction (fed 70% of *ad libitum* fed SED animals) 12-20 weeks (DR; n=10); or 3) voluntary wheel running and *ad libitum* fed, 12-20 weeks (RUN; n=10). Animals in the RUN group were housed with running wheels connected to a Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA, USA) for determination of daily running distance.

Our rationale for this design was that comparisons between SED, DR, and RUN provide insight into the efficacy of daily voluntary wheel running in preventing disease progression. Under sedentary conditions, OLETF rats exhibit early increases in serum insulin, have hyperglycemia and advanced insulin resistance by 12-20 weeks of age, and

continue to progress to overt type 2 diabetes between 20-40 weeks of age (32, 89, 101, 156, 162). In previous studies we have shown that diet restriction in OLETF rats attenuates gains in adiposity and mitigates the advancement of insulin resistance (120, 158). Therefore direct comparisons between DR and SED provide insight into the effects of attenuating gains in body mass and adiposity, whereas comparisons between DR (lowered adiposity but sedentary) and RUN (lowered adiposity plus daily wheel running) allow us to evaluate specific effects of daily voluntary wheel running, independent of changes in body composition.

At 20 weeks of age we anesthetized the rats with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), harvested tissues and euthanized the animals by exsanguination, in full compliance with the American Veterinary Medical Association Guidelines on Euthanasia. The wheels of the RUN group were locked and food was removed from the cages of all groups ~14 hrs before the rats were sacrificed.

Body Composition and Blood Parameters

On the day of experiments we measured body mass of the rats and determined body composition by using a dual energy x-ray absorptiometry machine (Hologic QDR-1000) calibrated for rodents. Subsequently, we collected blood samples for analysis and euthanized the rats by exsanguination. Serum samples were prepared by centrifugation and stored at -80°C until analysis. Glucose, cholesterol, triglycerides, and non-esterified fatty acids (NEFA) assays were performed by a commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO) on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA) using commercially available assays according to the manufacturer's guidelines. Plasma insulin concentrations were determined using a commercially available,

rat-specific ELISA (Alpco Diagnostics, Salem, NH). Samples were run in duplicate and manufacturer's controls and calibrators were used according to assay instructions. The homeostasis model assessment-insulin resistance (HOMA-IR) was calculated according to the formula of Matthews et al. (115): [fasting glucose (mg/dl) • fasting insulin (μIU/mL)]/405).

Assessment of Aortic Vascular Function.

Isolation and assessment of thoracic aortic function was determined as previously described (32, 78, 175). Briefly, immediately following exsanguination we removed the thoracic aorta and dissected and cleaned the vessel of all connective and adipose tissue in ice-cold Krebs-bicarbonate buffer (4°C). The thoracic aorta was segmented into aortic rings, and their outer diameter, inner diameter, and axial length were measured with an Olympus microscope and NIH ImageJ software. We mounted the aortic rings on myographs and submerged in 20-ml water baths containing physiological Krebs solution maintained at 37°C for 1 hour to allow for equilibration. We determined the optimal vessel diameter, which elicited a maximal response to 60 mM KCL (L_{max}); this ranged from 130-140% passive diameter. Aortic vasoreactivity was assessed with cumulative concentration-response curves to Acetylcholine (ACh, 10^{-10} to 10^{-4}M), insulin (10 to 10,000 μIU/mL), endothelin-1 (ET-1, 10^{-10} to 10^{-7} M), and sodium nitroprusside (SNP, 10^{-10} to 10^{-4}M). Mounted rings were pre-constricted with a submaximal concentration of phenylephrine (PE, $3E^{-7}$ M) prior to ACh, insulin and SNP dose response curves. The vasoreactivity to insulin was assessed at four concentrations (10 to 10,000 μIU/mL) of human insulin (Novolin) in 10-minute intervals. We evaluated the contribution of ET-1 in the aortic vasomotor response to insulin, by incubating separate aortic rings vessels with either tezosentan (3 μM), a non-selective ETA

and ETB receptor antagonist, or BQ-123 (1 μM), a specific ETA receptor antagonist, for 20 minutes, and assessed the vasoreactivity to insulin (10-10,000 μIU/mL). Following each drug dose response curve, we washed the vessel baths with warm Krebs-bicarbonate buffer (37 °C) and allowed a re-equilibration period of 30 minutes. For insulin, ACh, and SNP curves, relaxation at each concentration was measured and expressed as percent maximum relaxation, where 100% relaxation is equivalent to loss of all tension developed in response to phenylephrine (31).

In vitro insulin signaling

The abdominal aorta was dissected and cleaned of all connective and adipose tissue in ice-cold Krebs-bicarbonate buffer. We cut the abdominal aorta into two segments, and incubated each separately in MOPS-buffered PSS (37 °C) for 50 min. During the final 20 minutes of incubation we added insulin (100 μIU/mL) or vehicle (PSS containing 1% BSA) to the samples. The abdominal aortas were then removed and placed in Laemmli buffer (62.5 mMTris, pH 6.8, 6 M urea, 160 mM 1,4-dithiothreitol, 2% SDS, and 0.0001% bromophenol blue) supplemented with Sigma phosphatase inhibitor cocktails 1 and 2 (1:100), and the vessels were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Western blotting to determine protein expression in the abdominal aorta was performed as previously described (32, 120). Briefly, samples were boiled and sonicated; and protein was quantified using the Quant-iT protein assay (Invitrogen, Carlsbad, CA). Samples were then diluted with Price-Laemmli buffer, and 10ug protein of each sample was loaded onto into a SDS-PAGE gel for separation by electrophoresis. Next, proteins were transferred to polyvinylidenediflouride (PVDF) membranes and probed with primary antibodies: eNOS (1:1000, BD Biosciences, Sparks, MD), phospho-specific eNOS at Ser1177 (1:250, BD

Biosciences), p44/42-ERK (1:500, Cell Signaling), and phospho-specific p44/42-ERK at Thr202/Tyr204 (1:250, Cell Signaling). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies. Protein bands were quantified using a densitometer (Bio-Rad, Hercules, CA). Content of total and phospho-specific densities were quantified as previously described (154, 155). For comparison all total and phospho-specific proteins were normalized to the SED group.

Citrate Synthase Activity

We assessed citrate synthase activity in the red and white portion of the vastus lateralis muscle as a measure of the adaptive response to physical activity on skeletal muscle oxidative capacity, as previously described by our group (156) using the methods of Srere (170).

Drugs and Solutions

All drugs and solutions were obtained from Sigma (St. Louis, MO), except albumin (USB Corporation, Cleveland, OH). The Krebs-bicarbonate buffer solution contained (in mM) 131.5 NaCl, 5.0 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 11.2 glucose, 20.8 NaHCO3, 0.003 propranolol, and 0.025 EDTA. The solution was aerated with a 95% O2-5% CO2 mixture (pH7.4) and maintained at 37°C. We prepared ACh, ET-1, and tezosentan to the study, froze them, and thawed aliquots on each day of experiments. All drug solutions were prepared in Krebs-bicarbonate buffer, except insulin, which was prepared fresh daily in physiological saline solution with BSA (1g/100mL). We prepared SNP fresh daily and protected the serial dilutions from light.

Data Analysis and Statistics

Results are presented as mean ± SEM. We evaluated the effects of RUN and DR (compared to SED) on all dependent variables using a one-way analysis of variance (ANOVA). We analyzed the drug dose-response curves from vasomotor function experiments by repeated measures two-way (group x dose) ANOVAs, and evaluated main effects of group, interactions between group and dose, and simple effects. Basal and insulin stimulated protein expression was analyzed utilizing two-way (group x insulin stimulation) ANOVAs. When appropriate, Fisher's Least Significant Differences (LSD) post-hoc analysis was used. We used IBM SPSS Statistics 19 for Windows (Chicago, IL) for all statistical analyses, and established *a priori* statistical significance of $P < 0.05$.

RESULTS

Body Composition

Voluntary wheel running prevented significant gains in body weight, and reduced total fat mass between 12 and 20 weeks (Figure 3.1; $P < 0.05$ SED vs RUN). Similarly, diet restriction attenuated gains in body weight and adiposity (Figure 3.1; $P < 0.05$, SED vs. DR), but not to the same degree as the RUN rats. Animals that remained sedentary between 12 and 20 weeks of age gained significantly more weight than those with access to wheels or restricted access to food, owing largely to gains in body fat (Figure 3.1; $P < 0.05$, SED vs. RUN and DR). Voluntary wheel running increased heart weight-to-body weight ratios, a

classic marker of exercise training, compared to sedentary and diet restricted animals (Figure 3.1; $P < 0.05$, RUN vs. SED and DR).

Food intake was similar among all groups through 12 weeks of age (215 ± 4 g/wk, equivalent to 0.5 ± 0.01 g food/kg body weight). From 12-20 weeks of age, food intake was experimentally reduced to 147 g/wk (0.3 g/kg) in DR rats, and SED and RUN animals were maintained *ad libitum* fed. At 20 weeks food consumption in SED rats was 211 ± 14 g/wk (equivalent to 0.35 ± 0.02 g food/kg body weight), and in RUN rats was 221 ± 4 g/wk (equivalent to 0.54 ± 0.01 g food/kg body weight). DR had lower retroperitoneal, epididymal, and omental fat stores compared to SED animals (Figure 3.1; $P < 0.05$), while RUN resulted in further reductions in all the fat depots (Figure 3.1 $P < 0.05$, DR vs. RUN).

Voluntary Wheel Running

When given access to a running wheel over 8 weeks (12- 20 weeks), OLETF rats began running ~8km/day from week 12 to 15, after which running distance gradually declined to around ~6km/day at 20 weeks (Figure 3.1). This average daily voluntary running distance in these physically active OLETF (RUN) rats is consistent with data from our previous reports (101, 154).

Fasting Plasma Characteristics

Food restriction and voluntary wheel running lowered total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, NEFAs, and glucose compared to sedentary animals (Table 3.1; all $P < 0.05$ vs. SED). Importantly, voluntary wheel running lowered triglycerides, NEFA, and glucose to a greater extent than diet restriction (Table 3.1; $P < 0.05$

RUN vs. DR). Additionally, voluntary wheel running, but not diet restriction, significantly lowered fasting insulin, and HOMA-IR (Table 3.1; $P < 0.05$ vs. SED).

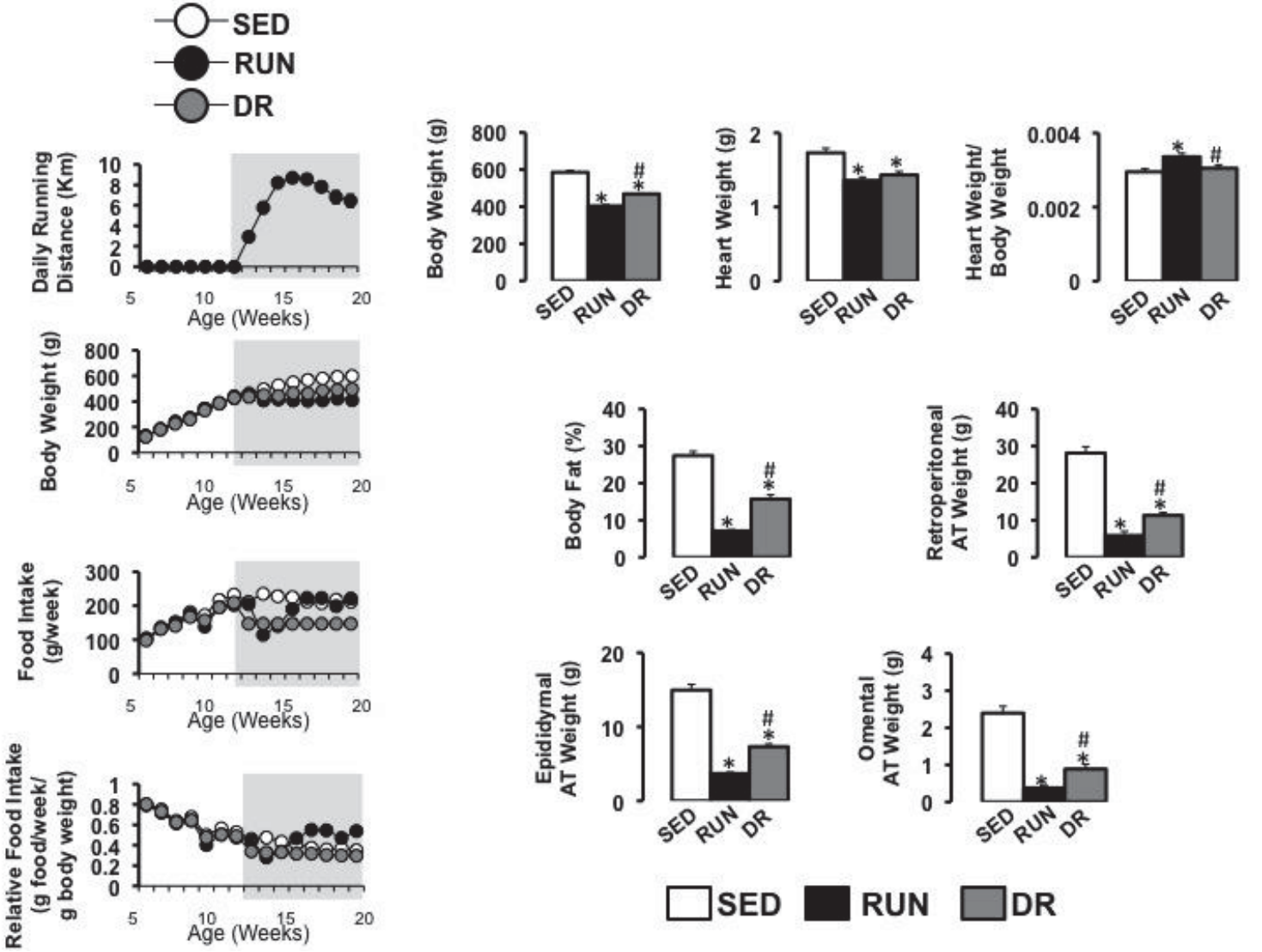

Figure 3.1 Body Composition and Food Intake in sedentary (SED), voluntary wheel running (RUN), and diet restriction (DR) OLETF rats. Values are expressed as means ± SEM. Body fat, heart weights, and fat pad weights were obtained at 20 weeks (time of sacrifice). *****Denotes difference ($P < 0.05$) from SED rats; [#]Denotes difference ($P < 0.05$) from RUN rats

Table 3.1 Fasting Plasma Characteristics and Citrate Synthase Activity			
Variable	**SED**	**RUN**	**DR**
Total Cholesterol ($mg.dL^{-1}$)	110.7±4.4	69.7±2.8*	78.0±1.1*
HDL-C ($mg.dL^{-1}$)	33.2±0.7	29.9±1.3*	29.1±0.5*
LDL-C ($mg.dL^{-1}$)	46.4±3.9	29.6±1.8*	34.5±0.8*
Triglycerides ($mg.dL^{-1}$)	155.0±6.5	51.1±2.7*	71.9±4.0*$^{\#}$
NEFA ($mmol.L^{-1}$)	0.57±0.05	0.19±0.01*	0.31±0.03*$^{\#}$
Glucose ($mg.dL^{-1}$)	311.6±9.5	196.1±4.9*	223.1±10.6*$^{\#}$
Insulin ($mg.dL^{-1}$)	30.8±10.1	6.9±0.7*	19.5±3.2
HOMA-IR Index (glucose*insulin/405)	24.1±8.2	3.4±0.4*	11.1±2.2
Citrate Synthase Activity in VLW ($nmol.min^{-1}.ug^{-1}$)	33.9±2.0	39.4±2.1†	34.8±1.7
Citrate Synthase Activity in VLR ($nmol.min^{-1}.ug^{-1}$)	141.4±9.8	158.0±5.7	129.5±9.3$^{\#}$

Data presented as mean ± SEM (n = 10/group). *****Denotes difference ($P < 0.05$) from SED rats; $^{\#}$Denotes difference ($P < 0.05$) from RUN rats. † $P = 0.06$ compared to SED rats. Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; NEFA; non-esterified fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance; VLW, vastus lateralis white muscle; and VLR, vastus lateralis red muscle.

Aortic Vasoreactivity

We observed no differences in vessel characteristics: outer diameter, inner diameter, vessel length, specific tension responses elicited by 60 mM KCl, and phenylephrine pre-constriction between groups. Overall endothelium dependent dilation, assessed by ACh-induced relaxation in thoracic aortic rings was not different between groups (Figure 3.2), except for a small, but significantly lower ACh-mediated relaxation at the highest dose of ACh (10^{-4}M) in diet restricted rats (Figure 3.2; $P = 0.024$ compared to SED and RUN). We

did not observe any differences in aortic vascular smooth muscle function, as assessed by SNP-mediated relaxation, between groups (Figure 3.2, $P > 0.05$).

Overall, RUN induced greater aortic relaxation in response to insulin than SED or DR animals. Specifically, insulin-stimulated aortic relaxation was significantly greater in RUN at 1,000 and 10,000 μIU/mL of insulin compared to SED (Figure 3.2, $P < 0.05$), and greater than DR at 10,000 μIU/mL of insulin (Figure 3.2, $P < 0.05$). Blockade of ET-1 receptors by tezosentan significantly enhanced insulin-stimulated relaxation within SED animals at 10,000 μIU/mL of insulin (Figure 3.2, $P = 0.057$, SED insulin vs. SED insulin + tezosentan). Additionally, in the presence of ET-1 blockade, insulin-stimulated relaxation of the aorta at 1,000 and 10,000 μIU/mL of insulin were not different between SED and RUN (Figure 3.2, $P > 0.05$). Conversely, treatment of aortic rings with tezosentan had little to no effect on insulin-simulated relaxation in the DR (Figure 3.2, $P > 0.05$). Blockade of ETA receptors with BQ-123 (data not shown) displayed similar insulin-stimulated vasorelaxation responses as tezosentan. ET-1 blockade removed differences in insulin-stimulated relaxation between RUN and SED, suggesting that insulin-induced ET-1 mediated aortic contraction contributes to the impaired relaxation to insulin in SED OLETF rats. We observed no differences in ET-1 mediated contraction among groups (Figure 3.2, $P > 0.05$), indicating that the vascular sensitivity to ET-1 was unaltered by physical activity or reduced adiposity.

Insulin Signaling

In general we did not observe robust insulin stimulation of eNOS or ERK1/2 in the abdominal aorta in any of our groups (Figure 3.3). Protein expression of eNOS and ERK1/2 (expressed as ratio to the sedentary basal condition) is shown in Figure 3. Basal levels of total and phosphorylated eNOS in the abdominal aorta were not different in RUN or DR

compared to SED (Figure 3.3A-E). In response to insulin the expression of total eNOS increased in SED and RUN, yet insulin stimulated phosphorylated eNOS only increased in RUN animals (Figure 3.3A and B). However the ratio of peNOS/eNOS in response to insulin was similar among groups, suggesting that insulin signaling to nitric oxide was not different between groups. Total basal ERK1/2 expression in the aorta of RUN animals was lower than SED animals (Figure 3.3D; $P < 0.05$), indicating that ERK1/2 expression and downstream ET-1 production may be upregulated in sedentary OLETF rats. In response to insulin total and phosphorylated ERK1/2 expression was enhanced above basal levels in DR and RUN rats (Figure 3.3D and E; $P < 0.05$). With insulin stimulation the ratio of pERK/ERK was elevated in the DR animals compared to SED (Figure 3.3F; $P < 0.05$), suggesting that insulin signaling to ET-1 may be elevated with diet restriction. Overall the ratio of peNOS/eNOS and pERK/ERK in response to insulin in the aorta was similar in RUN and SED animals, suggesting that aortic insulin signaling to nitric oxide and ET-1 was unaltered by physical activity.

Citrate Synthase Activity

Voluntary wheel running significantly increased citrate synthase activity in the white portion of the vastus lateralis muscle compared to sedentary animals (Table 3.1, $P = 0.06$, RUN vs. SED). This data provides important evidence that eight weeks of daily physical activity in OLETF rats elevated skeletal muscle oxidative capacity.

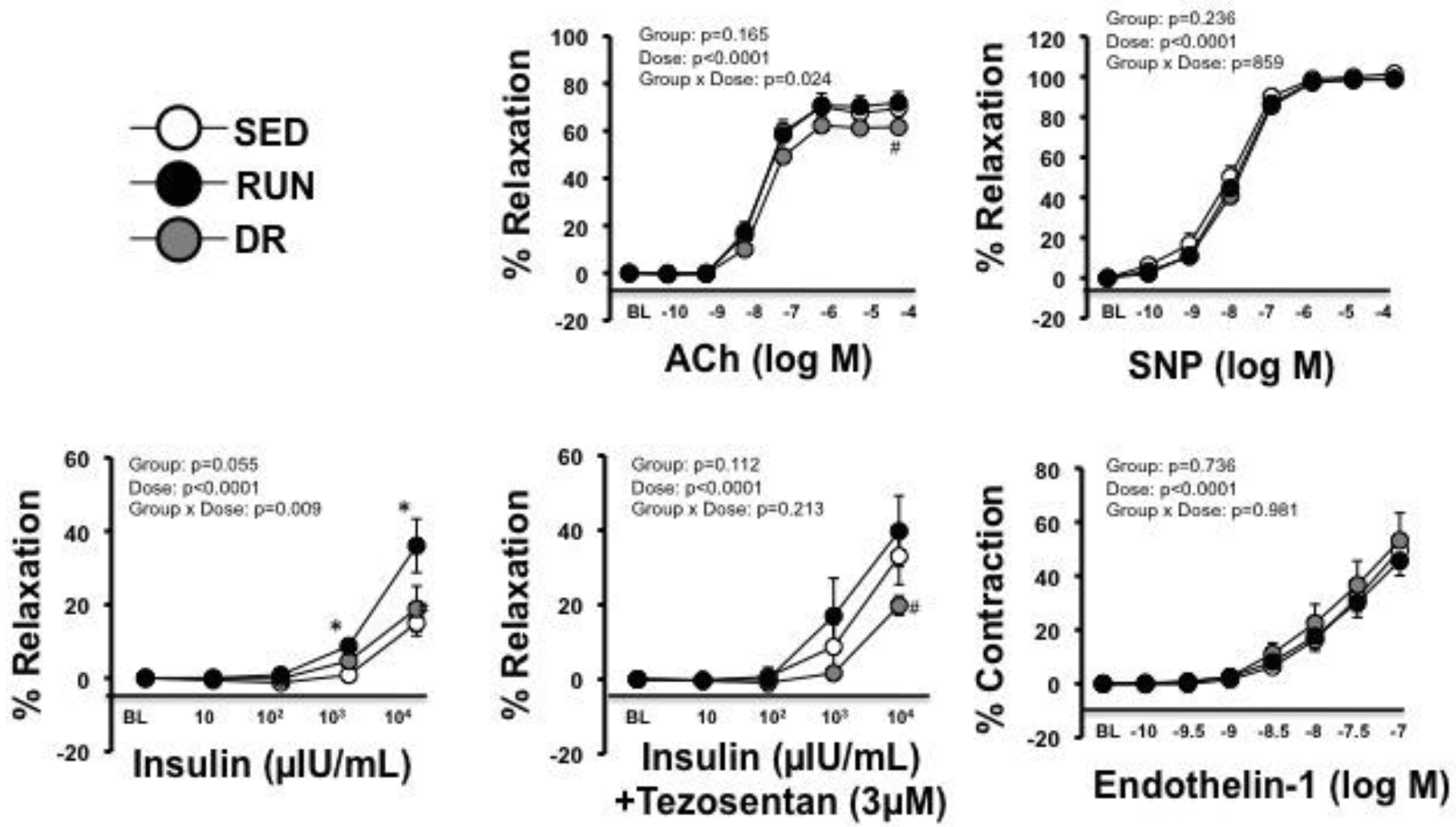

Figure 3.2 Vasomotor Function of thoracic aortic rings in sedentary (SED), voluntary wheel running (RUN), and diet restriction (DR) OLETF rats. Values are expressed as means ± SEM. *****Denotes difference ($P < 0.05$) from SED rats; $^{\#}$Denotes difference ($P < 0.05$) from RUN rats.

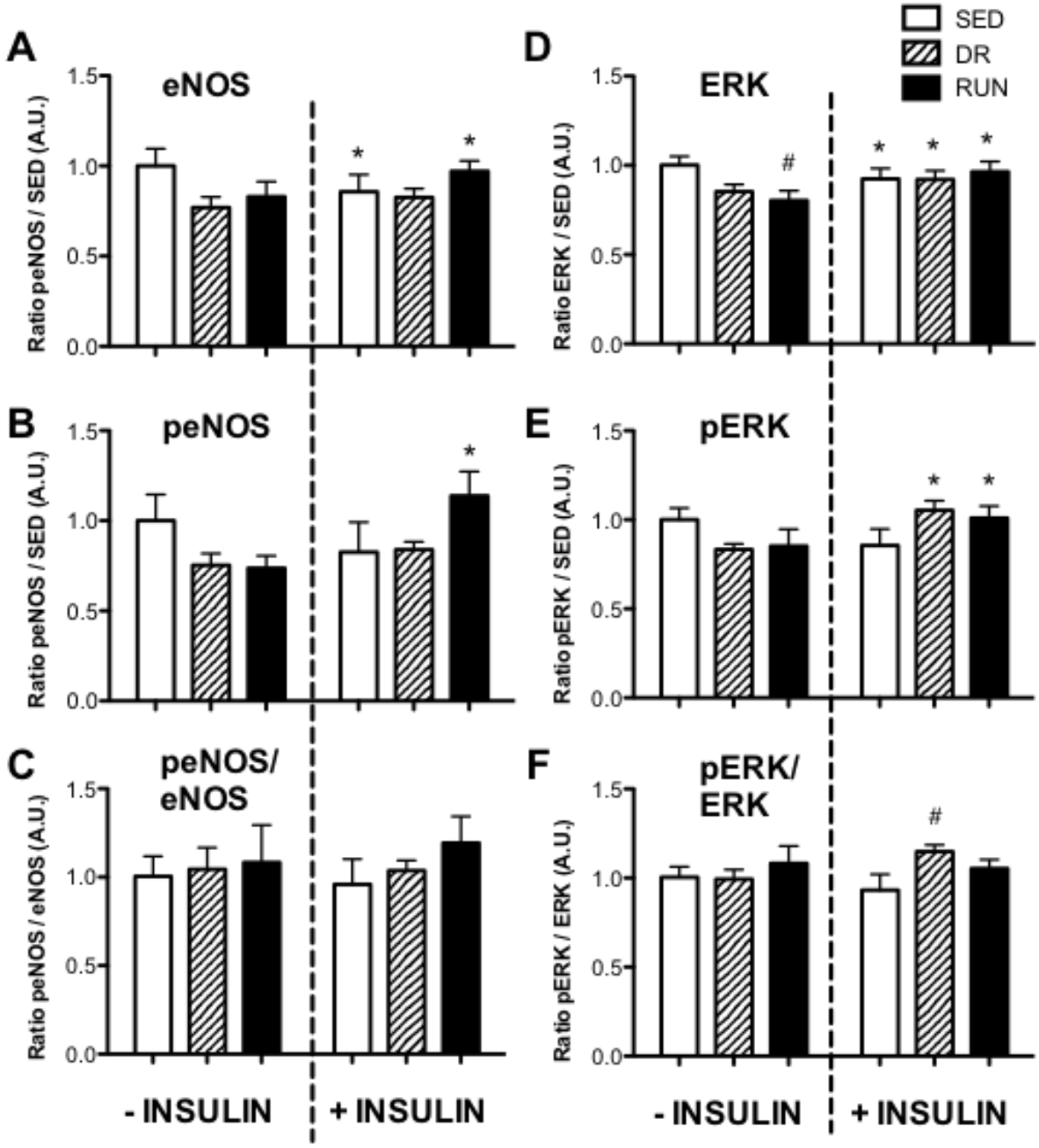

Figure 3.3 Protein Expression of Total and Phospho-specific eNOS and ERK1/2. Total eNOS (A), and ERK1/2 (D); phospho-specific eNOS (B), and ERK1/2 (E), and the ratio of phosph-specific/total eNOS (C), and ERK1/2 (F) in abdominal aortic rings in sedentary (SED), diet restriction (DR), and voluntary wheel running (RUN) 20-week old OLETF rats under basal (-Insulin) or insulin-stimulated conditions (+Insulin). All values are expressed as ratio to the sedentary basal condition as means ± SEM. *****Denotes difference between insulin compared to basal within group ($P < 0.05$); $^{\#}$Denotes difference ($P < 0.05$) within basal or insulin conditions compared to SED ($P < 0.05$).

DISCUSSION

The primary purpose of this study was to test the hypothesis that daily physical activity attenuates the vascular reactivity to insulin and insulin signaling in the aorta of insulin resistant, obese OLETF rats, independent of changes in body composition. The major findings of our study are that (i): both daily voluntary wheel running and 30% diet restriction regimen for eight weeks (12-20 weeks of age) resulted in marked reductions in adiposity, improved blood lipid profiles and systemic markers of insulin resistance in the obese insulin resistant OLETF rat model; yet (ii) only daily voluntary wheel running (not diet restriction) ameliorated impairments in aortic insulin-stimulated vasorelaxation; an effect that was in part mediated by a decrease in insulin-mediated ET-1 vasocontraction; and (iii) the enhanced insulin-mediated relaxation in the aorta was not due to changes in insulin signaling to nitric oxide and endothelin-1.

Our results show that physical activity-induced improvements in aortic insulin-stimulated relaxation in the aorta of obese insulin resistant rats appear to be independent of changes in adiposity. Insulin-stimulated relaxation of the aorta was greater in rats given access to a running wheel over eight weeks, but not in sedentary diet restricted rats, with similar decreases in body weight. This finding is consistent with previous data from our group showing that insulin-stimulated vasodilation in gastrocnemius muscle arterioles is enhanced following voluntary wheel running, but not in diet restricted 20 week old OLETF rats (120). Herein, we also report that differences in insulin-stimulated vasorelaxation in aortic rings between physically active and sedentary rats were largely abolished after treatment with ET-1 receptor blockers. ET-1 blockade had little to no effect on insulin-induced relaxation in the aorta of voluntary wheel running rats, but produced nearly a two

fold increase in insulin-induced relaxation in sedentary rats. This finding strongly suggests that (i) enhanced vasomotor reactivity to insulin with physical activity was mediated in part by a decrease in insulin-induced ET-1 contraction and/or (ii) impaired insulin-induced aortic relaxation in sedentary animals was mediated by increased in insulin-induced ET-1 contraction. Additionally, our data indicate that insulin-stimulated ET-1 vasocontraction is primarily mediated through ETA receptors given that improvements in insulin-stimulated vasodilation in sedentary rats occurred to a similar degree by blockade of ETA and ETB receptors (with tezosentan) and selective blockade of ETA (with BQ-123), Furthermore, to evaluate whether greater insulin-stimulated relaxation in the aorta of voluntary wheel running rats was due to reduced vascular sensitivity to ET-1 and/or decreased vascular production of ET-1, we examined aortic responsiveness to exogenous ET-1 in all three groups of animals. We found similar ET-1 mediated aortic contraction among groups suggesting that it is local vascular ET-1 production and/or insulin-stimulated ET-1 vasocontraction, and not the vascular sensitivity to ET-1 that is modulated by physical activity.

Insulin's cardiovascular actions are complex and are thought to be balanced by endothelial signaling pathways controlling the production of the vasodilator, nitric oxide (NO) (39, 183), and the vasoconstrictor endothelin-1 (ET-1) (125, 126). Current conjecture is that insulin-mediated blood flow is impaired in insulin resistance and obesity as a result of aberrations in the PI3K/Akt/eNOS branch of the insulin signaling pathway concurrent with overstimulation of the MAPK/ET-1 branch, resulting in diminished production of NO and overproduction of ET-1 (53, 61, 84, 120, 148). However, not all data are in agreement (104, 112). Consistent with our previous report in skeletal muscle arterioles (122) overall we did not observe differences in basal or insulin-stimulated expression of total or phosphyorylated

eNOS in the abdominal aorta between groups. These data suggest that enhanced aortic insulin-mediated vasorelaxation with voluntary wheel running does not appear to be due to insulin-mediated changes in eNOS content or activity.

However, we cannot rule out the possibility that we did not observe significant insulin stimulation due to the fact that we assessed *in-vitro* insulin signaling in the isolated aorta with intact vascular smooth muscle and endothelial cells. Although insulin has been shown to primarily signal through endothelial cells, it can also signal and act through vascular smooth muscle cells (168). Therefore, it is possible that altered endothelial insulin signaling may have been masked by largely unchanged vascular smooth muscle insulin signaling or vice versa. In future studies *in-vitro* insulin stimulation should be performed separately on isolated aortic endothelial cells and vascular smooth muscle cells to distinguish how insulin signaling is modulated in each cell type. Moreover, isolated vessels *in-vitro* are not exposed to the same circulating factors, pressure, stretch, or blood flow that they are exposed to *in-vivo*, thereby limiting the physiological applicability of our *in-vitro* findings. Given that we have observed similar difficulties with *in-vitro* insulin stimulation in the past (122), future studies should attempt to avoid these problems by performing *in-vivo* insulin stimulation.

Previously, we have shown that basal phosphorylated eNOS is higher in the aorta of OLETFs given access to a running wheel for 36 weeks (4 to 40 weeks of age), but not in rats given access to a running wheel for 16 weeks (4 to 20 weeks of age) (32). Our finding that eight weeks of voluntary wheel running from 12 to 20 weeks of age in OLETF rats did not induce changes in basal levels of total or phsosphorylated eNOS, further indicates that the age of the animals, severity of insulin resistance, and/or duration of physical activity may be important moderators of basal phosphorylated eNOS in the aorta.

Of additional importance is our finding that insulin activation of total or phosphorylated ERK1/2 was not different among groups. Although, basal protein expression of ERK1/2 was elevated in the sedentary compared to the voluntary wheel running animals, providing further evidence that basal local vascular production of ET-1 is upregulated in obese sedentary OLETF rats, and/or reduced by physical activity. Collectively, our data suggests that while insulin signaling to nitric oxide and endothelin-1 may not be altered by obesity, physical activity, or diet restriction in 20-week OLETF rats, the local vascular production of ET-1 in sedentary obese insulin resistant rats appears to be enhanced. Thus, the impaired insulin-induced aortic relaxation in sedentary animals appears to be mediated by greater overall vascular production of ET-1, possibly through chronic insulin activation of ET-1 in the hyperinsulinemic state.

Our finding that physical activity, but not diet restriction improved aortic insulin-stimulated vasorelaxation as a result of decreased insulin-stimulated ET-1 vasocontraction, may be significant in light of evidence indicating that excess ET-1 signaling is an important contributor to the pathogenesis of macro-vascular disease (145). A potential mechanism by which physical activity may exert an insulin sensitizing effect on the aorta and result in a decrease in ET-1 vasocontraction, independent of changes in adiposity, may be related to the repeated bouts of blood flow and thus shear stress to which the artery wall is exposed during exercise (81, 141). This hypothesis is supported by evidence that (i) shear stress reduces expression of ET-1 in cultured endothelial cells (177), (ii) cessation of voluntary wheel running for 7 days increases expression of ET-1 in the rat iliac artery (139), (iii) rat skeletal muscle feed arteries chronically exposed to high levels of blood flow (e.g., soleus feed artery) display greater insulin-stimulated dilation, as a result of reduced insulin-induced ET-1

activation, than arteries chronically exposed to lower levels of flow (e.g., gastrocnemius feed artery) (83), and (iv) inactive lower limbs of spinal cord injury patients, chronically exposed to low blood flow and shear stress (12), exhibit enhanced ET-1 mediated basal vascular tone (174).

Another interesting finding of the present study is that the physical activity-related increase in insulin-stimulated relaxation in the aorta occurred in the absence of changes in ACh-mediated relaxation, a response largely mediated by nitric oxide (NO). Incidentally, compelling evidence from studies using the Zucker obese rat model indicates that impairments in insulin-mediated vasodilation occurs prior to impairments in ACh-mediated vasodilation in both skeletal muscle (52) and coronary arterioles (87, 136). However, it is also possible that ACh-mediated vasorelxation was not improved in our voluntary wheel running animals, relative to the sedentary obese OLETF animals, because ACh-mediated dilation in the 20-week-old sedentary obese OLETF animals was not impaired. Consistent with this hypothesis, previous data in our lab have shown that while ACh-mediated aortic relaxation is not different between physical active and sedentary OLETF animals at 20 weeks it is different at 40 weeks (32), suggesting a temporal decline, likely related to age and disease progression, in endothelium dependent relaxation in the aorta. Moreover, data in animals and humans suggest that exercise training does not lead to improvements in endothelium-dependent dilation in subjects with a healthy endothelium, likely due to a ceiling effect (80, 140). These data highlight the need for further investigation of the temporal effects on the development and progression of endothelial dysfunction and impairments in insulin-mediated blood flow with obesity.

In summary, our data demonstrate that physical activity in the form of daily voluntary

wheel running for eight weeks in obese insulin resistant OLETF rats effectively reverses insulin resistance and improves insulin-stimulated relaxation in the aorta, without concomitant changes in vascular insulin signaling to nitric oxide or endothelin-1. Our findings support the idea that the insulin sensitizing effects of physical activity on the vasculature are largely independent of reductions in adiposity, given that improvements in aortic insulin-mediated relaxation were not observed with diet restriction.

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GRANTS

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CHAPTER 4

STUDY THREE:

DIVERGENT ROLES OF NITRIC OXIDE IN INSULIN-STIMULATED AORTIC VASORELAXATION OF LOW AND HIGH INTRINSIC AEROBIC CAPACITY RATS

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ABSTRACT

Intrinsically low aerobic capacity increases risk for cardiovascular and metabolic diseases and is a strong predictor of early mortality. Rats selectively bred for low or high running capacity (LCR and HCR) display contrasting levels of intrinsic aerobic capacity and provide a unique polygenic model to examine the underlying mechanism(s) by which aerobic capacity impacts disease risk. **OBJECTIVE:** We tested the hypothesis that low fit LCR display vascular dysfunction, and impaired vascular reactivity to insulin in isolated aortic rings compared to high fit HCR. **METHODS:** Sedentary female LCR (n=21) and HCR (n=17) rats from generation 31 and 32 were utilized. **RESULTS:** At 44 weeks of age female LCR exhibited 13% greater body weight, 35% greater percent body fat, and greater subcutaneous, peri-gonadal and retroperitoneal adipose tissue depots, compared to HCR. Endothelial function assessed by acetylcholine-induced aortic vasorelaxation was not different between strains and was entirely nitric oxide (NO) mediated in both LCR and HCR. Paradoxically insulin stimulated aortic vasorelaxation was significantly greater in LCR (32.7 ± 4.1%) compared to HCR (16.7 ± 2.8%). Inhibition of eNOS generated NO entirely abolished insulin-mediated vasorelaxation in LCR animals, while in the HCR, eNOS inhibition did not alter insulin-stimulated vasorelaxation. **CONCLUSIONS:** Low intrinsic aerobic fitness in female rats is not associated with endothelial dysfunction in the aorta. . High aerobic fit HCR rats exhibit NO independent insulin-stimulated vasorelaxation in the rat aorta. Additionally, low fit LCR rats display reduced vascular mechanisms to stimulate aortic vasorelaxation in response to insulin.

INTRODUCTION

Aerobic capacity, independent of physical activity levels, is a strong predictor of cardiovascular disease and overall mortality (15, 35, 128, 143). Low levels of aerobic capacity are associated with greater risk of death than other more established risk factors including physical inactivity, hypertension, smoking, and type 2 diabetes (15, 16, 73). Indeed, cardiovascular risk and mortality is approximately eight-fold greater among sedentary individuals with low aerobic capacity, but only two-fold greater in individuals with low levels of physical activity (16, 73, 149). This strong, graded and consistent inverse relationship between aerobic capacity (also termed cardiorespiratory fitness) and mortality is seen in both men and women, and is not confounded by age or other risk factors. Clearly, low aerobic capacity is an important mediator of cardiovascular disease and lifespan.

Genetics putatively accounts for 60-70% of the variation in intrinsic aerobic capacity in humans (26), although the volume and intensity of daily physical activity level also plays an important role. Recent evidence associating low intrinsic aerobic capacity with chronic disease has emerged from a novel rodent model of inherited low endurance running capacity and impaired metabolic health, independent of physical activity effects (76, 93, 189). Koch and Britton developed two strains of rats with marked differences in intrinsic aerobic endurance exercise capacity. Over several generations rats were selectively bred for high or low treadmill endurance running capacity (run time to exhaustion on a graded treadmill exercise test). The strains are referred to as high-capacity runners (HCR) or low-capacity runners (LCR). Importantly, both strains are sedentary and receive normal caged activity-only, removing the potential confounding influences of daily physical activity and exercise training. Previous studies have shown that the LCR rats, with inherently low aerobic

capacity, display cardiovascular risk factors associated with increased mortality and susceptibility to insulin resistance (129, 133, 176, 189).

Insulin resistance is a major cardiovascular disease risk factor and is associated with all-cause mortality (33, 57, 159). Insulin resistance is characterized by a blunted ability of peripheral tissues to regulate glucose homeostasis in response to insulin. While endothelial dysfunction is well established as a classic marker of vascular disease, evidence now indicates that insulin resistance is also a prominent component of hypertension, coronary artery disease, and atherosclerosis (153). In healthy persons insulin has important cardiovascular actions, including increased blood flow, vasodilation and capillary recruitment in skeletal muscle (125). Abnormalities in the vascular reactivity to insulin are present in obese, physically inactive, and type 2 diabetic humans and animals. Impairments in the vascular sensitivity to insulin with disease contribute to reduced skeletal muscle glucose uptake by limiting perfusion and glucose and insulin delivery (90, 97, 110, 120, 121, 185). While, it is well known that increased physical activity improves the vasomotor response to insulin in diseased animals and humans (47, 62, 86, 120, 122), the role of intrinsic aerobic capacity, independent of physical activity levels, is relatively unknown. Understanding the role of intrinsic aerobic capacity and etiology of early markers of insulin resistance in the vasculature is important for uncovering insulin-sensitizing approaches and mechanisms to ameliorate cardiovascular abnormalities associated with worsening insulin resistance linked to chronic disease. Hence, the purpose of this study was to test the hypothesis that intrinsic low aerobic capacity in rats is associated with endothelial dysfunction, and impaired vasoreactivity to insulin in isolated rat aortic rings.

METHODS

Animals

Koch and Britton (76, 93, 189) originally developed two strains of rats divergent in intrinsic aerobic endurance exercise capacity. The founder population (N:NIH stock) and generations of the HCR and LCR strains have been previously reported (189). Briefly, the 13 lowest and 13 highest running capacity rats of each sex were selected from the founder population and randomly paired for mating. By *generation 6,* there was a 171% divergence in running capacity, with most of the change in running capacity relative to the founder population occurring in the HCR group (13% in LCR and 136% in HCR) (28).

In the present investigation, we used female LCR (n=21) and HCR (n=17) rats from generations 31 and 32, as body weight differences between phenotypes are less marked in females than in males (76), and thus body weight was less likely to represent a confounding influence on our results. Eleven-week-old female rats were phenotyped for intrinsic endurance running capacity (103, 161, 189). After which they remained sedentary and underwent no further exercise training or testing (cage activity-only) to factor out the confounding influences of daily exercise. We housed the animals in pairs maintained in temperature (21°C) and light controlled, 12:12-hr light-dark cycle (lights off at 1800h) animal quarters, and provided all rats with *ad libitum* access to water and standard rodent chow (Formulab 5008, Purina Mills, St. Louis, MO) comprised of ~ 26% protein, 18% fat, and 56% carbohydrate. The University of Missouri Institutional Animal Care and Use Committee approved all experimental protocols.

Intraperitoneal Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests (IPGTT) were performed at 39 and 43 weeks of age (n=6 per group), as previously described (158). Briefly, food was removed from the cages 12 hours before each rat received an intraperitoneal injection of dextrose (50% solution, 2 g/kg body weight). Venipuncture blood samples were collected from the lateral tail vein immediately before (0 minutes) dextrose administration and 15, 30, 45, 60, and 120 minutes after injection. Insulin and glucose responses were quantified by the product of the area under the curve (AUC) for glucose and insulin using the trapezoidal method (173).

Body Composition and Blood Parameters

Following an overnight fast, we anesthetized the rats at 44 weeks of age with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). We harvested tissues, collected blood and euthanized the animals by exsanguination, in full compliance with the American Veterinary Medical Association Guidelines on Euthanasia. We measured body mass of the rats and determined body composition by using a dual energy x-ray absorptiometry machine (Hologic QDR-1000) calibrated for rodents. Subsequently, we collected blood samples, harvested tissues, and euthanized the rats by exsanguination. Serum samples were prepared by centrifugation and stored at -80°C until analysis. Glucose, cholesterol, triglycerides, and non-esterified fatty acids (NEFA) assays were performed by a commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO) on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA) using commercially available assays according to the manufacturer's guidelines. Plasma insulin concentrations were determined using a commercially available, rat-specific ELISA (Alpco Diagnostics, Salem, NH). Samples were run in duplicate and manufacturer's controls and calibrators were used

according to assay instructions. The homeostasis model assessment-insulin resistance (HOMA-IR) was calculated according to the formula of Matthews et al. (115): [fasting glucose (mg/dl) • fasting insulin (μIU/mL)]/405).

Assessment of Aortic Vascular Function.

Isolation and assessment of thoracic aortic function was determined as previously described (32, 78, 175). Briefly, immediately following exsanguination we removed the thoracic aorta and dissected and cleaned the vessel of all connective and adipose tissue in ice-cold Krebs-bicarbonate buffer (4°C). The thoracic aorta was segmented into aortic rings, and their outer diameter, inner diameter, and axial length were measured with an Olympus microscope and NIH ImageJ software. Aortic rings were mounted on myographs and submerged in 20-ml water baths containing physiological Krebs solution maintained at 37°C for 1 hour to allow for equilibration. We determined the optimal vessel diameter, which elicited a maximal response to 60 mM KCL (L_{max}); which was around 150% of the passive diameter. Aortic vasoreactivity was assessed with cumulative concentration-response curves to Acetylcholine (ACh, 10^{-10} to 10^{-4}M), insulin (10 to 10,000 μIU/mL), and sodium nitroprusside (SNP, 10^{-10} to 10^{-4}M). Mounted rings were pre-constricted with a submaximal concentration of phenylephrine (PE, $3E^{-7}$ M) prior to ACh, insulin and SNP dose response curves.

The vasoreactivity to insulin was assessed (10 to 1,000 μIU/mL) with human insulin (Novolin, Novo Nordisk) in 10-minute intervals. We evaluated the contribution of ET-1, and NO in the aortic vasomotor response to ACh and insulin, by incubating separate aortic rings with tezosentan (3 μM), a non-selective ETA and ETB receptor antagonist, or L-NG-Nitroarginine methyl ester (L-NAME, 300 μM)), an endothelial nitric oxide synthase

inhibitor, for 20 minutes prior to insulin and ACh curves. Following each drug dose response curve, we washed the vessel baths with warm Krebs-bicarbonate buffer (37 °C) and allowed a re-equilibration period of 30 minutes. For insulin, ACh, and SNP curves, relaxation at each concentration was measured and expressed as percent maximum relaxation, where 100% relaxation is equivalent to loss of all tension developed in response to phenylephrine (31).

Drugs and Solutions

All drugs and solutions were obtained from Sigma (St. Louis, MO), except albumin (USB Corporation, Cleveland, OH). The Krebs-bicarbonate buffer solution contained (in mM) 131.5 NaCl, 5.0 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 11.2 glucose, 20.8 NaHCO3, 0.003 propranolol, and 0.025 EDTA. The solution was aerated with a 95% O^2 - 5% CO^2 mixture (pH7.4) and maintained at 37°C. All drug solutions were prepared in Krebs-bicarbonate buffer, except insulin, which was prepared fresh daily in physiological saline solution with BSA (1g/100mL). We also prepared SNP fresh daily and protected the serial dilutions from light.

Data Analysis and Statistics

Results are presented as mean ± SEM. We evaluated overall group effects (LCR vs. HCR) on all dependent variables using a one-way analysis of variance (ANOVA). We analyzed all drug dose-response curves from vasomotor function experiments by repeated measures two-way (group x dose) ANOVAs, and evaluated main effects of group, interactions between group and dose, and simple effects. When appropriate, Fisher's Least Significant Differences (LSD) post-hoc analysis was used. We used IBM SPSS Statistics 19

for Windows (Chicago, IL) for all statistical analyses, and established *a priori* statistical significance of $P < 0.05$.

RESULTS

Animal Characteristics

During the graded exercise test performed at 11 weeks of age, HCR rats ran significantly further distance (~10-fold, P <0.001), time (~5-fold P <0.001), and speed (~3-fold P <0.001) to exhaustion compared to LCR rats (Table 4.1). LCR rats were ~13% heavier in body weight, owing to ~35% greater percent body fat compared to HCR animals determined by DEXA (Figure 4.1A and B). The increased adiposity in the LCR rats is evident by ~34% more visceral (peri-gonadal and retroperitoneal) adipose tissue than HCR rats (Figure 4.1C). Although heart weight was slightly different between strains (Figure 4.1D), no differences were observed when heart weight was normalized to body weight (Figure 4.1E). Fasting serum triglycerides, non-esterified fatty acids, glucose, insulin, and HOMA-IR were not significantly different in LCR and HCR female rats at 44 weeks of age (Table 4.1, P > 0.05). The lack of strain differences in serum triglycerides and fatty acids in this study agrees with our previous report in 31-week-old female LCR and HCR rats (129). Also in agreement with the aforementioned study, glucose responses to the IPGTT at 39 and 43 weeks were not different between strains (Figure 4.2A and D). Although LCR and HCR animals did not exhibit divergent glucose responses following IPGTT, insulin responses (Figure 4.2B) and insulin AUC (Figure 4.2C) was significantly elevated in LCR animals at 39 weeks, corresponding to our previous data (129). These glucose tolerance data indicate that at this age LCR hypersecrete insulin in response to a glucose load to maintain blood glucose levels to those observed in the HCR rats. However, four weeks later, at 43 weeks of

age, one week prior to sacrifice, insulin responses (Figure 4.2E) and insulin AUC (Figure 4.2F) following IPGTT were not different between groups. At this second time point, insulin responses in the HCR animals were elevated to those observed in LCR animals, suggesting that postprandial glycemic control is altered with age in these animals.

Table 4.1 Animal Characteristics		
Variable	**LCR**	**HCR**
Max Distance Run to Exhaustion at 11wks (m)	238 ± 8	2303 ± 68*
Time to Exhaustion at 11wks (min)	17.2 ± 0.4	78.7 ± 1.4*
Speed at Exhaustion at 11 wks (m/min)	18.1 ± 0.2	48.8 ± 0.7*
Triglycerides ($mg.dL^{-1}$)	44.9 ± 6.8	29.2 ± 2.7
Non-Esterified Fatty Acids ($mmol.L^{-1}$)	44.3 ± 3.3	39.9 ± 2.0
Glucose ($mg.dL^{-1}$)	150 ± 5	173 ± 15
Insulin ($mg.dL^{-1}$)	1.74 ± 0.29	1.78 ± 0.46
HOMA-IR Index	0.67 ± 0.11	0.87 ± 0.33

Values are expressed as means ± SEM. *Denotes strain difference ($P < 0.05$). LCR n=21, and HCR n=17. Abbreviations: LCR, low capacity runner; HCR, high capacity running; HOMA-IR, homeostasis model assessment of insulin resistance.

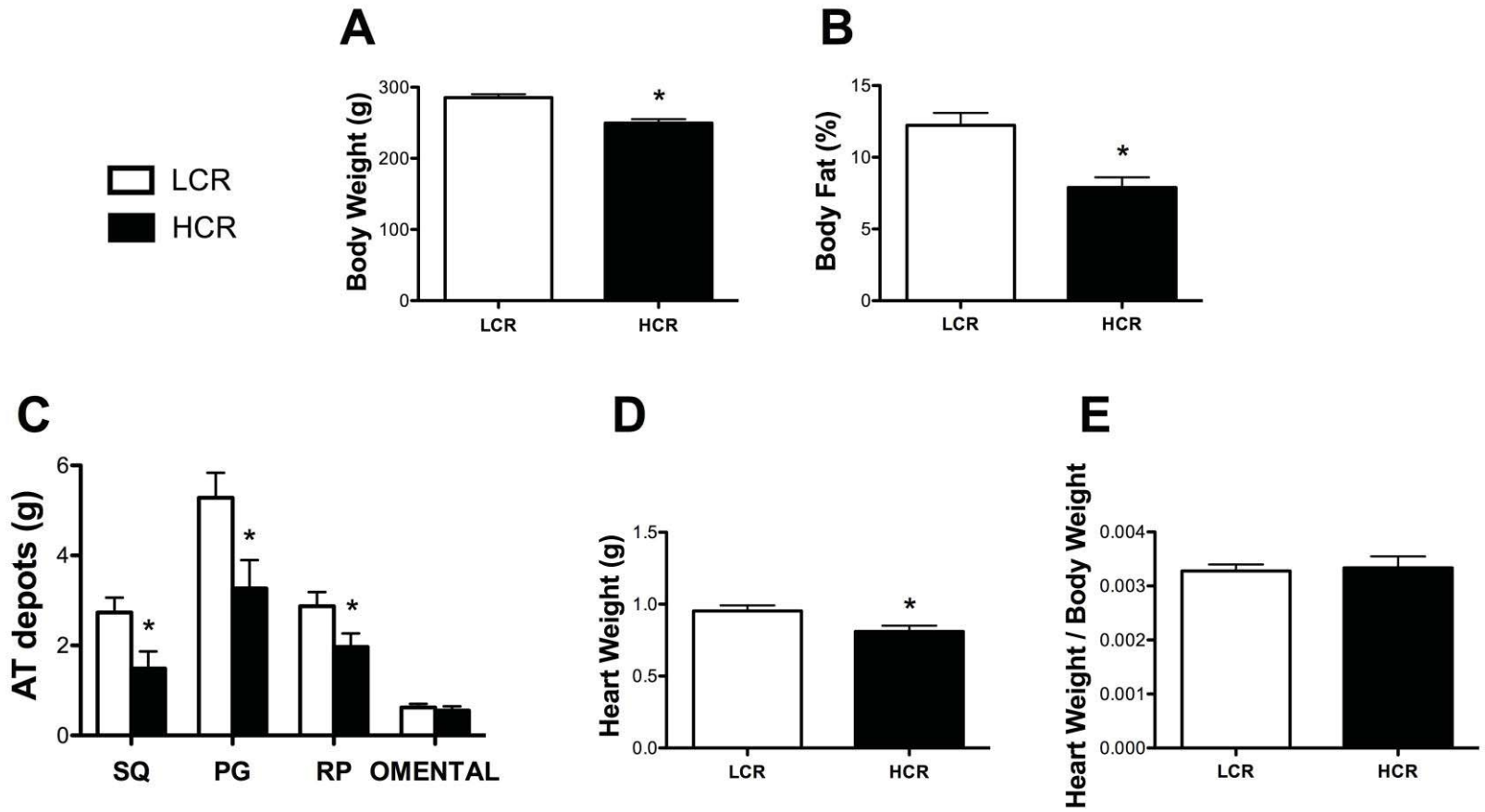

Figure 4.1 Body Composition in low capacity running (LCR), and high capacity running (HCR) rats. AT; adipose tissue, SQ; subcutaneous; PG; peri-gonadal; and RP; retroperitoneal. Values are expressed as means ± SEM. Body fat, heart weights, and fat pad weights were obtained at 44 weeks . *****Denotes strain difference ($P < 0.05$

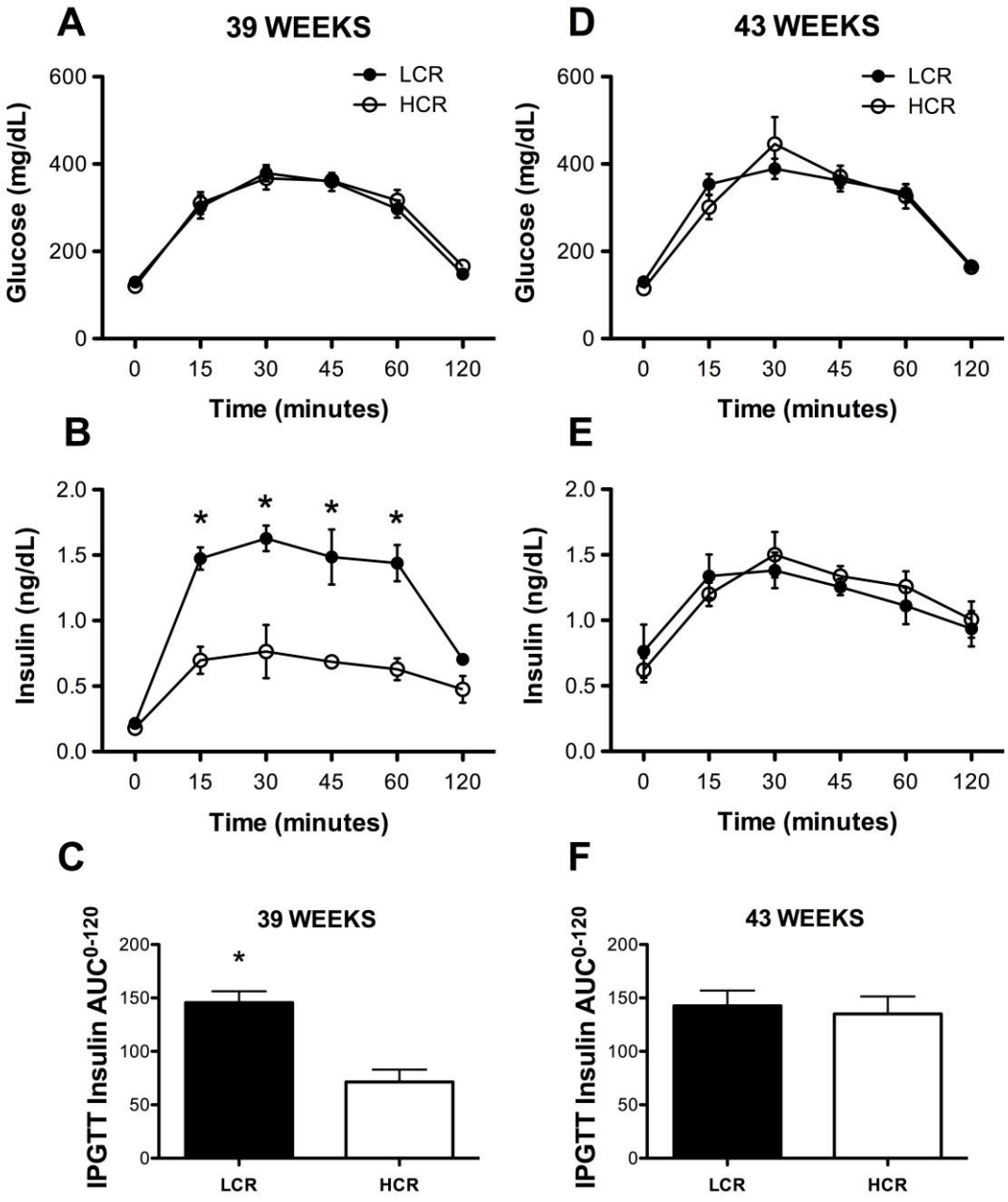

Figure 4.2 Intraperitoneal Glucose Tolerance Test: Glucose and insulin responses to an intraperitoneal glucose tolerance test in low capacity running (LCR), and high capacity running (HCR) rats, n=6/group at 39 and 43 weeks of age. AUC; area under the curve. All data are expressed as means ± SEM. *Denotes strain difference ($P < 0.05$).

Aortic Vasoreactivity

We observed no differences in vessel characteristics: outer diameter, inner diameter, vessel length, specific tension responses elicited by 60 mM KCl, and phenylephrine preconstriction between strains (Table 4.2). Overall, endothelium dependent dilation, assessed by acetylcholine (ACh)-induced vasorelaxation in thoracic aortic rings was statistically greater in LCR compared to HCR animals (Figure 4.3A, Group effect $P < 0.05$). The maximum aortic relaxation to ACh was ~55% in HCR, and ~65% in LCR rats. Similarly, insulin stimulated vasorelaxation was significantly greater in LCR than HCR across all insulin doses (Figure 4.3B, Group $P = 0.003$) with a significant group x dose interaction ($P = 0.03$). Insulin induced an approximate two fold higher maximal vasorelaxation of the aorta in LCR (32.7 ± 4 .1%) compared to HCR (16.7 ± 2.8%) rats. Additionally, we did not observe any strain differences in aortic vascular smooth muscle function, as assessed by SNP-mediated relaxation.

Table 4.2 Vessel Characteristics

Group	**OD** (mm)	**ID** (mm)	**Length** (mm)	**SA** (mm^2)	**Wall Thickness** (mm)	**60mM KCL** (g)	**Pre-PE** (g)
LCR	1.74 ± 0.02	1.34 ± 0.02	2.43 ± 0.04	0.97 ± 0.03	0.20 ± 0.01	5.0 ± 0.2	4.1 ± 0.1
HCR	1.75 ± 0.02	1.39 ± 0.02	2.50 ± 0.05	0.90 ± 0.03	0.18 ± 0.01	5.2 ± 0.2	4.2 ± 0.1

Values are expressed as means ± SEM. Abbreviations: LCR, low capacity runner; HCR, high capacity runner; OD, outer diameter of the aorta; ID, inner diameter of the aorta; SA, aorta surface area; 60mM KCL, contraction tension in response to 60mM potassium chloride; and Pre-PE, pre-constriction tension produced by phenylephrine.

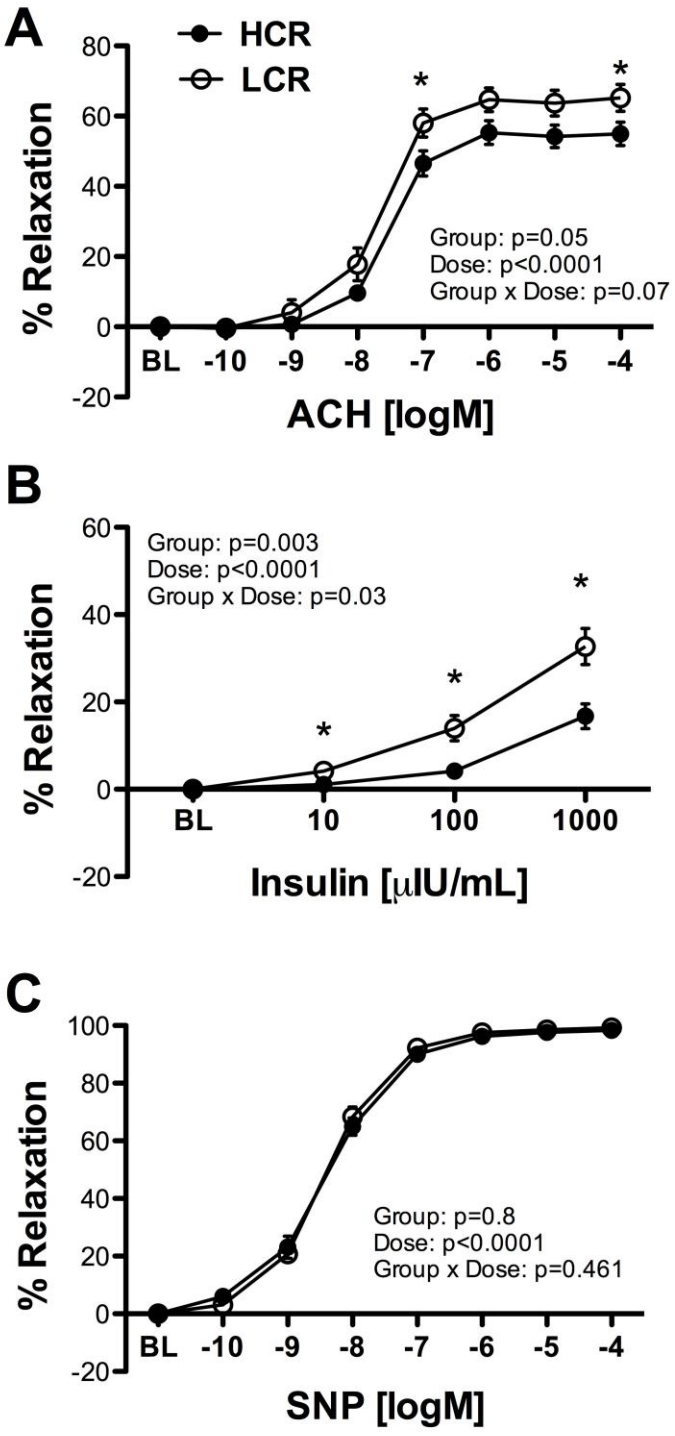

Figure 4.3 Vasomotor Function: of thoracic aortic rings in low capacity running (LCR), and high capacity running (HCR) rats. Values are expressed as means ± SEM. *Denotes strain difference ($P < 0.05$).

Role of Endothelin-1 and Nitric Oxide in Aortic Vasomotor Responses

ACh-induced aortic vasorelaxation of both strains of animals was unaltered by ET-1 blockade, with tezosentan (Figure 4.4A and D). In contrast, inhibition of eNOS, with L-NAME, completely blocked aortic ring relaxation responses to ACh in both LCR and HCR animals, indicating that ACh mediated vasorelaxation in the thoracic aorta is entirely nitric oxide mediated (Figure 4.4B and D). Analogous to ACh, ET-1 blockade had no effect on insulin-stimulated relaxation in the aorta of LCR or HCR animals (Figure 4.5A and C), suggesting that ET-1 release and/or ET-1 mediated contraction do not account for differences in the vasomotor response to insulin between strains. However, in the presence of NO blockade insulin-mediated relaxation was completely abolished in LCR animals (Figure 4.5D). In contrast, inhibiting eNOS, and removing the contribution of NO did not alter insulin-stimulated vasorelaxation in HCR rats (Figure 4.5B). Importantly, these data suggest that insulin induces relaxation in the aorta of HCR rats by mechanisms not involving eNOS generated NO, while insulin stimulated vasorelaxation is entirely NO dependent in LCR rats. To determine the relative contribution of the endothelium in the observed vasoreactivity to insulin, we have previously shown that insulin-mediated vasorelaxation is completely attenuated in the denuded endothelium of the rat aorta (138) indicating that an intact endothelium is obligatory for insulin-induced vasorelaxation of thoracic aortic rings.

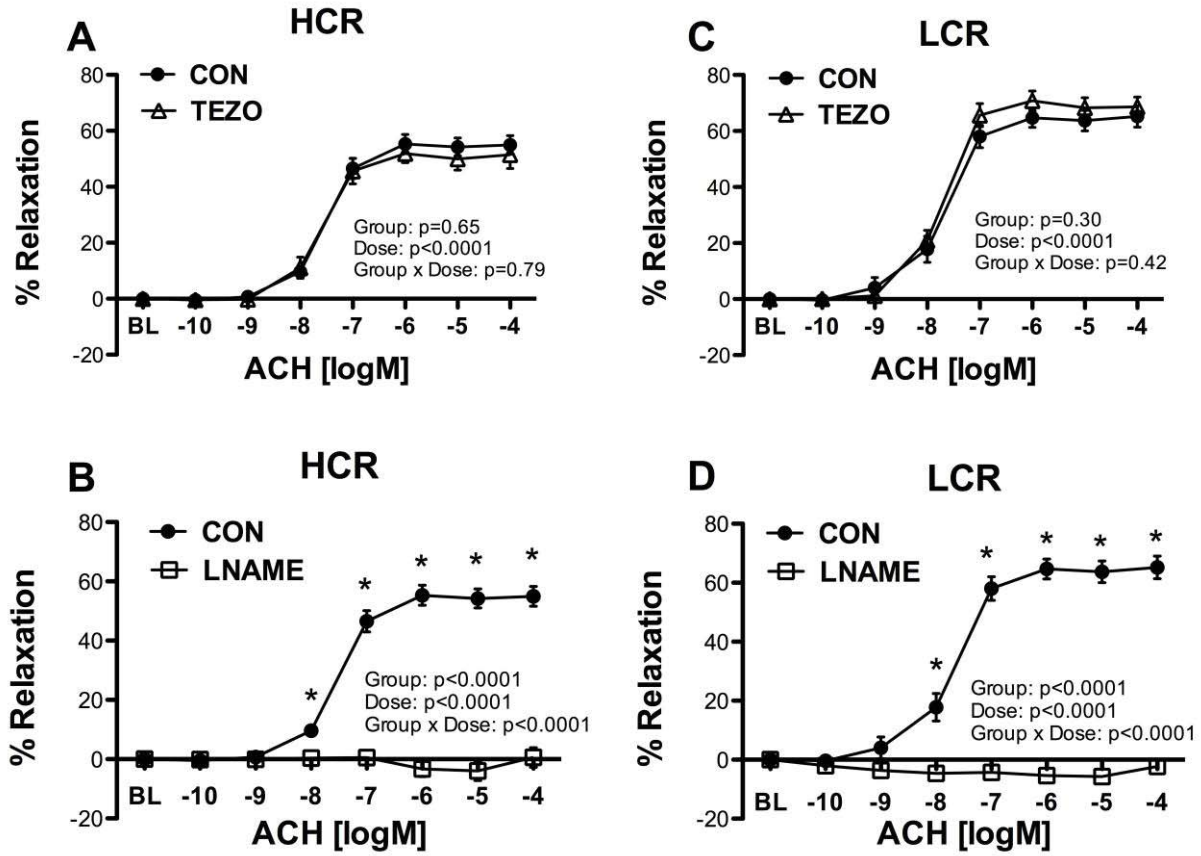

Figure 4.4 Effects of ET-1 and NO blockade on ACh-induced vasorelaxation: of thoracic aortic rings of low capacity running (LCR) and high capacity running (HCR) rats. The left panel (A and B) present data from acetylcholine (ACh) dose-response curves in HCR, and right panel (C and D) present data from ACh dose-response curves in LCR. Closed circles represent % relaxation in response to ACh alone, open triangles represent % relaxation to ACh in the presence of 3 μM tezosentan, a non-specific inhibitor of endothelin-1 (ET-1) receptors (top panel, A and C) N=9-16/group, and open squares represent % relaxation to insulin in the presence of 300 μM L-NAME, an endothelial nitric oxide synthase inhibitor, (bottom panel, B and D) N=6-8/group. Values are expressed as means ± SEM *Denotes strain difference ($P < 0.05$).

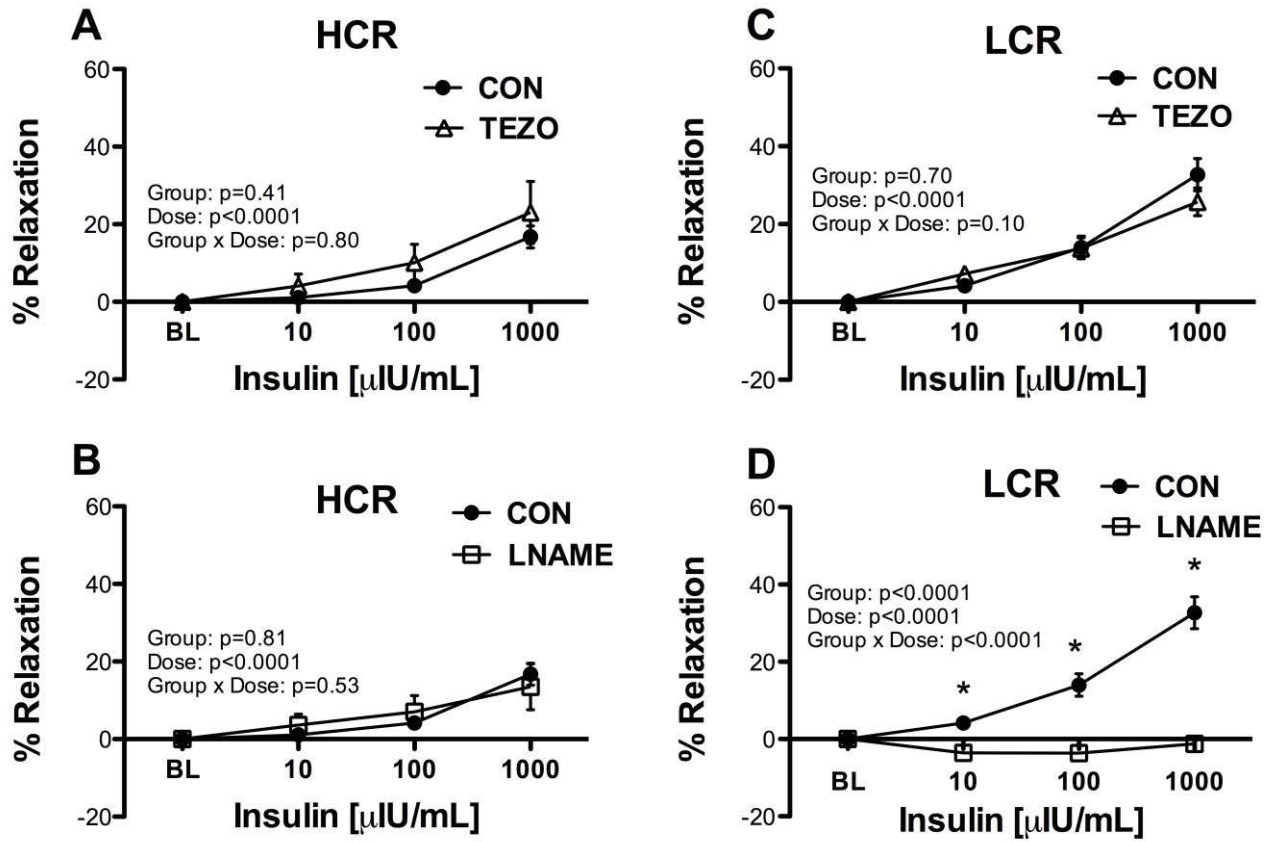

Figure 4.5 Effects of ET-1 and NO blockade on Insulin-Mediated Vasorelaxation of thoracic aortic rings of low capacity running (LCR) and high capacity running (HCR) rats. The left panel (A and B) present data from insulin dose-response curves in HCR, and right panel (C and D) present data from insulin dose-response curves in LCR. Closed circles represent % relaxation in response to insulin alone, open triangles represent % relaxation to insulin in the presence of 3 μM tezosentan, a non-specific inhibitor of ET-1 receptors (top panel, A and C) N=9-16/group, and open squares represent % relaxation to insulin in the presence of 300 μM L-NAME, an endothelial nitric oxide synthase inhibitor, (bottom panel, B and D) N=6-8/group. Insulin μIU/mL, micro international units per milliliter. Values are expressed as means ± SEM. *Denotes strain difference ($P < 0.05$).

DISCUSSION

Evidence in humans indicates that low aerobic capacity, or low cardiorespiratory fitness, independent of physical activity levels, is a strong predictor of early mortality (17, 128) and is linked to cardiovascular disease (128). Data from a novel rodent model suggest that a low capacity for aerobic exercise can be inherited, and leads to impaired metabolic health and risk factors for cardiovascular disease (103). Using this model we provide new insight into the relationships between intrinisc aerobic capacity on vascular insulin resistance and dysfunction, independent of physical activity levels. More specifically, the primary purpose of this study was to test the hypothesis that rats with intrinsic low aerobic capacity exhibit vascular dysfunction, in the form of reduced endothelial dependent vasorelaxation and insulin-stimulated vasorelaxation of the thoracic aorta. Contrary to our hypothesis, our findings show that intrinsic low aerobic capacity in sedentary rats was not associated with reduced endothelial function assessed by acetylcholine-induced aortic vasorelaxation. Additionally, low aerobic capacity was paradoxically associated with increased vasorelaxation in response to insulin in the aorta; an effect that was entirely mediated by eNOS generated nitric oxide. In contrast, high intrinsic aerobic capacity was associated with other alternant (nitric oxide-independent) endothelial mechanisms to induce insulin-stimulated vasorelaxation in the rat aorta. Overall our data suggest that rats with low aerobic capacity display a compensatory elevation in insulin-mediated vasorelaxation, and are completely reliant on nitric oxide to stimulate vasorelaxation in response to insulin.

In agreement with previous reports (49, 160), our results indicate that low aerobic capacity female rats were heavier with greater overall percent body fat compared to high aerobic capacity animals. Unlike previous reports in low aerobic capacity male rats (133,

176, 189), we did not observe characteristics of overt metabolic disease in low aerobic capacity female rats. Analogous to our prior study investigating female LCR and HCR rats (129), we observed no differences in fasting glucose or insulin (and HOMA-IR). We also report similar glucose responses to a glucose tolerance test between strains, yet insulin responses and AUC were significantly higher in LCR rats, suggestive of mild insulin resistance in the LCR at 39 weeks of age. Interestingly, at 43 weeks of age HCR animals secreted similar amounts of insulin to LCR following the IPGTT, indicating that differences in glycemic control between strains may be confounded by aging-related influences on insulin sensitivity. Collectively, low aerobic capacity female rats appear to be somewhat protected against markers of metabolic disease compared to males. Based on our findings that postprandial glycemic control was altered with age, it is likely that overtime impairments in fasting glucose and insulin in LCR may develop, as well as other markers of metabolic disease.

Endothelial dysfunction is an independent predictor of long-term cardiovascular disease progression and cardiovascular events (163). To assess endothelial function in this study we evaluated acetylcholine (ACh)-mediated vasorelaxation in aortic ring segments from 44-week-old female LCR and HCR rats. Although ACh-induced vasorelaxation was statistically greater in LCR (~65%) compared to HCR (~55%) animals, this difference is likely not physiologically significant. Evidence for this comes from prior experiments in our laboratory reporting a range of 55-75% ACh-induced aortic vasorelaxation in healthy sedentary rats (32, 48). The variable response to ACh-mediated vasorelaxation in these studies appears to be dependent on the age, and specific animal strain studied in the sedentary state. To our knowledge only two other studies have assessed isolated artery responses to

ACh-stimulated relaxation in the LCR and HCR animal model. Similar to our findings Ritchie et al. report no strain differences in the vascular sensitivity to ACh in the aorta at 35 wks (160). In contrast, Wisloff et al. observed lower maximal and EC_{50} acetylcholine-induced vasorelaxation in common carotid arteries of younger female LCR rats (16-24 weeks of age) compared to HCR rats (189). While it is possible that these differences could simply be due to a differential response between the carotid artery and the aorta, it is very likely that we are observing an aging effect on endothelial function. At 16-24wks maximal ACh-vasorelaxation was ~60% in LCR and ~80% in HCR (189), while at 44 weeks ACh-vasorelaxation was ~65% in LCR and ~55% in HCR. Taken together this data implies that endothelial function in the LCR animals remains blunted, while in the HCR animals it is reduced (to levels observed in LCR) with age. Furthermore, in agreement with Ritchie et al. (160) we show that endothelial dependent relaxation in the rat aorta is entirely nitric oxide (NO) mediated in both LCR and HCR animals. Therefore, in the present study, both sedentary LCR and HCR exhibit reduced NO-endothelial dependent vasorelaxation.

Contrary to our hypothesis insulin-stimulated vasorelaxation of the aorta was greater in LCR rats. Insulin's cardiovascular actions are complex and are thought to be balanced in part, by vasorelaxation through the endothelial phosphatidylinositol 3-kinase (PI3K) signaling pathway producing the vasodilator nitric oxide (NO) (39, 90, 183), and vasocontraction via mitogen-activated protein kinase (MAPK) signaling to produce the vasoconstrictor endothelin-1 (ET-1) (90, 125, 126). Therefore we evaluated the role of ET-1 and NO in insulin-mediated aortic vasorelaxation. While we observed no effect of ET-1 blockade on insulin stimulated aortic vasorelaxation in LCR or HCR, NO blockade entirely eliminated all vasorelaxation in response to insulin in LCR. These data suggest that in 44-

week-old female LCR and HCR rats, insulin does not appear to signal through the MAPK-ET-1 pathway to induce aortic vasocontraction. Additionally, our findings that nitric oxide completely mediated vasorelaxation responses to acetylcholine and insulin in LCR aorta reveal an exclusive dependence on nitric oxide in aortic vasomotor function within LCR animals. It is possible that in LCR animals there is a compensatory up-regulation (or greater absolute stimulation) of insulin-mediated activation of the PI3K/NO-signaling pathway, leading to greater NO-mediated insulin-stimulated blood flow.

Perhaps in the initial stages of insulin resistance, characterized by recurrent postprandial episodes of hyper-insulinema, the sensitivity of the vasculature to insulin may become somewhat enhanced. Both enhanced sensitivity and greater amounts of insulin may lead to greater vascular insulin signaling through eNOS and nitric oxide to increase insulin-mediated vasorelaxation. Furthermore, with regular episodic bouts of postprandial hyperinsulinemia, increased insulin-induced phosphorylation of eNOS may lead to greater eNOS expression to produce more nitric oxide. Evidence for this hypothesis comes from studies where (i) insulin increased the expression of eNOS mRNA and protein by two-fold in aortic endothelial cells (6, 55, 94), and (ii) insulin enhanced eNOS gene expression in microvessels isolated from lean Zucker rats (94). Clearly insulin's ability to modulate eNOS expression could enhance NO production induced by insulin. It is probable that in the early stages of insulin resistance, characterized by increased release of insulin in response to a meal (observed in LCR animals at 39 weeks of age), the sensitivity of the vasculature to insulin may be enhanced. Thus, enhanced vascular sensitivity to insulin and increased insulin-stimulated eNOS expression and NO production is a possible mechanism to explain the compensatory elevation in insulin-stimulated vasorelaxation observed in the LCR

animals. Importantly overtime, with the progression from postprandial hyperinsulinemia to more severe insulin resistance (ie. impaired postprandial glucose tolerance, and fasting hyperinsulinemia and hyperglycemia) there is clear evidence that the vasculature becomes de-sensitized to increased secretion of insulin leading to impairments in insulin-stimulated vasorelaxation. In overtly insulin resistant states in both humans and animals insulin-mediated blood flow is impaired as a result of aberrations in the PI3K/eNOS branch of the insulin signaling pathway concurrent with overactivation of the MAPK/ET-1 branch, resulting in diminished production of NO and an overproduction of ET-1 (53, 61, 84, 120, 148), leading to an overall loss of insulin's vasodilatory effects.

A further novel finding of this study was that in healthy sedentary rats with high intrinsic aerobic capacity insulin induced vasorelaxation of the aorta by non-nitric oxide endothelial factors. In the presence of an eNOS inhibitor (LNAME), the aortic vasorelaxation to insulin was still present in the HCR, providing evidence of the existence of insulin-mediated NO-independent dilatory mechanism(s). This effect was only existent in the HCR, but not the LCR. Previously, we have shown that vasoreactivity to insulin is completely abolished in denuded rat aortic rings (138). In light of this data and our findings that vascular smooth muscle function was unaltered by intrinsic aerobic capacity, the differential responses to the aortic insulin-stimulated vasorelaxation between LCR and HCR rats appear to be primarily due to differences in endothelial derived factors. However, since we did not directly assess the vascular reactivity to insulin in the absence of an intact endothelium, it is possible that insulin directly activates vascular smooth muscle relaxation in HCR rats. Within the endothelium there are three primary factors known to elicit relaxation of the vascular smooth muscle: (i) nitric oxide (NO), (ii) prostacyclin (PGI_2), and (iii) putative endothelium-

derived hyperpolarizing factor(s) (EDHF) (13). Our data suggest that LCR rats have an impaired ability to induce insulin-stimulated vasomotor relaxation of the aorta via PGI_2 and EDHF-mediated mechanisms. In agreement with our findings, Ritchie et al. report that EDHF-mediated ACh relaxation was impaired in LCR, despite intact NO-mediated vasorelaxation (160). Taken together it seems probable that insulin's ability to stimulate PGI_2 and EDHF pathways is reduced with low aerobic fitness, thereby leading to a compensatory response of enhanced insulin-stimulated NO production. There is also evidence that NO exerts a constant inhibition on EDHF pathways (11), suggesting that a greater reliance on NO, may lead to an even further decrements in EDHF-mediated vasorelaxation in response to insulin with low aerobic capacity. Future studies investigating the influence of aerobic fitness on the balance between NO, PGI_2 and/or EDHF endothelial mediated mechanism(s) of insulin-stimulated vasomotor relaxation are warranted. Additionally, future work is necessary to elucidate the direct actions of insulin on the endothelium and vascular smooth muscle in the aorta of LCR and HCR rats. In summary, intrinsic low aerobic capacity impairs insulin's ability to induce vasomotor relaxation via nitric oxide independent mechanisms. This exciting finding opens the door for future studies to investigate mechanisms of insulin-stimulated vasoreactivity modified by inherited exercise capacity.

Despite these intriguing findings we cannot rule out the possibility that observed differences between insulin-stimulated vasorelaxation in the aorta of LCR and HCR rats may merely be associated with the artificial selection of high or low running capacity. In other words perhaps the particular gene involved regulating intrinsic aerobic capacity may also control the vascular reactivity to insulin and the stimulation of eNOS generated NO in these animals. Thus, it is possible that intrinsic aerobic capacity may not be causal to the vascular

reactivity to insulin in conduit arteries.

In conclusion, this investigation tested the hypothesis that low intrinsic aerobic capacity, independent of physical activity, is associated with endothelial dysfunction, and impaired vasoreactivity to insulin in the rat aorta. Our data show that low aerobic capacity in mature adult sedentary female rats was associated with greater body weight and adiposity, and a paradoxical elevation of insulin-stimulated aortic vasorelaxation. Interestingly high aerobic capacity was associated with nitric oxide independent insulin-mediated vasorelaxation of the aorta. Collectively our data suggest that an inherited impairment in exercise capacity leads to an exclusive dependence on nitric oxide in aortic vasomotor function, and that low aerobic capacity is associated with reduced endothelial mechanisms to stimulate vasorelaxation in response to insulin in the rat aorta.

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CHAPTER 5

DISCUSSION

The complex vascular and metabolic actions of insulin are fundamental to cardiometabolic health. Although the association between insulin resistance and cardiovascular disease has been well established, it is poorly understood. Insulin has important hemodynamic actions in vascular tissue, including capillary recruitment, peripheral vasodilation, and enhanced regional blood flow (8, 39, 125, 183). Importantly insulin-mediated increases in skeletal muscle blood flow accounts for as much as 40-50% of insulin-stimulated glucose disposal (8, 95, 132) in healthy individuals. It follows that aberrations in the vascular reactivity to insulin that occur with obesity and type 2 diabetes (96, 97, 110, 120, 122, 185) contribute to reduced glycemic control and insulin sensitivity by limiting the perfusion and subsequent delivery of glucose and insulin to skeletal muscle (37, 38, 97, 185). Available evidence indicates that coordinate impairments in cardiovascular and metabolic actions of insulin play key roles in the etiology of obesity, type 2 diabetes and cardiovascular disease.

Our main findings indicate that (i) metformin does not enhance the microvascular reactivity to insulin, (ii) obesity-related impairments in the micro- and macro-vasomotor reactivity to insulin are largely independent of changes in body composition and adiposity, (iii) aberrations in the vascular reactivity to insulin in obesity are in part due to enhanced insulin-induced endothelin-1 (ET-1) activation, (iv) physical activity ameliorates obesity-related impairments in insulin-stimulated vasorelaxation, an effect that is mediated by reduced insulin-stimulated ET-1 vasocontraction, and (v) low intrinsic aerobic exercise

capacity is paradoxically associated with a compensatory increase in insulin-mediated vasorelaxation and a greater reliance on nitric oxide-mediated mechanisms to stimulate vasorelaxation in response to insulin. Collectively these data shed new light on mechanisms underlying the influence of intrinsic aerobic fitness on the vasoreactivity to insulin, and provide strong evidence that insulin-sensitizing effects on the vasculature with obesity are specific to physical activity.

Despite the well-known health benefits of regular physical activity, humans have become progressively more and more inactive over the past several decades. The recommended physical activity levels to promote and maintain health, defined by the US Surgeon General, the American College of Sports Medicine, Centers for Disease Control, and the American Heart Association, include: at least 30 minutes of moderate-intensity physical activity five days of the week, or vigorous-intensity aerobic physical activity for 20 minutes, three days each week (72, 143). Adherence to these recommendations is appalling, as a mere 3.5% of Americans actually meet the recommendations (179). Lack of regular physical activity and over-nutrition with obesity are implicated as key contributors to the development of metabolic syndrome, type 2 diabetes, and cardiovascular disease (20, 68).

Metformin

Given the limited number of healthy individuals meeting physical activity guidelines it is no surprise that the majority of patients with type 2 diabetes also fail to engage in regular physical activity (109, 123). For these reasons the medical community has sought to identify drugs that "mimic" the beneficial effects of physical activity (130), to treat obesity-related diseases, including type 2 diabetes. Metformin has been purported to be one such "exercise

mimetic" (130) drug. Metformin is a widely used anti-hyperglycemic drug, and is well established as a first line treatment for type 2 diabetes (1, 3). Metformin is also increasingly prescribed as a preventative therapy in pre-diabetic individuals. Results from the Diabetes Prevention Program show that treatment with metformin reduced the incidence of type 2 diabetes by 31% in non-diabetic impaired glucose tolerant individuals (92). Although metformin is one of the most commonly used drugs for the treatment of diabetes its mechanisms of action in the body are diverse, varied and not completely understood. Many of metformin's therapeutic effects are thought to be due in part to its ability to stimulate AMP-activated protein kinase (AMPK) in a variety of tissues (28, 192). Evidence indicates that in addition to metformin's ability to reduce chronic hyperglycemia and enhance insulin sensitivity via AMPK in muscle (127), it has also been reported to have vasculoprotective effects (77, 88, 91, 113) linked to reduced cardiovascular events (142). Metformin's vascular effects may be associated with AMPK activation in the vasculature. AMPK has been shown to phosphorylates eNOS in both rat and human endothelial cells (36, 124), leading to enhanced production of NO and vasodilation. Furthermore, although metformin has been shown to reverse vascular dysfunction in conduit, large arteries, and the mesenteric circulation (79, 88, 113, 114); its ability to ameliorate or reverse vascular dysfunction in the skeletal muscle resistance vasculature was unknown. Given these gaps in the literature, we investigated metformin's effects on vascular insulin action in the skeletal muscle microcirculation.

While only a handful of studies have investigated vascular effects of metformin in humans and animals, none have evaluated the vascular reactivity to insulin in response to metformin treatment. Additionally we have previously shown that physical activity and

exercise can both prevent and reverse impairments in insulin-stimulated vasomotor function in the skeletal muscle microvasculature (110, 120, 122) of a rodent model of hyperphagia-induced obesity and diabetes (OLETF rats). Therefore in Study 1 we sought to identify whether metformin treatment promotes physical activity and exercise-like effects to enhance the vascular reactivity to insulin in resistance arteries of skeletal muscle in pre-diabetic OLETF rats between 12 and 32 weeks of age. We now have evidence that unlike daily physical activity (120, 122) and exercise training (110) metformin does not enhance insulin-stimulated vasodilation of skeletal muscle resistance arteries in obese insulin resistant OLETF rats. Importantly, metformin's beneficial effects to lower food consumption, body weight, and adiposity did not influence the microvascular reactivity to insulin. These findings are similar to previous data from our laboratory showing that insulin-stimulated vasodilation in gastrocnemius muscle arterioles is unaltered by reductions in body weight and adiposity (induced by caloric restriction) of OLETF rats (120). Taken together these data imply that simply correcting for obesity, by attenuating adiposity and body mass is not sufficient to reverse and/or protect against impairments in the microvascular reactivity to insulin.

Many studies utilizing drug treatments uncover opposing incongruous findings, due to varied doses, length of treatment and use in different populations. Data on metformin's ability to improve metabolic and cardiovascular dysfunction in type 2 diabetes have yielded inconsistent and conflicting results (5, 43, 45, 51, 70, 74, 131, 150). A strong predictor of whether metformin treatment improved vascular function in these studies is the level of insulin resistance following treatment (70, 113, 114). Conversely, we showed that although metformin treatment for three months significantly lowered glycosylated hemoglobin

(HbA1c), it had no effect on the skeletal muscle microvascular reactivity to insulin. Importantly our data suggest that metformin-induced improvements in glycemic control through reduced body weight and adiposity and not though improvements in insulin-induced vasodilation in skeletal muscle resistance arteries. Overall favorable effects on body composition and glycemic control due to metformin treatment are not associated with improvements in the skeletal muscle microvascular reactivity to insulin. In conclusion, metformin treatment does not produce "physical activity-like" insulin-sensitizing effects in the skeletal muscle microvasculature.

Physical Activity

While physical activity and exercise are recommended as a key component of a healthy lifestyle for everyone, they are particularly important in patients with type 2 diabetes (40). Physical activity and exercise can have a profound influence on either maintaining or improving vascular function in obesity and type 2 diabetes (50, 62), and are associated with improvements in cardiovascular mortality and morbidity (18, 63-65, 85, 111). Furthermore, the Centers for Disease Control and National Institutes of Health recognize that lifestyle interventions which incorporate physical activity are more successful and cost-effective in preventing and treating type 2 diabetes than pharmaceutical therapies (40). Additional evidence from the Diabetes Prevention Program show that physical activity interventions in impaired glucose tolerant individuals decreased incidence of type 2 diabetes 58% more than metformin treatment (92). Strongly implicating the importance of a physically active lifestyle over medications for high risk impaired glucose tolerant individuals.

Although the effect of physical activity and exercise on the vasomotor response to insulin in diseased animals and humans has previously been investigated (47, 62, 86, 110,

120, 122), the role of physical activity, independent of changes in body composition in preventing impairments in the vascular reactivity to insulin in conduit arteries with obesity was not known. Given our important findings in Study 1 that lowered body mass and adiposity following metformin treatment did not alter the microvascular reactivity to insulin in OLETF rats (42), we set out to distinguish the effects of daily physical activity (voluntary wheel running) from those of reduced adiposity to the vascular reactivity to insulin in the rat aorta. In Study 2, we assessed the effects of daily voluntary wheel running in OLETF rats between 12 and 20 weeks of age on insulin-stimulated aortic vasorelaxation to those of diet restriction, to reduce body weight to comparable levels as the running group. Voluntary wheel running and diet restriction attenuated gains in adiposity relative to sedentary, *ad libitum* fed OLETF rats. Insulin-stimulated vasorelaxation of the aorta was similar in sedentary and diet-restricted rats, but was significantly enhanced in rats given access to a running wheel for eight weeks. These data combined with our previous findings indicate that physical activity improvements in the vascular reactivity to insulin occur in skeletal muscle resistance arteries (120, 122) and the aorta, largely independent of changes in body composition. Thus, improvements in the vascular reactivity to insulin with obesity is physical activity-specific and occurs along the arterial tree, in both large conduit arteries to enhance total blood flow, and in skeletal muscle resistance arteries to enhance nutritive flow.

Role of Endothelin-1

Excess endoethlin-1 (ET-1) signaling is an important contributor to the pathogenesis of vascular disease (145). Together data from Study 1 (metformin) and Study 2 (physical activity) strongly indicate that insulin-mediated ET-1 vasocontraction in the macro- and microvasculature is enhanced with obesity. In both of these studies the vascular reactivity to

insulin was blunted in part due to increased insulin-stimulated ET-1 contraction in both the aorta and skeletal muscle resistance arteries of obese, insulin resistant OLETF rats. In Study 2, we evaluated whether the impaired vascular reactivity to insulin was due to reduced vascular sensitivity to ET-1 and/or decreased vascular production of ET-1 by assessing they aortic responsiveness to exogenous ET-1. Obesity did not alter ET-1 mediated aortic contraction, indicating that the local vascular ET-1 production and/or insulin-stimulated ET-1 activation is enhanced with obesity. In agreement with this theory, we observed enhanced expression of ERK1/2 (MAPK/ET-1 signaling) in sedentary obese OLETF rats. Thus, reduced insulin-stimulated vasorelaxation with obesity appears to be mediated by greater overall vascular production of ET-1, possibly through insulin activation of ET-1 in the hyper-insulinemic state. Supporting this hypothesis, ET-1 production is augmented in response to an oral glucose load in insulin resistant, but not healthy individuals (146), suggesting the ET-1 branch of the insulin signaling pathway is overstimulated in insulin resistance and obesity. Collectively our findings agree with the prevailing concept in the literature that insulin-mediated blood flow is impaired in obesity as a result of overstimulation of the MAPK/ET-1 signaling pathway, resulting in overproduction of local vascular ET-1 (53, 61, 84, 120, 148).

Nevertheless, we need to re-think and better appreciate the underlying role of elevated local vascular ET-1 production in impaired vascular insulin action and endothelial function. Evidence for this comes from our finding that ET-1 blockade in isolated *in-vitro* skeletal muscle microvessels prior to insulin exposure resulted in significantly greater vasodilation in obese compared to lean rats (Study 2, Table 2.2). Thus, elevated local vascular ET-1 production (not systemic circulating levels of ET-1) chronically "brakes" skeletal muscle vasodilation (independent of insulin) in obese OLETF rats. This concept has

important clinical relevance, indicating that chronic over-activation of local vascular ET-1 may be associated with endothelial dysfunction and insulin-stimulated impairments in vasomotor function with obesity and disease. Future animal and human work should further investigate the role of chronic basal elevations in local vascular ET-1 production on obesity-related impairments in skeletal muscle blood flow.

Given the observed role of greater insulin-stimulated ET-1 activation with obesity, we evaluated the contribution of ET-1 to the vasomotor reactivity to insulin with metformin and physical activity. In the skeletal muscle microcirculation, metformin treatment was associated with enhanced insulin-mediated ET-1 vasoconstriction, blunting insulin-stimulated vasodilation in OLETF rats (Study 1). Conversely, physical activity reduced insulin-stimulated ET-1 vasocontraction in the aorta, despite no change in vascular ET-1 mediated aortic contraction (Study 2). These data suggest that metformin enhances, and physical activity blunts the local vascular ET-1 production and/or insulin-stimulated ET-1 vasoconstriction. Of further importance is our observation that reductions in body mass and adiposity with diet restriction, did not alter obesity-related increases in insulin-mediated ET-1 vasocontraction. Overall these findings indicate that improvements in the vascular reactivity to insulin with physical activity are in part due to decreased insulin-mediated ET-1 vasocontraction, and independent of changes in body composition induced by metformin or diet restriction.

Physical activity results in repeated bouts of blood flow and shear stress to the artery wall (81, 141), which may exert an insulin sensitizing effect on the vasculature to decrease ET-1 vasocontraction by altering the local vascular production of ET-1. In support of this idea there is evidence that shear stress reduces ET-1 expression in cultured endothelial cells

(177). Similarly, cessation of voluntary wheel running reduced shear stress and increased ET-1 expression in the rat iliac artery (139). Recently we have also shown that postural rat skeletal muscle feed arteries regularly exposed to high levels of blood flow (e.g., soleus feed artery) display greater insulin-stimulated vasodilation, as a result of reduced insulin-induced ET-1 vasoconstriction, than arteries exposed to lower levels of flow (e.g., gastrocnemius feed artery) (83). This vascular heterogeneity of insulin-stimulated vasodilation may be related to differences in the local vascular production ET-1. It is possible that differential expression in skeletal muscle myosin heavy chain proteins may be associated with factors regulating vascular ET-1 production. Additionally, in the presence of hyperinsulinemia, with insulin resistance, greater amounts of blood flow to the soleus may expose the vessel to more total insulin leading to enhanced ET-1 activation and local vascular ET-1 production. This could explain our finding in Study 1 that enhanced insulin-stimulated ET-1 vasocontraction with metformin treatment was only observed in the soleus and not the gastrocnemius muscle microcirculation. Overall these data suggest that inherent differences in regional skeletal muscle blood flow, concomitant with increased levels of shear induced by physical activity, play important roles in the local vascular production of ET-1, and improvements in the vascular reactivity to insulin.

Intrinsic Aerobic Fitness

In Study 1 and Study 2 we showed that improvements in the vascular reactivity to insulin with obesity are largely specific to physical activity, independent of changes in adiposity. Increased physical activity levels and exercise training often lead to improvements in aerobic fitness, which is an independent and strong predictor of cardiovascular disease and overall mortality (15, 35, 128, 143). Although obesity is also associated with cardiovascular

risk and mortality, studies that fail to account for the influence of underlying aerobic fitness distort the relationship between obesity and mortality (118, 119). Moreover, the role of enhanced aerobic fitness, which occurs concomitant with increased physical activity, to modulate the vascular reactivity to insulin was unknown. For these reasons in Study 3 we set out to uncover the potential underlying influence of aerobic fitness on insulin-stimulated vasorelaxation, by removing the confounding influence of daily physical activity. To elucidate the role of intrinsic aerobic fitness on endothelial function and the vascular reactivity to insulin we characterized vasomotor function in female rats selectively bred for low (LCR) or high (HCR) running capacity. Despite greater body weight and adiposity low aerobic capacity was paradoxically associated with enhanced vasorelaxation in response to insulin in the aorta, an effect that was entirely nitric oxide-mediated. Additionally, the vascular reactivity to insulin with intrinsic high aerobic capacity occurred independent of eNOS generated nitric oxide, providing evidence for the existence of alternant mechanism(s) to induce insulin-stimulated vasomotor relaxation. In addition to the endothelial actions of insulin, insulin has been shown to promote vasorelaxation via direct actions in vascular smooth muscle cells (168). Thus, future work evaluating the direct actions of insulin on the endothelium and vascular smooth muscle is justified.

In opposition to our findings with metformin and physical activity, intrinsic aerobic fitness was not associated with alterations in insulin-stimulated ET-1 vasocontraction. In fact our data indicate that insulin does not appear to signal through the MAPK/ET-1 pathway in the aorta of female LCR and HCR rats, This data suggest that vascular ET-1 release and/or ET-1 mediated vasocontraction do not account for differences in the aortic vasomotor response to insulin between LCR and HCR rats. Given that both low fit LCR and high fit

HCR rats in our study were mostly healthy, it is likely that insulin-mediated ET-1 vasocontraction is linked to the progression of a diseased vascular phenotype, present in conditions of insulin resistance, and obesity.

Further our data suggest that inherited aerobic capacity, independent of daily physical activity, is associated with divergent roles of NO in the vascular reactivity to insulin. Whilst NO entirely mediated insulin-stimulated vasorelaxation with low aerobic capacity, high aerobic capacity was associated with insulin-mediated NO-independent vasorelaxation. Although these results appear to be at odds with the current understanding of insulin's endothelial actions to elicit vasorelaxation through the PI3K/eNOS signaling pathway to produce NO (39, 90, 183), and vasocontraction through the MAPK signaling to produce ET-1 (90, 125, 126), they unveil the existence of other unknown mechanisms by which insulin stimulates vasorelaxation. Based on the literature three primary endothelial factors are known to elicit vascular smooth muscle relaxation: (i) nitric oxide (NO), (ii) prostacyclin (PGI_2), and the (iii) putative endothelium-derived hyperpolarizing factor(s) (EDHF) (13). Thus it is possible that high aerobic fitness may induce insulin-stimulated vasomotor relaxation via PGI_2 and/or EDHF-mediated mechanisms. In contrast insulin's ability to stimulate PGI_2, EDHF, and/or other alternant pathways appear to be reduced with low aerobic fitness, possibly leading to a compensatory over-activation of insulin-stimulated NO signaling. Future studies are necessary to investigate the influence of intrinsic aerobic fitness on the balance between endothelial NO, PGI_2, EDHF, and direct vascular smooth muscle mechanism(s) responsible for insulin-stimulated vasoreactivity.

Overall we speculate that greater vascular reactivity to insulin with low aerobic fitness may be due in part to a compensatory (albeit acute) increase in the vascular sensitivity

to insulin. Similar to past investigations we reported elevated insulin responses to a glucose tolerance test in LCR rats (129, 133). It is my hypothesis that in the early initial stages of insulin resistance recurrent postprandial episodes of hyperinsulinemia may enhance insulin-stimulated vasorelaxation and bulk flow in conduit arteries to ultimately augment downstream skeletal muscle blood flow. Intrinsic low exercise capacity in LCR rats is associated with 32% lower capillary density (75), and reduced skeletal muscle insulin sensitivity with a high fat diet (133). To counteract reduced skeletal muscle perfusion with low aerobic capacity, enhanced vascular reactivity to insulin following a meal may augment the delivery of glucose and insulin to help maintain skeletal muscle glucose disposal. One possible mechanism by which post-absorptive hyperinsulinemia may lead to greater insulin-stimulated vasorelaxation is founded on insulin's ability to modulate eNOS expression and regulate NO production. Insulin has been shown to increase the expression of eNOS in endothelial cells (6, 55, 94), and isolated microvessels (94). Thus, low aerobic fitness may be associated with a compensatory up-regulation of insulin-mediated activation of the PI3K/eNOS-signaling pathway, enhancing eNOS expression and producing greater NO-mediated insulin-stimulated blood flow.

Conclusion

The association between insulin resistance and cardiometabolic disease has been well established but is poorly understood. Coordinate impairments in the cardiovascular and metabolic actions of insulin play key roles in the etiology of obesity, type 2 diabetes, and cardiovascular disease. The purpose of this dissertation has been to expand our understanding of mechanisms regulating the vascular reactivity to insulin with disease. Previously we have demonstrated that exercise effectively prevents and reverses impairments in skeletal muscle

microvascular reactivity to insulin in rats prone to obesity and diabetes. Here we provide new evidence that unlike exercise, metformin does not enhance insulin-stimulated vasodilation in microvessels of obese insulin resistant rats, despite beneficial effects on body weight, food composition, and glycemic control. We extended these and our previous findings with exercise in the same rodent model to demonstrate that regular daily physical activity improves insulin-mediated vasorelaxation of the aorta, largely independent of changes in body composition. Although metformin, physical activity, and diet restriction treatments were associated with lower adiposity, and improvements in some markers of glycemic control, only physical activity enhanced the vascular reactivity to insulin. Overall these data strongly indicate that improvements in the vascular reactivity to insulin with obesity are specific to physical activity in both large conduit arteries and skeletal muscle resistance arteries.

Here we have also shown that intrinsic aerobic fitness, in the absence of exercise, modulates the vascular reactivity to insulin in rats selectively bred for divergent endurance running capacity. Taken as a whole, our data indicate that an inherited impairment in aerobic capacity is associated with an exclusive dependence on nitric oxide in aortic vasomotor function, and reduced mechanisms to stimulate vasorelaxation in response to insulin in the rat aorta. More research is needed to investigate the precise mechanisms of insulin-stimulated vasoreactivity in endothelial and vascular smooth muscle cells modified by aerobic fitness. Collectively, the data presented in this dissertation provide strong evidence that physical activity has powerful insulin-sensitizing effects on the vasculature with cardiometabolic disease.

FUTURE DIRECTIONS

Metformin and Exercise Combination Therapy

Given that both metformin and physical activity are well established as effective glucose-lowering and insulin-sensitizing approaches when prescribed individually, studies are needed to investigate the effects of the combined treatments on ameliorating metabolic and vascular dysfunction. Although not part of this dissertation we have recently investigated the combination therapy of metformin treatment and endurance or interval sprint training on the microvascular reactivity to insulin in OLETF rats prone to obesity and diabetes. Importantly our results indicate that metformin treatment abolished the beneficial effects of exercise on insulin-stimulated vasodilation in skeletal muscle resistance arteries. These clinically relevant findings agree with other recent studies demonstrating that metformin blunts exercise-induced adaptations (30, 106-108, 165). Currently I am in midst of writing this manuscript to submit to the American Journal of Physiology-Heart and Circulatory Physiology as a clinically significant Rapid Report.

In-vivo Insulin Stimulation of Vascular Tissue

In Study 2, overall we did not observe robust insulin stimulation of isolated abdominal aortic rings, and report no differences in basal or insulin-stimulated expression of total or phosphyorylated eNOS. This findings are consistent with our previous report in skeletal muscle arterioles (122) reporting a lack of insulin stimulation to modify eNOS content or activity. It is important to note that we cannot rule out the possibility that we did not observe significant insulin stimulation due to the fact that we assessed *in-vitro* insulin signaling in the isolated aorta with intact vascular smooth muscle and endothelial cells.

Although insulin has been shown to primarily signal through endothelial cells, it can also signal and act through vascular smooth muscle cells (168). Therefore, it is possible that altered endothelial insulin signaling may have been masked by largely unchanged vascular smooth muscle insulin signaling or vice versa. Thus, in future studies insulin stimulation should be performed separately on isolated aortic endothelial cells and vascular smooth muscle cells to distinguish insulin signaling in each cell type. Moreover, isolated vessels *in-vitro* are not exposed to the same circulating factors, pressure, stretch, or blood flow that they are exposed to *in-vivo*, thereby limiting the physiological applicability of our *in-vitro* findings. Given that we have observed similar difficulties with *in-vitro* insulin stimulation in skeletal muscle arterioles in the past (122), future studies should attempt to avoid these problems by performing *in-vivo* insulin stimulation of vascular tissue (e.g. hyperinsulinemic euglycemic clamp).

Physical Activity and Adiposity

Herein we have demonstrated that the vascular reactivity to insulin appears to be largely independent of changes in body mass and adiposity. However, in order to definitively make the claim that insulin-sensitizing effects on the vasculature are independent of changes in body weight and adiposity additional studies investigating the effects of physical activity in obesity without concomitant changes in body composition are necessary. In other words if an individual is physical active and fit, but classified as obese (based on adiposity) will they exhibit greater vascular reactivity to insulin than an obese sedentary physically inactive individual? This type of study would have important implications in understanding the "fat but fit" paradigm as it relates to the vascular insulin sensitivity. There is strong evidence, which Blair and colleagues have well documented, that obese and fit individuals have

substantially lower mortality risk than normal-weight but unfit individuals (116, 117). Additionally, there is a growing body of evidence indicating phenotypic and functional heterogeneity among different adipose tissue (AT) depots including visceral, subcutaneous, brown and perivascular adipose tissue (56, 59, 137, 147). We recently submitted a paper for publication (Appendix A) where we report that reduced adiposity, owing to either increased physical activity or diet restriction, in the obese insulin resistant OLETF rat model, leads to a marked reduction in the expression of inflammatory genes and markers of immune cell infiltration in visceral and perivascular AT. These anti-inflammatory effects of physical activity and diet restriction on AT were accompanied with a more athero-protective gene expression profile in the rat aorta. Therefore, it is possible that physical activity in the presence of greater adiposity may also promote an overall healthier adipose tissue phenotype associated with a less pro-atherogenic vascular phenotype and greater vascular sensitivity to insulin. Further research evaluating the effects of physical activity in the absence of changes in adiposity is needed to fully elucidate the insulin-sensitizing effects of physical activity on the vasculature.

Role of Intrinsic Aerobic Fitness

Future studies evaluating the time course of vascular insulin reactivity between low (LCR) and high capacity running (HCR) rats is warranted. Rationale for this is in part due to the observed age-related differences in glycemic control between LCR and HCR rats. Our data indicate that LCR animals display signs of reduced insulin sensitivity (greater insulin response to a glucose load) at an earlier age than HCR, yet overtime HCR "catch up" to LCR and exhibit a similar response. While LCR display a greater insulin response compared to HCR during the glucose tolerance test at 39 weeks of age, this difference disappears at 43

weeks of age, due to increased insulin secretion in HCR. Thus, one possibility for our finding that HCR exhibited lower insulin-stimulated aortic vasorelaxation compared to LCR is that the HCR vasculature may have become slightly desensitized to greater amounts of insulin over this time. Perhaps, if we assessed the vascular reactivity to insulin at 39 weeks of age when HCR animals had lower insulin responses during the glucose tolerance test, we may have observed similar or greater insulin-stimulated vasorelaxation compared to LCR. Alternatively, it is possible that recurrent postprandial episodes of hyperinsulinemia present in the LCR at 39 to 43 weeks of age may have increased the sensitivity of the LCR vasculature to insulin, leading to greater vascular insulin signaling and the observed enhanced (compensatory) insulin-stimulated vasorelaxation. Since, the enhanced insulin response to the glucose tolerance test was first observed at 43 weeks of age in HCR, it is likely that a later time point a similar compensatory augmentation of the vascular reactivity to insulin may also occur in HCR animals. It is also likely that as these animals continue to age, the vascular insulin sensitivity could continue to change, supporting the need to characterize the time-course of vascular reactivity to insulin in LCR and HCR rats. In summary, it is probable that glycemic control is associated with changes in vascular insulin sensitivity with intrinsic aerobic capacity, yet further research is needed to better understand the time course of these events.

In light of our observed paradoxical finding that insulin induced greater vasorelaxation in the aorta of LCR rats, it is important to remember that vasomotor reactivity and function is heterogeneous along the arterial vascular tree, and our findings in the aorta may not extend to other vascular beds. Although physical activity exerts strong insulin-sensitizing effects on both large conduit arteries and the skeletal muscle microcirculation, we

do not know how intrinsic aerobic fitness may influence insulin-mediated vasodilation in skeletal muscle resistance arteries. We know that insulin-stimulated vasodilation is important for optimal skeletal muscle perfusion, nutritive flow, and delivery of insulin and glucose to skeletal muscle. These insulin-mediated increases in skeletal muscle blood flow are estimated to account for as much as 40-50% of insulin-stimulated glucose disposal (8, 95, 132). Given that LCR exhibit reduced skeletal muscle insulin sensitivity with a high fat diet (133), future studies assessing the role of intrinsic aerobic fitness on insulin-stimulated vasodilation in the skeletal muscle microcirculation are of value.

Provided that the current obesity and type 2 diabetes epidemics appear to be largely related to physical inactivity and over-nutrition (21-23, 34, 172), it would be of interest to evaluate the effects of diet-induced obesity with intrinsic aerobic fitness on the vascular reactivity to insulin. Moreover, given that female LCR and HCR rats herein appeared to be somewhat protected against markers of metabolic and cardiovascular disease, the addition of a high fat diet may reveal metabolic and vascular dysfunction previously observed in males in this rodent model (133, 176, 189). Thus, consumption of a high fat diet may initiate a greater divergence in the vascular phenotype allowing for a better understanding of mechanisms involved in vascular insulin reactivity of LCR and HCR animals.

Finally, a completely unexpected finding in Study 3 was that high intrinsic aerobic fitness was associated with insulin-induced vasorelaxation of the aorta by non-nitric oxide (NO) mechanisms in healthy sedentary rats. Our data provides the first evidence for the existence of an insulin-mediated NO-independent dilatory mechanism(s). This surprising finding opens the door for prospective studies to elucidate the precise mechanism(s) of insulin-stimulated vasoreactivity modified by intrinsic aerobic fitness. Uncovering alternant

mechanisms and pathways by which insulin can induce vasodilation in the endothelium and vascular smooth muscle has important therapeutic benefits to enhance insulin-stimulated blood flow and skeletal muscle glucose uptake with obesity and type 2 diabetes.

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APPENDIX A

Adipose tissue and vascular phenotypic modulation by voluntary physical activity and dietary restriction in obese insulin resistant OLETF rats

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Running Title: Adipose tissue and vascular effects of physical activity and diet

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ABSTRACT

Adipose tissue (AT)-derived cytokines are proposed to contribute to obesity-associated vascular insulin resistance. We tested the hypothesis that voluntary physical activity and diet restriction-induced maintenance of body weight would both result in decreased AT inflammation and concomitant improvements in insulin-stimulated vascular relaxation in the hyperphagic, obese OLETF rat. OLETF rats (age 12 wk) were randomly assigned to sedentary (SED, n=10), wheel running (WR, n=10), and diet restriction (DR, n=10; fed 70% of SED) for 8 weeks. WR and DR rats exhibited markedly lower adiposity (7.1±0.4 and 15.7±1.1% body fat, respectively) relative to SED (27±1.2% body fat), as well as improved blood lipid profiles and systemic markers of insulin resistance. Less adiposity in both WR and DR was associated with decreased AT expression of inflammatory genes (e.g., MCP-1, TNF-α, IL-6) and markers of immune cell infiltration (e.g., CD4, CD8, CD11c, F4/80). The extent of these effects were most pronounced in visceral AT (retroperitoneal) compared to subcutaneous and periaortic AT. Markers of inflammation in brown AT were upregulated with WR but not DR. In periaortic AT, WR and DR-induced reductions in mRNA expression and secretion of cytokines were accompanied with a more athero-protective gene expression profile in the adjacent aortic wall. WR, but not DR, resulted in greater insulin-stimulated relaxation in the aorta; an effect that was in part mediated by a decrease in insulin-induced endothelin-1 activation in WR aorta compared to SED. Collectively, we show in OLETF rats that lower adiposity leads to less AT and vascular inflammation as well as an exercise-specific improvement in insulin-stimulated vasorelaxation.

INTRODUCTION

More than one-third of Americans are obese (38) and the causes underlying the obesity epidemic appear to be largely related to physical inactivity and over-nutrition, a set of behaviors increasingly prevalent in our society (4-6, 10, 57). Cumulative evidence indicates that obesity is an important contributor to the development of whole body insulin resistance, type 2 diabetes, and cardiovascular disease (20). A critical link between obesity and its associated metabolic and cardiovascular diseases is thought to be chronic low-grade systemic inflammation (20). In this regard, recent studies implicate adipose tissue (AT) as a local and systemic source of inflammatory cytokines that may be involved in the instigation of vascular insulin resistance and atherosclerosis associated with obesity (11, 12, 18, 19, 30, 32-35, 45, 46, 51, 55, 56). Indeed, excessive lipid accumulation and enlargement of adipocytes in obesity is associated with infiltration of immune cells into AT, contributing to AT inflammation and subsequent secretion of pro-inflammatory cytokines (52). A deeper understanding of the influence of lifestyle modifications on AT inflammation and vascular insulin resistance may lead to more effective strategies aimed at prevention and treatment of obesity-related metabolic and cardiovascular diseases.

Accordingly, we tested the hypothesis that treatment with increased voluntary physical activity or diet restriction-induced maintenance of body weight would result in decreased AT inflammation and concomitant improvements in vasomotor reactivity to insulin in obese, insulin-resistant rats. Given the growing appreciation for phenotypic and functional heterogeneity among AT depots including visceral, subcutaneous, brown and perivascular AT (15, 16, 40, 48), we also reasoned that the extent of the effects of physical activity and diet restriction on AT inflammation would be AT depot-specific. Furthermore, we tested the hypothesis that reduced

AT expression and secretion of cytokines caused by physical activity or dietary restriction would be accompanied by a less pro-atherogenic vascular phenotype and enhanced insulin-stimulated vascular relaxation.

METHODS

Animals

All animal protocols were approved by the University of Missouri Institutional Animal Care and Use Committee. Male OLETF rats were obtained at 4 weeks of age (Japan SLC, Inc. 3371-8, Kotoh-Cho, Hamamatsu, Shizuoka, Japan) and housed individually in cages maintained in temperature-controlled (21ºC) animal quarters with light from 06:00 to 18:00 h and dark from 18:00 h to 06:00 h. At 12 weeks, rats were randomized to one of the following three groups: (i) sedentary (SED; n=10); (ii) voluntary wheel running (WR, n=10); or (iii) sedentary + diet restriction (DR, fed 70% of ad libitum-fed SED animals; n=10). Animals in the WR group were housed with running wheels connected to a Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA, USA) for determination of daily running distance. All groups were provided with standard rodent chow (Formulab 5008, Purina Mills, St. Louis, MO) with approximately 26% protein, 18% fat, and 56% carbohydrate. SED and WR groups had *ad libitum* access to food. Body weights and food intakes were recorded on a weekly basis. At 20 weeks of age, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Tissues were harvested and the animals were euthanized by exsanguination in full compliance with the American Veterinary Medical Association Guidelines on Euthanasia. The wheels of the WR group were locked and food was removed from the cages of all groups ~14 hrs before the rats were sacrificed.

Body composition and blood parameters

On the day of the experiments, body mass was measured to the nearest 0.01 g and, following anesthetization, body composition was determined using a dual energy x-ray absorptiometry instrument (Hologic QDR-1000) calibrated for rodents. In addition, retroperitoneal, epididymal, and omental AT weights were measured to the nearest 0.01 g. Plasma samples were prepared by centrifugation and stored at -80ºC until analysis. Glucose, cholesterol, triglycerides, and non-esterified fatty acids (NEFA) assays were performed by a commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO) on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA) using commercially available assays according to manufacturer's guidelines. Plasma insulin concentrations were determined using a commercially available, rat-specific ELISA (Alpco Diagnostics, Salem, NH). In addition, plasma and periaortic AT-conditioned medium samples were assayed for concentrations of leptin, MCP-1, TNF-α, and IL-6 using a multiplex cytokine assay (Millipore Milliplex, cat no. RCYTOMAG-80K; Billerica, MA, USA) on a MAGPIX instrument (Luminex Technologies; Luminex Corp., Austin, TX, USA) according to the manufacturer's instructions (24, 40).

Tissue sampling

Perivascular AT surrounding the thoracic aorta, retroperitoneal white AT, inguinal subcutaneous white AT, and interscapular brown AT were quickly excised from the anesthetized rat. For each fat depot, a portion was flash frozen and kept at -80ºC for examination of gene expression and a portion was fixed in neutral-buffered 10% formalin for histology analysis. A portion of perivascular AT surrounding the thoracic aorta was used for in vitro assessment of cytokine secretion as described below. A segment of the thoracic aorta cleaned of perivascular

AT and excess adventitia was sectioned into 2 mm rings in cold Krebs for subsequent assessment of vasomotor function. In addition, isolated thoracic aortic segments were kept in RNAlater (Ambion, Austin, TX) for 24 h at 4ºC, then removed from the RNAlater solution and stored at -80ºC until processing.

Cytokine secretion from periaortic AT

A portion of perivascular AT surrounding the thoracic aorta was incubated in Medium 199 at pH 7.4 for 24 hrs (100 mg of AT per 300 ul) under standard culture conditions (37ºC, 5% CO_2) to obtain the corresponding secretomes (SED, WR, and DR) (1). After 24 hrs of incubation, the conditioned media from the AT explants were stored at -80ºC until analysis.

RNA extraction and real-time PCR

AT and aortic samples were homogenized in TRIzol solution using a tissue homogenizer (TissueLyser LT, Qiagen, Valencia, CA). Total RNA was isolated using the Qiagen's RNeasy Lipid Tissue Kit and assayed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) to assess purity and concentration. First-strand cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed as previously described (40-42) using the CFX Connect™ Real-Time PCR Detection System (BioRad, Hercules, CA). Primer sequences (Table 1) were designed using the NCBI Primer Design tool. All primers were purchased from IDT (Coralville, IA). A 20-µl reaction mixture containing 10 µl iTaq UniverSYBR Green SMX (BioRad, Hercules, CA) and the appropriate concentrations of gene-specific primers plus 4 µl of cDNA template were loaded in each well of a 96-well plate. All PCR reactions were performed in duplicate. PCR was performed with thermal conditions as follows: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 45 s. A

dissociation melt curve analysis was performed to verify the specificity of the PCR products. 18S primers were used to amplify the endogenous control product. Our group has established that 18S is a suitable house-keeping gene for real-time PCR when examining AT and vascular gene expression. In the present study, 18S CTs were not different among the three groups of animals for any of the different tissues examined. mRNA expression values are presented as $2^{\Delta CT}$ whereby ΔCT = 18S CT - gene of interest CT (40-42). mRNA levels were normalized to the SED group of rats, which was always set at 1.

Histology assessments

Formalin-fixed AT samples were processed through paraffin embedment, sectioned at five microns, stained with hematoxylin and eosin for morphometric determinations. Sections were examined using an Olympus BX60 photomicroscope (Olympus, Melville, NY) and photographed at 40x magnification using with a Spot Insight digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) (24, 40).

Functional assessment of isolated aortic rings

A segment of the thoracic aorta, trimmed of fat and connective tissue, was sectioned into 2 mm rings in cold Krebs. Rings were then mounted on wire feet connected to isometric force transducers and submerged in 20mL baths containing physiological Krebs solution maintained at 37ºC for 1 hour to allow for equilibration. Aortic rings were stretched to optimal length which ranged from 130 to 140% of passive diameter. Vasoreactivity was assessed with cumulative concentration-response curves of acetylcholine (ACh, 10^{-10} to 10^{-4} M), insulin (10 to 10,000 µIU/mL), sodium-nitro-prusside (SNP, 10^{-10} to 10^{-4} M), and endothelin-1 (ET-1, 10^{-10} to 10^{-7} M). A submaximal concentration of phenylephrine ($3e^{-7}$ M) was used to preconstrict all vessels prior to acetylcholine, insulin and SNP relaxation curves. The contribution of ET-1 in altering insulin-

stimulated relaxation was assessed by incubating the rings with tezosentan (3 μM for 20 min), a nonselective ET-1 receptor blocker. For insulin, ACh, and SNP curves, relaxation at each concentration was measured and expressed as percent maximum relaxation, where 100% is equivalent to loss of all tension developed in response to phenylephrine (9).

Statistical analysis

The effects of WR and DR on all dependent variables were evaluated using a one-way ANOVA. Dose-response curves from vasomotor function experiments were analyzed using a two-way (group x dose) ANOVAs. When appropriate, the Fishers protected least significant difference post hoc was used. All data are presented as mean ± standard error (SE). For all statistical tests, the alpha level was set at 0.05. All statistical analyses were performed with SPSS V21.0.

RESULTS

As shown in Figure 1, rats with access to running wheels increased daily running distance from week 12 to week 15 (~8km/day), after which running distance gradually declined to ~6km/day at 20 weeks. These are similar voluntary running distances as we have previously reported in this animal model (31, 36, 37, 50). WR and DR rats weighed less and had improved body composition profiles (e.g., lower total percent body fat and less visceral adipose tissue mass) compared to SED rats (all $p<0.05$). As summarized in Table 2, compared to the SED group, WR and DR plasma had lower fasting total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, NEFAs, glucose, and leptin (all $p<0.05$). In addition, WR had improved HOMA-IR, lower plasma insulin, and lower circulating MCP-1 compared to SED rats (all $p<0.05$).

Figure 2 illustrates representative histological photographs of retroperitoneal AT, subcutaneous AT, interscapular brown AT, and periaortic AT. As shown, WR and DR had less lipid deposition in brown and periaortic AT as well as decreased adipocyte size in white AT. Consistent with our previous report (40), a clear structural similarity between thoracic perivascular AT and interscapular brown AT was noted.

Retroperitoneal AT of WR and DR rats exhibited reduced expression of leptin, MCP-1, TNF-α, IL-6 (Figure 3), PAI-1, ICAM-1 (Figure 4), CD4, CD8, CD11c, F4/80, FOXP3 (Figure 5), p22 phox, and p47phox (Figure 6) relative to SED (all $p<0.05$). Furthermore, WR resulted in increased expression of PPARGC-1α, and DR resulted in decreased expression of GRP78 and CHOP (all $p<0.0.5$). Subcutaneous AT of WR and DR rats exhibited reduced expression of leptin and MCP-1 relative to SED (Figure 3; all $p<0.05$). Interscapular brown AT of WR and DR rats exhibited reduced expression of leptin (Figure 3) and PAI-1 (Figure 4) relative to SED (all $p<0.05$). Furthermore, WR resulted in increased expression of MCP-1, TNF-α ($p=0.06$), IL-6 (Figure 3), E-selectin (Figure 4), CD4 ($p=0.06$), F4/80 ($p=0.06$) (Figure 5), and p47phox (Figure 6; all $p<0.05$ unless otherwise indicated); whereas DR resulted in reduced expression of UCP-1 (Figure 7; $p<0.05$). Periaortic AT of WR and DR rats exhibited reduced expression of leptin, MCP-1, TNF-α (Figure 3), CD11c (Figure 5), and UCP-1 (Figure 7) relative to SED (all $p<0.05$). Furthermore, WR resulted in reduced expression of PAI-1 (Figure 4), CD8 (Figure 5) and increased expression of VCAM-1 (Figure 4; all $p<0.05$). On the other hand, DR resulted in reduced expression of IL-6 (Figure 3), E-selectin, ICAM-1 (Figure 4), CD4, F4/80 (Figure 5), p22phox, and p47phox (Figure 6; all $p<0.05$).

Aorta of WR and DR rats exhibited reduced expression of IL-6 (Figure 3), E-selectin, VCAM-1 (Figure 4), CD4, CD8, F4/80 (Figure 5), GRP78 and CHOP (Figure 6) relative to SED

(all $p<0.05$). Furthermore, WR resulted in reduced expression of leptin (Figure 3); whereas DR resulted in reduced expression of MCP-1 (Figure 3), CD11c (Figure 5), and increased expression of PAI-1 (Figure 4; all $p<0.05$).

As illustrated in Figure 7, WR and DR rats exhibited reduced periaortic AT-derived secretion of leptin, IL-6 (all $p<0.05$), and MCP-1 ($p=0.09$ and $p=0.11$, respectively) relative to SED. Periaortic-derived secretion of TNF-α was similar among groups ($p>0.05$).

Insulin-stimulated aortic relaxation was significantly greater in WR rats relative to SED and DR rats (Figure 9). Treatment of aortic rings with tezosentan, a non-selective ET-1 receptor blocker, largely removed differences in insulin-stimulated relaxation between WR and SED rats. That is, tezosentan increased insulin-stimulated relaxation in the SED ($p=0.057$) but had no effect in WR or DR ($p>0.05$). A small decrease in ACh-mediated relaxation was observed in DR rats at the highest dose of ACh. In addition, we observed no differences in ET-1-mediated contraction or SNP-mediated relaxation among groups ($p>0.05$).

DISCUSSION

The primary findings of the present study are as follows: (i) treatment with voluntary WR and 30% DR regimen for 8 weeks (starting at 12 weeks of age) resulted in marked reductions in adiposity, improved blood lipid profiles and systemic markers of insulin resistance in the obese OLETF rat model; (ii) reductions in adiposity, through both WR and DR, were associated with decreased AT expression of inflammatory genes and markers of immune cell infiltration, effects that were most pronounced in visceral AT compared to subcutaneous and periaortic AT; (iii) in contrast to other depots, markers of inflammation/immune function in brown AT were upregulated with WR but not DR; (iv) WR and DR-induced reductions in mRNA expression and secretion of cytokines in periaortic AT were accompanied with a more athero-protective gene

expression profile in the adjacent aortic wall; and (v) WR, but not DR, resulted in increased insulin-stimulated relaxation in the aorta; an effect that was in part mediated by a decrease in insulin-induced ET-1 mediated activation/contraction in the aorta of WR.

Existing evidence in animals and humans indicates that exercise results in reduced expression of inflammatory genes and markers of immune cell infiltration in white AT (8, 17, 27, 29, 60, 61). We found that voluntary WR-induced decreases in adiposity produced, by and large, similar down-regulation of inflammatory genes in white AT (retroperitoneal) to that produced by decreases in adiposity with 30% DR regimen. Thus, our data support the notion that changes in adiposity appear to be the main driving force for the decreased inflammation in white AT. Further research evaluating the effects of physical activity in the absence of changes in adiposity is needed to elucidate any exercise-specific effects on AT. A unique aspect of the present study was the examination of the effects of WR and DR in several AT depots beyond visceral fat, i.e., subcutaneous, brown, and perivascular AT depots. Overall, our data indicate that the greatest effects of WR and DR on AT gene expression were in visceral AT. While visceral AT is highly susceptible to obesity-mediated inflammation/infiltration of immune cells (17, 27, 29, 60, 61), these data suggest that the phenotype of visceral AT is also highly amenable to reductions in adiposity. Given this close link between visceral AT expansion and inflammation in obesity (17, 27, 29, 60, 61), it is not surprising that measures of central adiposity in humans relate with metabolic and cardiovascular outcomes (20) and that interventions that result in weight loss are associated with improvements in insulin sensitivity and cardiovascular health (21).

An intriguing finding of the present study is that WR and DR produced differential effects on brown AT gene expression. While both WR and DR produced a down-regulation of leptin and PAI-1 mRNA, WR resulted in the induction of pro-inflammatory genes, markers of

immune cell infiltration, and oxidative stress. The finding that WR markedly increased expression of IL-6 in brown AT is of particular interest considering the recent study by Stanford and colleagues (54) demonstrating that brown AT-derived IL-6 is required for the profound effects of brown AT transplantation on glucose homeostasis and insulin sensitivity. Indeed, those authors found that the beneficial metabolic effects of brown AT transplantation were lost when AT used for transplantation was obtained from IL-6 knockout mice (54). Our data combined with the results of Standford et al. (54) suggest that the beneficial metabolic effects of exercise may be in part mediated by an increased expression of IL-6 in brown AT. To our knowledge, this is the first study to provide evidence that physical activity, but not calorie restriction, is effective in inducing IL-6 expression in brown AT. Our finding that other pro-inflammatory markers (e.g., MCP-1, TNF-α) were also upregulated in brown AT warrants further investigation.

Given the increasing evidence implicating AT surrounding large arteries as a local source of inflammatory cytokines that may be involved in the instigation of vascular dysfunction and atherosclerosis (11, 12, 18, 19, 32, 34, 35, 45, 46, 56), a central focus of the present study was to examine the impact of WR and DR on the phenotypic modulation of periaortic AT. Periaortic AT from WR and DR rats clearly exhibited reduced lipid deposition relative to sedentary control rats (Figure 2) and this effect was associated with reduced expression of macrophage (CD11c) and T-cell specific genes (CD4 and CD8), suggesting a decrease in the infiltration of immune cells into periaortic AT. Notably, we also found that WR and DR caused a reduction in periaortic AT-derived secretion of cytokines, such as leptin, IL-6, and MCP-1. To our knowledge this is the first evidence that physical activity and dietary restriction can effectively reduce expression and secretion of cytokines from perivascular AT. The robust reduction in leptin secretion from periaortic AT induced by WR and DR deserves specific attention given

direct evidence implicating perivascular AT-derived leptin as a potential contributor to vascular dysfunction. In this regard, Payne et al. (45) elegantly demonstrated that perivascular AT-induced impairment of coronary artery function in metabolic syndrome pigs was reversed with a recombinant leptin antagonist. Furthermore, there is increasing evidence that leptin, when in excess, induces a pro-inflammatory and pro-oxidant vascular phenotype (3, 7, 13, 28, 49, 53, 62). Given our findings that WR and DR reduced systemic inflammation and secretion of cytokines from perioarotic AT, we hypothesized that these effects would be accompanied by an athero-protective phenotype in the adjacent aortic wall. Indeed, we found that both WR and DR resulted in down-regulation of inflammatory genes (e.g., IL-6, E-selectin, VCAM-1), markers of immune cell infiltration (e.g., CD4, CD8, F4/80), and makers of endoplasmic reticulum stress (e.g., GRP78 and CHOP) in the aorta. These findings are consistent with the hypothesis that at the transcriptional level, the effects of physical activity on vascular cell phenotype may be driven by changes in adiposity and the consequent alterations in cytokine secretion from AT.

In contrast to the results where WR and DR had similar effects on gene expression in AT, our results from the aortic vasomotor function experiments indicate that physical activity improved insulin-induced vasorelaxation while DR had no effect. In particular, we found that insulin-stimulated relaxation of the aorta was increased with physical activity but not by a lowering of body weight evoked through dietary restriction. This finding is consistent with previous data from our group showing that insulin-stimulated dilation in skeletal muscle arterioles was enhanced in WR, but not DR, OLETF rats (36). Herein we also report that differences in insulin-stimulated vasorelaxation between WR and SED rats were largely abolished after treatment of aortic rings with a nonselective ET-1 receptor blocker. ET-1 blockade had little to no effect on insulin-induced relaxation in the aorta from WR animals but produced nearly a 2-fold increase in insulin-induced relaxation in SED. This finding suggests

that increases in vasorelaxation to insulin with physical activity were mediated in part by a decrease in ET-1 signaling. Furthermore, to evaluate whether greater insulin-stimulated relaxation in the aorta of WR rats was due to reduced vascular sensitivity to ET-1 and/or decreased vascular production of ET-1, we examined aortic responsiveness to exogenous ET-1 in all three groups of animals. We found similar ET-1 mediated constriction among groups suggesting that it was local insulin-stimulated ET-1 activation, and not the vascular sensitivity to ET-1 *per se* that is likely modulated by physical activity.

Our findings that physical activity, but not DR, increases insulin-stimulated vasorelaxation as a result of decreased insulin-mediated ET-1 activation may be significant in light of evidence indicating that excess ET-1 signaling is an important contributor to the pathogenesis of macro-vascular disease (47). Exercise-induced increases in blood flow and thus shear stress to the artery wall is a likely mechanism by which physical activity exerts an insulin sensitizing effect on the aorta and a decrease in ET-1 (23, 44). This hypothesis is supported by evidence that (i) shear stress reduces expression of ET-1 in cultured endothelial cells (59), (ii) removal of WR for 7 days increases expression of ET-1 in the rat iliac artery (42), (iii) rat soleus muscle feed arteries, known to be chronically exposed to high levels of blood flow display greater insulin-stimulated dilation, as a result of reduced ET-1 activation, than gastrocnemius feed arteries, known to be chronically exposed to lower levels of flow (25), and (iv) inactive lower limbs of spinal cord injury patients, chronically exposed to low blood flow and shear stress (2), exhibit an enhanced ET-1 mediated basal vascular tone (58).

Another interesting finding of the present study is that the WR-related increase in insulin-stimulated relaxation in the aorta occurred in the absence of changes in ACh-mediated relaxation, a response largely mediated by nitric oxide in rat aorta. Incidentally, compelling

evidence from studies using the Zucker obese rat model indicates that impairments in insulin-mediated dilation occurs prior to impairments in ACh-mediated dilation in both skeletal muscle (14) and coronary arterioles (26, 39). It is possible that ACh-mediated dilation was not improved in our WR animals, relative to the sedentary OLETF animals, because no impairment in ACh-mediated dilation may have been present in these 20 week old sedentary rats. Consistent with this hypothesis, data in animals and humans suggest that exercise training does not further improve endothelium-dependent dilation in subjects with a healthy endothelium, likely due to a ceiling effect (22, 43). We did observe, unexpectedly, that DR resulted in a small but significant decrease in ACh-mediated dilation relative to the SED group fed *ad libitum*; this effect was not observed in WR animals.

In summary, we provide evidence that reduced adiposity, owing to either increased physical activity or diet restriction, in the obese, insulin resistant OLETF rat model leads to a marked reduction in the expression of inflammatory genes and markers of immune cell infiltration in visceral and periaortic AT. Our data also demonstrate unique AT depot-specific effects of both increased physical activity and dietary restriction. The anti-inflammatory effects of physical activity and diet restriction on AT were accompanied with a more athero-protective gene expression profile in the contiguous aorta. Importantly, our results indicate that physical activity enhanced aortic insulin-induced relaxation while diet restriction did not, suggesting that the insulin sensitizing effect on the vasculature is exercise-specific.

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Table 1. Forward and reverse primer sequences for quantitative real-time PCR

	Primer sequence (5'→3')	
Gene	**Forward**	**Reverse**
18S	GCCGCTAGAGGTGAAATTCTTG	CATTCTTGGCAAATGCTTTCG
Leptin	GACACCCTTAGAGGGGGCTA	AACCCAAGCCCCTTTGTTCA
MCP-1	CTGTCTCAGCCAGATGCAGTTAA	AGCCGACTCATTGGGATCAT
TNF-α	AACACACGAGACGCTGAAGT	TCCAGTGAGTTCCGAAAGCC
IL-6	AGAGACTTCCAGCCAGTTGC	AGCCTCCGACTTGTGAAGTG
E-Selectin	GCCATGTGGTTGAATGTAAAGC	GGATTTGAGGAACATTTCCTGACT
VCAM-1	GAAGGAAACTGGAGAAGACAATCC	TGTACAAGTGGTCCACTTATTTCAATT
ICAM-1	CACAAGGGCTGTCACTGTTCA	CCCTAGTCGGAAGATCGAAAGTC
PAI-1	AGCTGGGCATGACTGACATCT	GCTGCTCTTGGTCGGAAAGA
CD4	ACCCTAAGGTCTCTGACCCC	TAGGCTGTGCGTGGAGAAAG
CD8	CACTAGGCTCCAGGTTTCCG	CGCAGCACTTCGCATGTTAG
CD11c	CTGTCATCAGCAGCCACGA	ACTGTCCACACCGTTTCTCC
F4/80	GCCATAGCCACCTTCCTGTT	ATAGCGCAAGCTGTCTGGTT
FoxP3	CTCCAGTACAGCCGGACAC	GGTTGGGCATCAGGTTCTTG
CHOP	AGAGCCAAAATAACAGCCGGA	ACCGGTTTCTGCTTTCAGGT
GRP78	GCAGTTGCTCACGTGTCTTG	TCCAAGGTGAACACACACCC
p22phox	ACCTGACCGCTGTGGTGAA	GTGGAGGACAGCCCGGA
p47phox	ACGCTCACCGAGTACTTCAACA	TCATCGGGCCGCACTTT
UCP-1	CCGGTGGATGTGGTAAAAAC	CTCCAAGTCGCCTATGTGGT
PPARGC-1-α	GGGGCACATCTGTTCTTCCA	GAGCTGTTTTCTGGTGCTGC

Table 2. Fasting plasma characteristics in sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats

Variable	SED	WR	DR
Total cholesterol, mg/dl	110.7±4.4	69.7±2.8*	78.0±1.1*
LDL cholesterol, mg/dl	46.4±3.9	29.6±1.8*	34.5±0.8*
HDL cholesterol, mg/dl	33.2±0.7	29.9±1.3*	29.1±0.5*
Triglycerides, mg/dl	155.0±6.5	51.1±2.7*	71.9±4.0*#
NEFA, mmol/l	0.57±0.05	0.19±0.01*	0.31±0.03*#
Insulin, ng/ml	30.8±10.1	6.9±0.7*	19.5±3.2
Glucose, mg/dl	311.6±9.5	196.1±4.9*	223.1±10.6*#
HOMA-IR index	24.1±8.2	3.4±0.4*	11.1±2.2
Leptin, ng/ml	260.1±39.0	2.2±0.3*	63.9±24.7*
MCP-1, pg/ml	197.1±8.5	144.3±9.4*	194.4±26.4#
TNF-α, pg/ml	6.5±0.3	5.3±0.5	6.6±0.5#
IL-6, pg/ml	204.4±66.0	173.6±56.7	194.5±66.3

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; NEFA; non-esterified fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance; MCP-1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6.

*****Denotes difference ($p<0.05$) from SED rats; #Denotes difference ($p<0.05$) from WR rats.

FIGURE LEGENDS

Figure 1. Body composition and food intake in sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. Body fat, heart weights, and fat pad weights were obtained at 20 weeks (time of sacrifice). *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Figure 2. Representative histology photographs (40X magnification) of retroperitoneal AT, subcutaneous AT, interscapular brown AT, and periaortic AT in sedentary (SED), wheel running, (WR), and diet restriction (DR) OLETF rats.

Figure 3. Expression of cytokine-related genes in ATs and aorta of sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. For each gene, SED is used as the reference group and set at 1. *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Figure 4. Expression of plasminogen activator inhibitor-1 (PAI-1) and adhesion molecules-related genes in ATs and aorta of sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. For each gene, SED is used as the reference group and set at 1. *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Figure 5. Expression of immune cell-related genes in ATs and aorta of sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. For each gene, SED is used as the reference group and set at 1. *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Figure 6. Expression of NADPH oxidase subunits and endoplasmic reticulum (ER) stress-related genes in ATs and aorta of sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. For each gene, SED is used as the reference group and set at 1. *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Figure 7. Expression of mitochondria-related genes in ATs and aorta of sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. For each gene, SED is used as the reference group and set at 1. *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Figure 8. Secretion of cytokines from periaortic AT explants in sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Figure 9. Vasomotor function of thoracic aortic rings in sedentary (SED), wheel running (WR), and diet restriction (DR) OLETF rats. Values are expressed as means ± SE. *****Denotes difference ($p<0.05$) from SED rats; $^{\#}$Denotes difference ($p<0.05$) from WR rats.

Fig 1

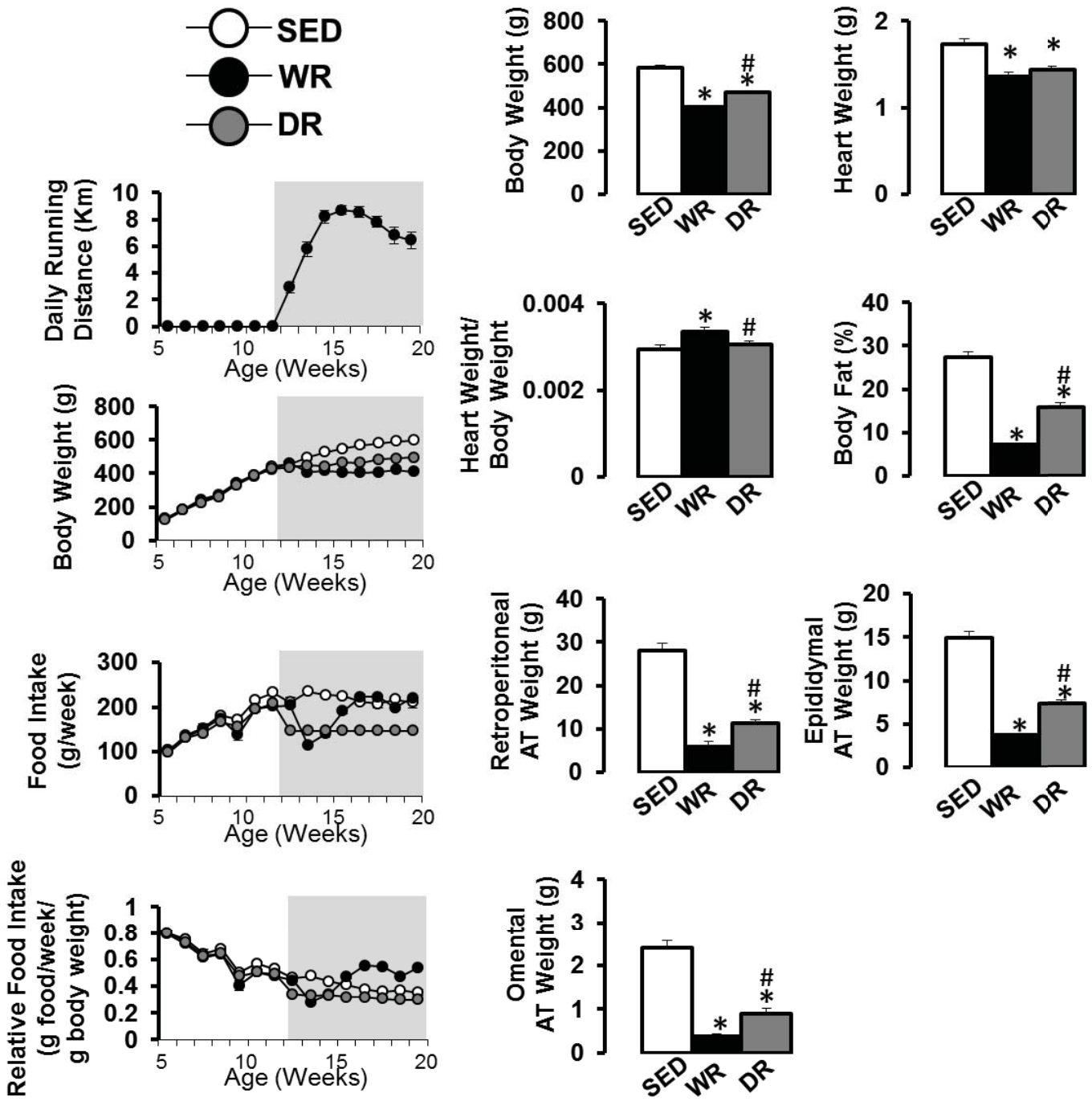

SED
WR
DR
Daily Running Distance (Km)
Age (Weeks)
Body Weight (g)
Food Intake (g/week)
Relative Food Intake (g food/week/ g body weight)
Heart Weight (g)
Heart Weight/ Body Weight
Body Fat (%)
Retroperitoneal AT Weight (g)
Epididymal AT Weight (g)
Omental AT Weight (g)

Fig 2

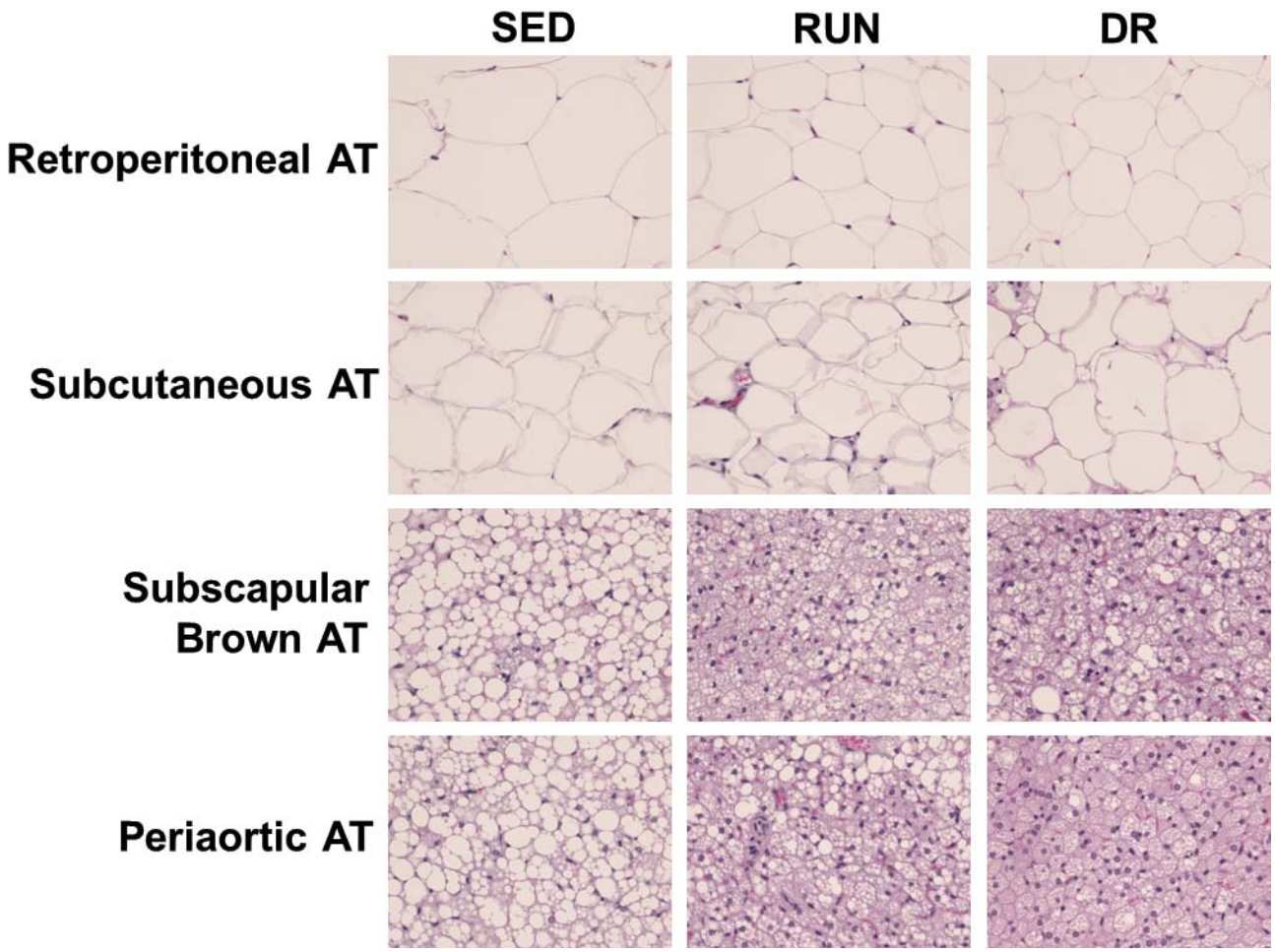

SED
RUN
DR
Retroperitoneal AT
Subcutaneous AT
Subscapular Brown AT
Periaortic AT

Fig 3

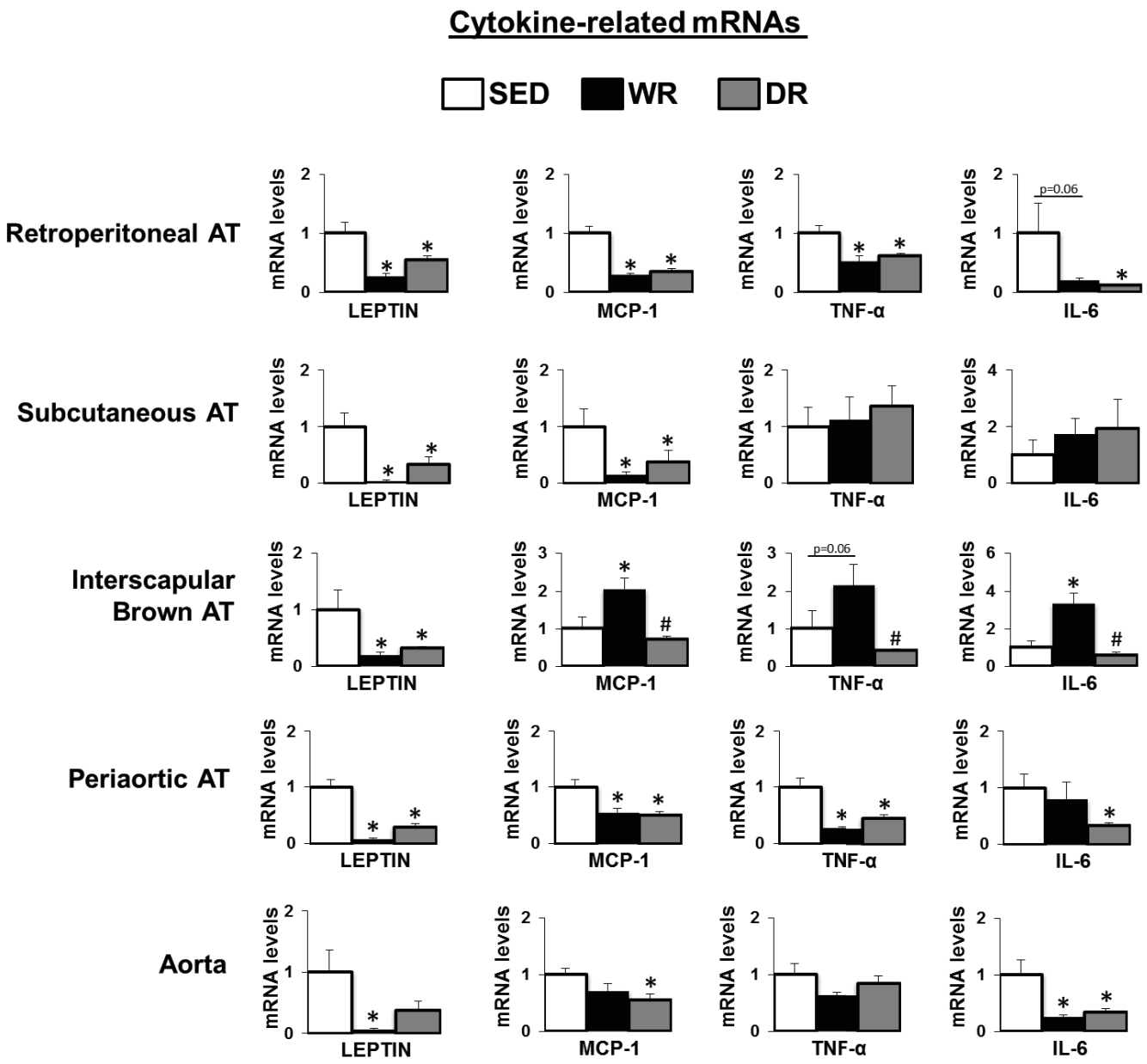

Cytokine-related mRNAs
SED
WR
DR
Retroperitoneal AT
Subcutaneous AT
Interscapular Brown AT
Periaortic AT
Aorta
mRNA levels
LEPTIN
MCP-1
TNF-α
IL-6
p=0.06
*
#

Fig 4

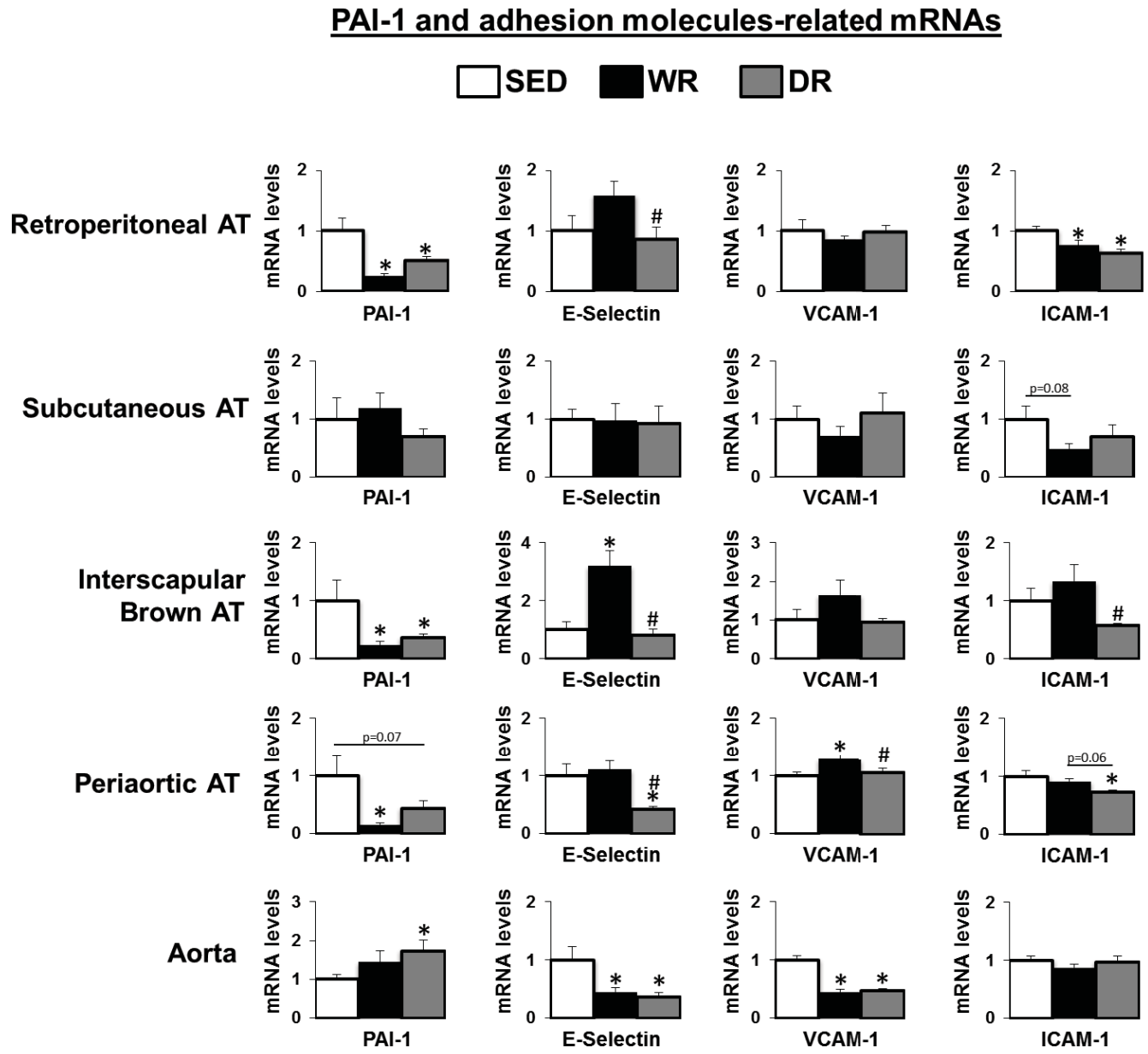

PAI-1 and adhesion molecules-related mRNAs
SED
WR
DR
Retroperitoneal AT
Subcutaneous AT
Interscapular Brown AT
Periaortic AT
Aorta
mRNA levels
PAI-1
E-Selectin
VCAM-1
ICAM-1
p=0.08
p=0.07
p=0.06

Fig 5

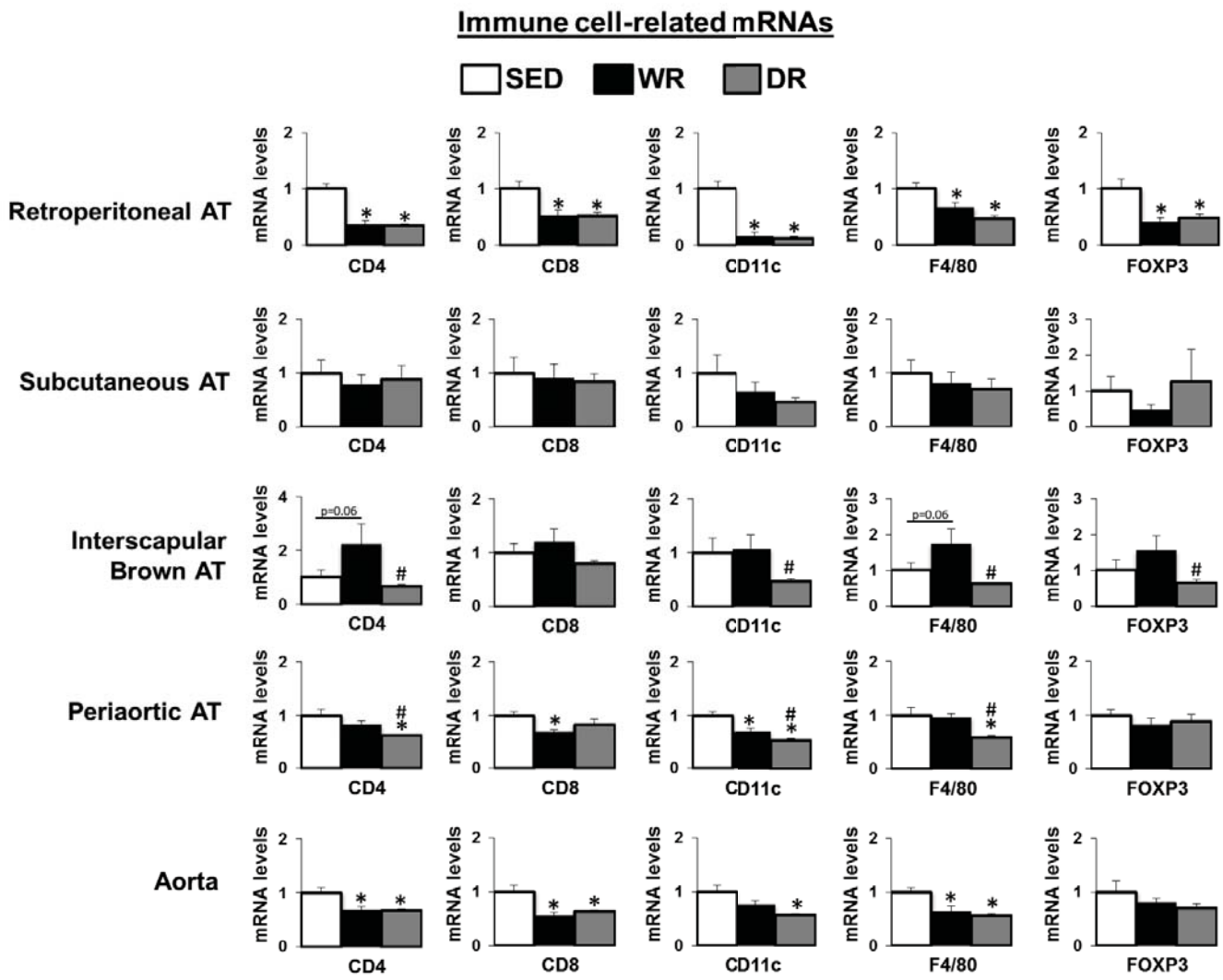

Immune cell-related mRNAs
SED
WR
DR
Retroperitoneal AT
Subcutaneous AT
Interscapular Brown AT
Periaortic AT
Aorta
mRNA levels
CD4
CD8
CD11c
F4/80
FOXP3
p=0.06
*
#

Fig 6

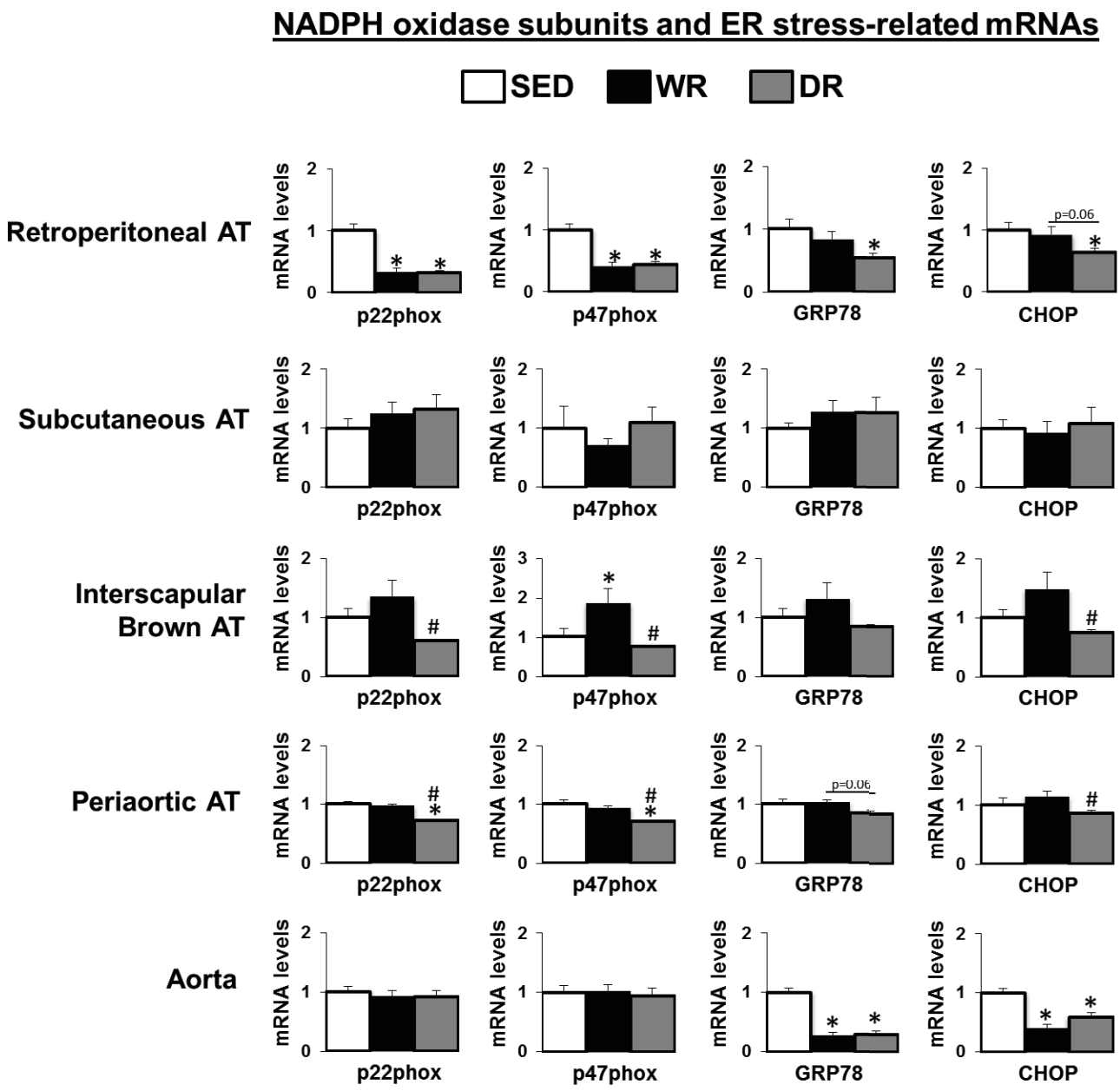

NADPH oxidase subunits and ER stress-related mRNAs
SED
WR
DR
Retroperitoneal AT
Subcutaneous AT
Interscapular Brown AT
Periaortic AT
Aorta
mRNA levels
p22phox
p47phox
GRP78
CHOP
p=0.06

Fig 7

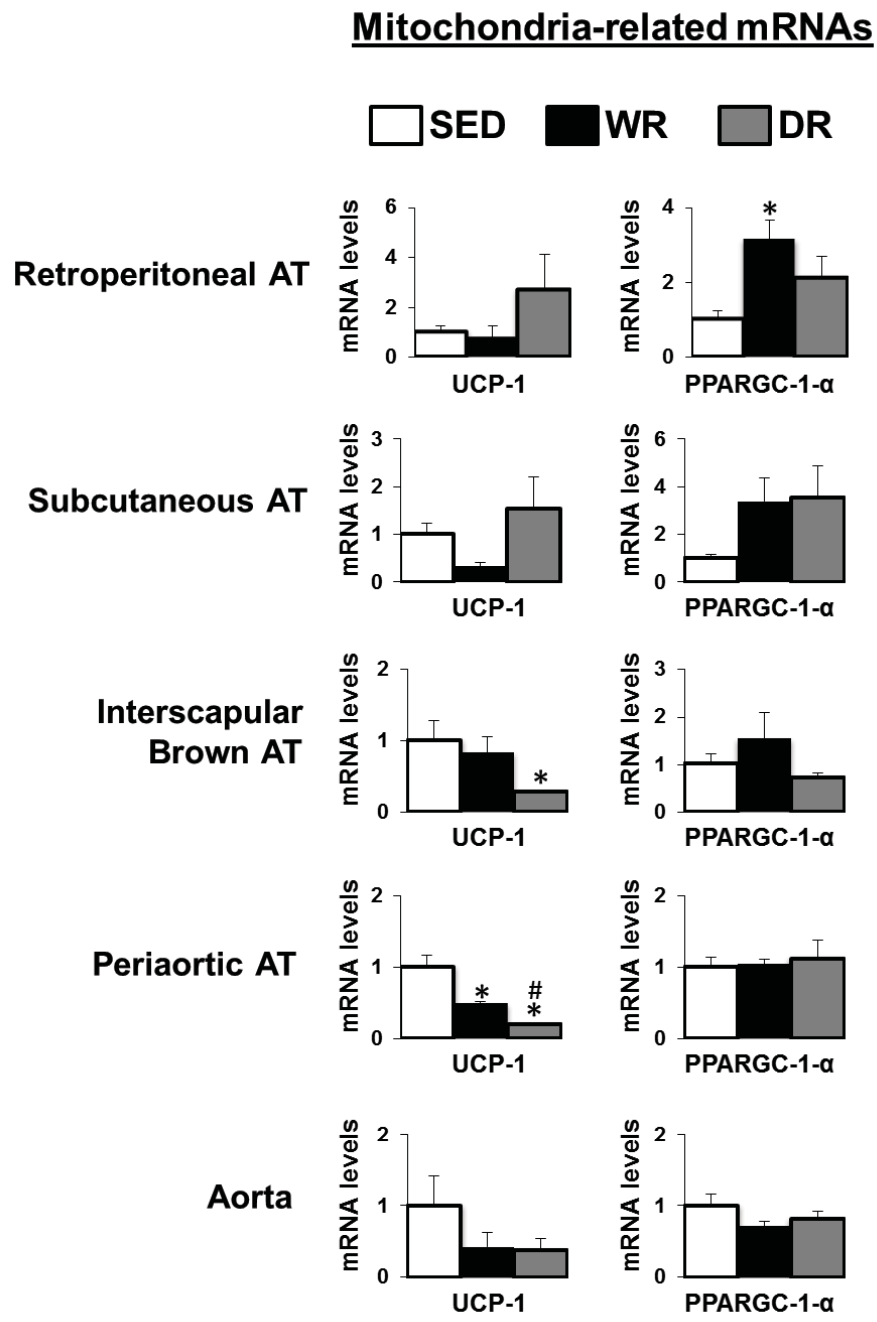

Mitochondria-related mRNAs
SED
WR
DR
Retroperitoneal AT
Subcutaneous AT
Interscapular Brown AT
Periaortic AT
Aorta
mRNA levels
UCP-1
PPARGC-1-α
*
#

Fig 8

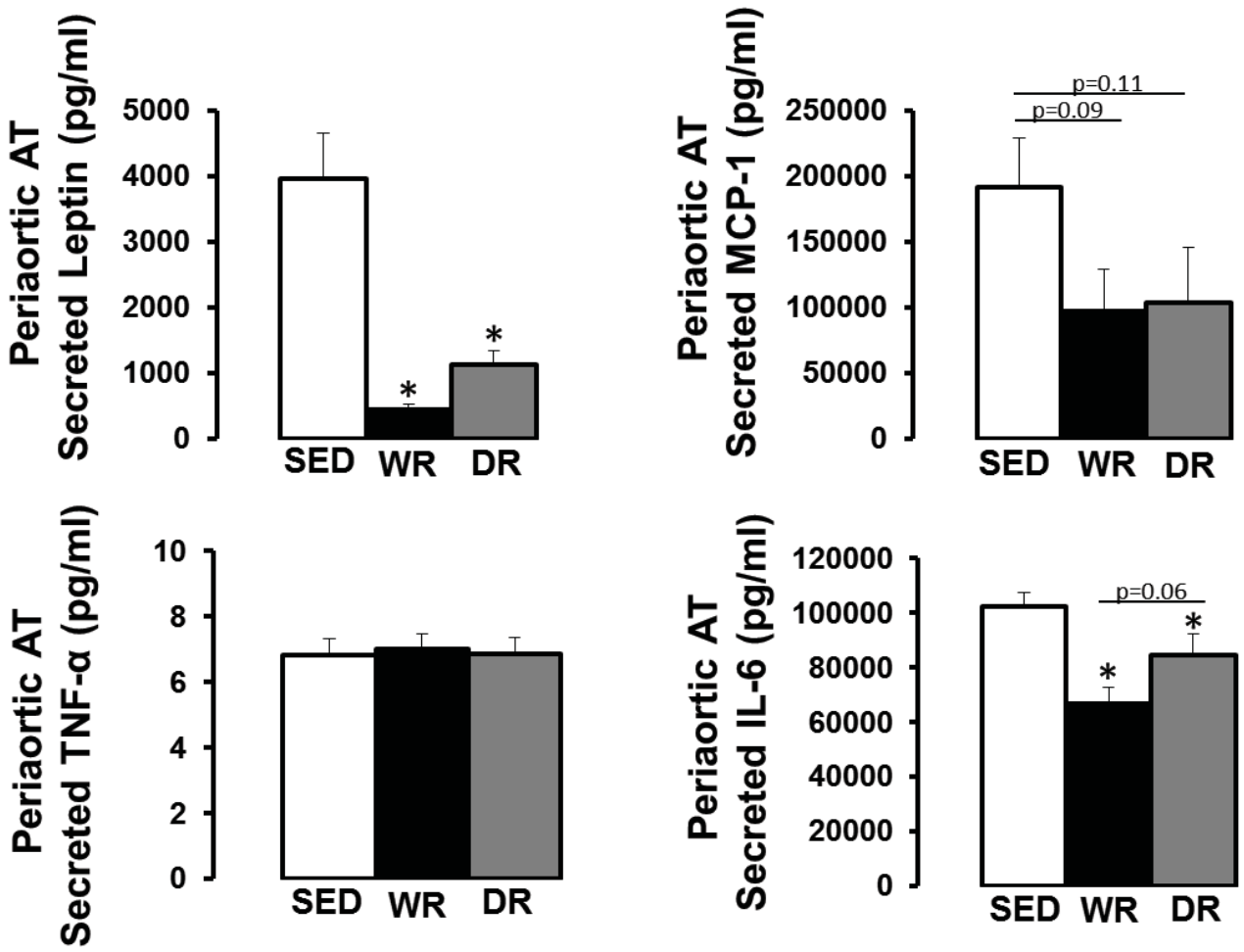
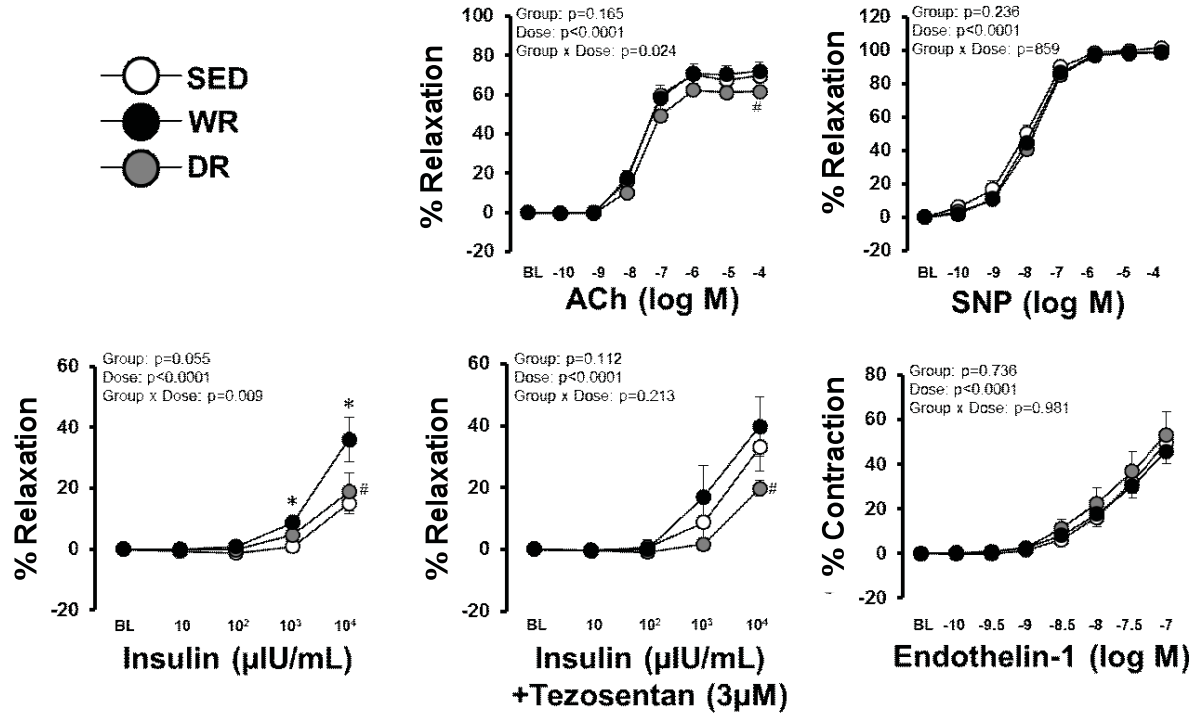

Periaortic AT Secreted Leptin (pg/ml)
5000
4000
3000
2000
1000
0
SED
WR
DR
Periaortic AT Secreted MCP-1 (pg/ml)
250000
200000
150000
100000
50000
0
p=0.11
p=0.09
Periaortic AT Secreted TNF-α (pg/ml)
10
8
6
4
2
0
Periaortic AT Secreted IL-6 (pg/ml)
120000
100000
80000
60000
40000
20000
0
p=0.06

Fig 9


SED
WR
DR
% Relaxation
Group: p=0.165
Dose: p<0.0001
Group x Dose: p=0.024
ACh (log M)
Group: p=0.236
Dose: p<0.0001
Group x Dose: p=859
SNP (log M)
Group: p=0.055
Dose: p<0.0001
Group x Dose: p=0.009
Insulin (µIU/mL)
Group: p=0.112
Dose: p<0.0001
Group x Dose: p=0.213
Insulin (µIU/mL)
+Tezosentan (3µM)
% Contraction
Group: p=0.736
Dose: p<0.0001
Group x Dose: p=0.981
Endothelin-1 (log M)

VITA

Jacqueline Marie Crissey was born November 12th, 1981 in Albuquerque, New Mexico. Jacqui's mother, Mary Crissey, was an active duty Air Force Officer, which necessitated several relocations during her childhood. After graduating from Antonian College Preparatory High School in 2000, she attended Trinity University in San Antonio, Texas. In 2004 Jacqui graduated with a Bachelor of Science degree in Biology, with minors in Chemistry and Studio Art. Jacqui conducted research in the Biology and Chemistry Departments throughout her undergraduate career. It was here that she developed a passion for scientific research, which led her to accept a position as a Research Associate (2004-2006) in the combat casualty care research unit at the US Army Institute of Surgical Research in Fort Sam Houston, Texas. Jacqui began her graduate research career at the University of Texas at Austin, where she earned her Masters degree in Exercise Physiology in 2008. That summer Jacqui married her husband, Dylan, and together they moved to Columbia Missouri, where Jacqui began her doctoral training in the Biomedical Sciences Program at the University of Missouri. She began her research investigating the detrimental effects of physical inactivity and sitting on human metabolism with Dr. Hamilton and Dr. Zderic. In 2009, Jacqui and Dylan welcomed their eldest son, Eoin Alexander, earthside. This momentous experience marked the beginning of Jacqui's most rewarding and challenging occupation ever, motherhood! This year she also transitioned her research to work with Dr. Brown on the role of estrogen in skeletal muscle, as Dr. Hamilton and Dr. Zderic moved their laboratory to the Pennington Research facility in Baton Rouge, Louisiana. Also, due to Dr. Brown approaching her retirement in 2011, Jacqui shifted her mentorship to Dr. Laughlin and Dr. Thyfault. The last several years of her doctoral training she focused on the vascular and metabolic actions of insulin in cardiometabolic disease. In 2012, Jacqui and Dylan welcomed their second son, Jack Elliott, with open arms. Jacqui earned her Ph.D. in Biomedical Sciences in Fall 2013. Jacqui has accepted a post-doctoral fellow position with Dr. Molly Bray in the Nutrition Sciences Department at the University of Texas at Austin.