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Reduction in Systolic Blood Pressure is Associated with Improvements in Metabolic Health in a Model of Diet Induced Obesity

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Reduction in Systolic Blood Pressure is Associated with Improvements in Metabolic Health in a Model of Diet Induced Obesity

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science in Quantitative and Systems Biology

by

Andrew Lee

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Professor Rudy M. Ortiz, Chair
Professor Nestor Oviedo
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2016
The thesis of Andrew Ying-Ping Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, Merced
2016
To my family and friends
for their love and support
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List of Abbreviations

4HNE, 4-hydroxynonenal
Acox1, Peroxisomal acyl-coenzyme A oxidase 1
AT1, Angiotensin Type 1 receptor
AT2, Angiotensin Type 2 receptor
Ang II, Angiotensin II
ARB, Angiotensin Type 1 receptor blocker
AUC, Area under the curve
BM, Body mass
CCB, Calcium channel blocker
CKD, Chronic kidney disease
CMC, Carboxymethyl cellulose
ETAN, Etanercept
GPx, Glutathione peroxidase
HDL, High-density lipoprotein
IRI, Insulin resistance index
LETO, Long Evans Tokushima Otsuka
MetS, Metabolic syndrome
NAFLD, Nonalcoholic fatty liver disease
NEFA, Non-esterified fatty acid
oGTT, Oral glucose tolerance test
OLETF, Otsuka Long Evans Tokushima Fatty
PPar-α, Peroxisome proliferator-activated receptor alpha
RAS, Renin angiotensin system
SBP, Systolic blood pressure
SE, Standard error
SOD, Superoxide dismutase
T2DM, Type 2 diabetes mellitus
TG, Triglycerides
TNF, Tumor necrosis factor
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Angiotensin Receptor and TNF-α Activation Contribute to Glucose Intolerance and Increased Insulin Resistance Independent of Systolic Blood Pressure in OLETF Rats

Published Abstracts

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Field of Study

Major Field: Quantitative and Systems Biology
Studies in Physiology
Professor Rudy M. Ortiz
Abstract

Reduction in Systolic Blood Pressure is Associated with Improvements in Metabolic Health in a Model of Diet Induced Obesity

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2016
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Pathological activation of the renin-angiotensin system (RAS) and inflammation are associated with hypertension and the development of metabolic syndrome (MetS). The progression of MetS is also associated with the development of chronic kidney disease (CKD) and nonalcoholic fatty liver disease (NAFLD). However, the contributions of the angiotensin type 1 receptor (AT1) receptor activation and tumor necrosis factor (TNF)-α-mediated inflammation have on the development of insulin resistance and how they impact hepatorenal health is not well defined. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a well-characterized model of diet-induced obesity that progresses similarly to humans. Using this model and its lean strain counterpart, the Long Evans Tokushima Otsuka (LETO) the progression of the disease was observed in six groups of 16 week old rats with 6 weeks of treatment: 1) lean strain-control LETO; n=5, 2) obese OLET; n=7, 3) OLET + angiotensin receptor blocker (ARB; 10 mg olmesartan/kg; n=8), 4) OLET + TNF-α inhibitor (ETAN; 1.25mg etanercept/kgd; n=6), 5) OLET + angiotensin receptor blocker + TNF-α inhibitor (ARB + ETAN; 10 mg olmesartan/kg + 1.25mg etanercept/kgd; n=6), and 6) OLET + calcium channel blocker (CCB; 5 mg amlodipine/kg; n=7).

All treatments resulted in similar reductions in blood pressure; however, the acute removal of etanercept resulted in an increase in blood pressure that was immediately reduced again once reintroduced. CCB treatment was not able to improve glucose tolerance or insulin resistance index, while ARB, ETAN, and ARB + ETAN were. Similarly, improvements in adiposity were observed with all treatment groups. Renal albumin excretion and glutathione peroxidase was recovered only with ETAN treatment. Renal 4-hydroxynonenal was reduced with ARB treatment. Hepatic non-esterified fatty acids were reduced with all treatments, however there was a greater reduction found with ETAN and ARB + ETAN treatments. Despite relatively similar reductions in systolic blood pressure and adiposity, the calcium channel blocker did not improve glucose tolerance or calculated insulin resistance index, while the other treatments did, demonstrating that improper RAS activation and inflammation are larger factors contributing to the development of impaired glucose and lipid metabolism and regulation during metabolic syndrome, than hypertension. These results suggest that AT1 activation and TNF-α mediated inflammation are mechanistically important in the development of metabolic syndrome and that the associated increase in systolic blood pressure (SBP) may be a consequence of the condition. They also suggest that there are other factors contributing to impaired glucose tolerance and adiposity since none of the treatments completely ameliorated the derangement.
Introduction

Metabolic Syndrome

Metabolic syndrome (MetS) is a relatively new topic of discussion. Abdominal obesity was associated with the development of cardiovascular disease and diabetes early on, but there were no clear metabolic characteristics identified to be associated with the development.\textsuperscript{1, 2} In the 1988 Banting Lecture Dr. Reaven started to associate individuals displaying a cluster of insulin resistance and compensatory hyperinsulinemia, high plasma triglycerides and low high-density lipoprotein (HDL) cholesterol levels, and hypertension proposing this as syndrome X.\textsuperscript{3} MetS was first introduced by the World Health Organization in 1999 where they defined the criteria based on insulin resistance.\textsuperscript{4} The current definition of MetS is commonly used today was defined in 2005 with the addition of abdominal obesity.\textsuperscript{5} To be diagnosed with MetS an individual would display three of the five following conditions including elevated arterial pressure, high fasting blood glucose, increased waist circumference, and dyslipidemia.\textsuperscript{6, 7} According to the National Health and Nutrition Examination Survey data from 2011-2012, the age-adjusted prevalence of MetS in adults is at 34.7\% in the United States.\textsuperscript{8} This is a major concern because those diagnosed with MetS increase the risk of developing cardiovascular diseases by two-fold and type 2 diabetes mellitus (T2DM) by five-fold, which are leading causes of premature deaths in the US.\textsuperscript{9, 10} Currently, it is not clear how metabolic syndrome develops. MetS is a collection of risk factors, not a single disease, probably due to underlying causes. Although these causes are not yet defined, some risk factors found to be associated with the development of MetS include an unhealthy lifestyle, insulin resistance, obesity, and hormonal imbalances.\textsuperscript{11}

The first line of treatment for patients with MetS involve lifestyle changes, including diet and exercise.\textsuperscript{11} Physical activity is a recognized lifestyle association with both the prevention and management of metabolic syndrome.\textsuperscript{12} However, there are cases in which individuals diagnosed with MetS are unable to change their lifestyles and are instead prescribed medicine to mitigate their symptoms. Currently there is no single treatment capable of completely alleviating all of the symptoms associated with MetS, but blood pressure control is an important clinical goal since it is a major risk factor for cardiovascular morbidity and mortality.\textsuperscript{13, 14} Multiple studies have linked insulin resistance and hyperinsulinemia to hypertension.\textsuperscript{15, 16} However, the relationship between insulin resistance and hypertension is still controversial.\textsuperscript{17, 18} Two organs that are associated with the development of MetS are the kidney and liver, due to their importance in regulating blood pressure and lipid metabolism.\textsuperscript{19} The progression of MetS is found to be strongly associated with chronic kidney disease (CKD) and nonalcoholic fatty liver disease (NAFLD).\textsuperscript{20, 21} Both of these derangements are influenced by the onset of the symptoms of MetS but are also associated to lead the final development of MetS itself by influencing systolic blood pressure (SBP) and proper glucose and lipid regulation.\textsuperscript{22, 23} Current studies have shown that the impact of kidney damage is directly related to the presence and severity of NAFLD.\textsuperscript{24} Similarly, inflammation is a major factor associated to the development of NAFLD.\textsuperscript{25} Two pathways commonly implicated in the pathogenesis of MetS are the renin-angiotensin system (RAS) and inflammation mediated by tumor necrosis factor (TNF)-\textalpha.\textsuperscript{26}
**Renin-Angiotensin System**

The RAS is a major regulator system for blood pressure and fluid balance.\(^27\) This pathway starts with angiotensinogen produced in liver which is cleaved into angiotensin I by renin, produced in the kidneys, which is then converted into the main biologically active hormone generated by this system, angiotensin II (Ang II) by angiotensin converting enzyme produced from the lungs.\(^28,29\) Ang II binds to Ang II type 1 receptor (AT1) and Ang II type 2 receptor (AT2), found in the brain, heart, kidney, vasculature, and even the immune system to maintain circulatory homeostasis.\(^28,30\) Improper activation of the RAS has been shown contribute to the development of hypertension, cardiac hypertrophy, and ultimately heart failure.\(^31-33\) AT1 receptors in the kidney are also primarily responsible for the actions of Ang II to cause hypertension.\(^34\) Because of this pharmacological inhibitors of AT1, angiotensin receptor blockers (ARB), are effective and widely used for the treatment of hypertension, congestive heart failure, and kidney diseases.\(^35,36\) Currently ARBs are one of the most frequently used antihypertensive drugs.\(^37\) ARBs are also being used to treat patients diagnosed with MetS to control SBP.\(^38\) Similarly, studies have shown that ARBs can improve glucose intolerance, insulin resistance, and even prevent the onset of type 2 diabetes.\(^39\) However, it is not known if the improvements in SBP are the result of the improvements found in glucose tolerance or if it is the reason for them.

**TNF-α Pathway**

TNF-α is a major signaling protein in the immune system that coordinates communication between immune cells and controls many of their functions.\(^40\) TNF-α is primarily involved in controlling whether a target cells lives or dies.\(^41\) Improper regulation of TNF-α have been shown to lead to the development of autoimmune diseases including rheumatoid arthritis, Crohn's disease, multiple sclerosis, lupus, and Type 1 diabetes.\(^41\) At this time systemic and local inflammation is thought to be a consequence of obesity, and is associated with MetS by impacting insulin resistance and increasing circulating inflammatory cytokines.\(^42,43\) Inflammatory cytokines like TNF-α have been found to be associated with obesity and the accompanying insulin resistance.\(^42,44\) Recent studies demonstrate that TNF-α is directly correlated with SBP\(^45,46\) and that immunosuppressive therapies can reduce SBP in hypertensive patients.\(^47\) However, the impact TNF-α has on the development of the other conditions of MetS are still unknown.

**Models of Metabolic Syndrome**

To study MetS many animal models have been used to simulate the manifestation of the symptoms. However, each model can be categorized as the diet-induced obesity, hypercholesterolemic, diabetic, or hypertensive type.\(^48\) Animal models with a mutation in the leptin-signaling pathway develop into a morbidly obese phenotype.\(^49\) Some animal models include the ob/ob and the db/db mouse, which lack leptin production or have leptin receptor mutations respectively, that allows the obese phenotype to manifest.\(^50,51\) These models are unable to develop the full phenotype of MetS and type II diabetes and when supplemented with leptin are able to normalize the phenotype.\(^49,50\) Other models, such as Dahl-S, rat a hypertensive MetS model, develop hypertension and insulin resistance when fed a high salt diet.\(^52,53\) Still
these models are unable to completely parallel the characteristics of human obesity and development of MetS.

One animal model that is used for studying MetS is the Otsuka Long-Evans Tokushima Fatty (OLETF) rat. The OLETF rat is a well-characterized model of diet-induced obesity that develops dyslipidemia, hypertension, late-onset hyperglycemia, increased RAS, and insulin resistance. The OLETF rat is the selectively bred counterpart of the Long-Evans Tokushima Otsuka (LETO) rat for the null expression of the cholecystokinin-1 receptor, allowing OLETF rats to exhibit a defect for satiety, resulting in hyperphagia and obesity, and the spontaneous development of insulin resistance and T2DM. The pathological conditions of the OLETF rat also resemble human metabolic syndrome and T2DM. Similarly, when OLETF rats are exposed to exercise the derangements of MetS will be recovered. Due to these reasons the OLETF rat an excellent animal model choice for studying the pathogenesis of MetS.

**Research Aims**

Metabolic syndrome is a suite of complications that makes it challenging to target the specific contributions to its progression. Currently blood pressure is a primary treatment target due to its relationship with cardiovasculature health. Inappropriate activation of AT1 and TNF-α are associated with developing hypertension and metabolic syndrome. While the blockade of the RAS or TNF-α separately, can improve the derangements of MetS, the interaction of these factors and whether combined blockade can improve MetS independently of blood pressure remains unclear. However, whether improving blood pressure improves the impaired glucose and lipid metabolism associated with metabolic syndrome or if blood pressure is the result of the metabolic improvements is not known. Similarly, it is unclear how TNF-α impacts the manifestation of metabolic complications. The purpose of this study was to delineate the contributions of AT1 and TNF-α activation on blood pressure regulation and on hepatorenal health. Using the OLETF rat we tested the hypothesis that decreasing systolic blood pressure will improve metabolic outcomes associated with metabolic syndrome as well as improve kidney and liver health by reducing damage in a model of diet induced obesity.
Methodology

Animals
All experimental procedures were reviewed and approved by the institutional animal care and use committees of Kagawa Medical University (Kagawa, Japan), and the University of California, Merced (Merced, CA).

Male, lean strain-control LETO (279 ± 7 g) and obese OLETF (359 ± 3 g) rats were studied (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan). At 10 weeks of age rats were assigned to one of the following groups: 1) untreated LETO (n=5), 2) untreated OLETF (n=7), 3) OLETF + angiotensin receptor blocker (ARB; 10 mg olmesartan/kg/d; n=8), 4) OLETF + TNF-α inhibitor (ETAN; 1.25 mg etanercept/kg/d; n=6), 5) OLETF + ARB + ETAN (n=6), and 6) OLETF + calcium channel blocker (CCB; 3-5 mg amlodipine/kg/d; n=7). At the onset of the study mean body mass of the OLETF groups were comparable. Carboxymethyl cellulose (CMC; 5%) solution was administered by gavage to the untreated LETO and OLETF groups daily as a vehicle control. ARB (olmesartan; Daiichi-Sankyo, Tokyo, Japan) was administered daily by gavage and prepared with equal amounts of CMC to achieve the final dosage.

Etanercept (Enbrel, Wyeth) was initially administered subcutaneously by osmotic minipump (2006; 0.15 μl/hr x 42 days; DURECT Corporation, USA) until the third week of the study; at which time the etanercept lost its potency due to the drug’s lifespan (denoted as ETAN removed on Figure 1B). Subsequently, the drug was administered by daily intraperitoneal injection to complete the treatment of the last 2 weeks of the study. CCB (Amlodipine; LKT Laboratories, St Paul, MN) was initially administered daily by gavage and prepared with equal amounts of CMC so that the final dosage was approximately 3 mg/kg; however, after the first week of treatment the dose was observed to be not sufficient enough to lower the SBP so the dose was increased to 5 mg/kg/d for the remainder of the study. The doses of ARB, ETAN, and CCB were determined on the basis of previous studies on rats. Animals were maintained in groups of three or four per cage in a specific pathogen-free facility under controlled temperature (23°C) and humidity (55%) with a 12-h light, 12-h dark cycle. All animals were given free access to water and standard laboratory rat chow (MF; Oriental Yeast Corp., Tokyo, Japan). Rats were placed in metabolic cages overnight in the initial week and in the final week of the study to obtain individual urine samples and measure water consumption.

Body Mass and Food Intake
Body mass was measured daily to calculate the appropriate amount of ARB, ETAN, and CCB to administer, and mean food intake was measured daily at the onset of the treatments to help confirm consistent dosing.

Blood pressure
SBP was measured weekly starting one week before the first administration of treatment till the end of the 6 weeks of treatment in conscious rats by tail cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan). Animals were allowed to acclimate to the tail-cuff apparatus before measuring the blood pressure.
**Oral Glucose Tolerance Test (oGTT)**

One week before the end of the experimental period, oGTT were performed. Rats were fasted overnight and glucose was administered by gavage (2 g/kg). Fifteen minutes before the collection of samples from the tail vein, a small region in the side of the tail was cleaned with alternating wipes of isopropyl alcohol and betadine, followed by a subcutaneous injection of 200–300 ± 1 of lidocaine (Henry Schein, Melville, NY). Blood glucose was measured before gavage, time 0, and at 15, 30, 60, and 120 min after the glucose infusion. In addition, the 0, 15, 30, and 120 minute samples were collected to measure plasma insulin concentration. The glucose area under the curve ($AUC_{glucose}$) and the insulin area under the curve ($AUC_{insulin}$) were calculated by the trapezoidal method. The insulin resistance index (IRI) was calculated as the product of area under the glucose and insulin curves ($AUC_{glucose} \times AUC_{insulin}$) as previously described.

**Sample Collection**

After 6 weeks of treatment, all animals were fasted overnight prior to dissection. Following body mass measurements, animals were decapitated and trunk blood was collected into chilled vials containing 50 mM EDTA and protease inhibitor cocktail and processed as previously described. Kidneys and livers were rapidly removed, weighed, and snap-frozen in liquid nitrogen. Frozen samples were kept at −80°C until analyzed. Retroperitoneal and epididymal fat masses were removed and weighed.

**Western Blot**

Renal cortical protein expression of TNF-α, hepatic peroxisome proliferator-activated receptor alpha (PPar-α), and peroxisomal acyl-coenzyme A oxidase 1 (Acox1) were determined using standard Western blot methods, as described previously. Blots were incubated with an anti-TNF-α antibody (1:250 Santa Cruz Biotechnology), anti–PPar-α antibody (1:1,000 Santa Cruz Biotechnology), anti–Acox1 antibody (1:1,000 Santa Cruz Biotechnology), and/or an anti–β-actin (1:5,000; Abcam) as a loading control. Secondary antibodies used were IR700-conjugated donkey anti-rabbit IgG, IR700-conjugated donkey anti-goat IgG, and IR800-conjugated donkey anti-mouse IgG (1:20,000; Rockland Immunologicals). Antibody labeling was visualized using the Odyssey Infrared Scanner (LI-COR). Data are presented in arbitrary units of protein optical density band normalized to β-actin or with Ponceau S staining if proteins used for normalization changed with the treatments. Percent change from LETO was calculated by normalizing all the normalized densitometry data with the average LETO value and multiplying by 100%.

**Enzyme Activities, and Antioxidant Measurements**

Renal and hepatic glutathione peroxidase (GPx), catalase, and/or superoxide dismutase (SOD) activities were measured using commercial kits (Cayman Chemical). Frozen tissue samples were homogenized in 50 mM potassium phosphate buffer containing 1 mM EDTA, 1% Triton X-100, 1% phenylmethylsulfonylfluoride, and 1% PIC. Supernatants were immediately used to measure catalase, GPx, and SOD activities. 4-hydroxynonenal (4HNE) and nitrotyrosine content was measured using dot blots. Ten micrograms of total protein were loaded onto 0.45 μm PVDF membranes using a Bio-Dot SF microfiltration apparatus (Bio-Rad Laboratories). Membranes were blocked, probed with a 4HNE antibody (1:2000; Millipore, Billerica, MA) or a
nitrotyrosine antibody (diluted 1:1000; Cell Signaling, Danvers, MA) and developed similar to Western blot (described above). Uniform protein loading was confirmed with Ponceau S staining.

**Plasma and Urine Analyses**
Plasma triglycerides (TG) and glucose concentrations were measured using an auto analyzer (Hitachi-Roche 912; Roche, Indianapolis, IN). Plasma non-esterified fatty acids (NEFA; Wako Chemicals; Richmond, VA), TNF-α (Phoenix Pharmaceuticals, Burlingame, CA), and urine albumin excretion (Bethyl, Montgomery, TX) were measured using commercial ELISA kits.

**Statistics**
Means (± standard error (SE)) were compared by one way ANOVA followed by a Fisher LSD post hoc test. For the oGTT studies, areas under the curve (AUC) were calculated to assess the integrated response to glucose and calculate insulin resistance index (IRI). IRI was calculated for each animal as the product of AUC_glucose and AUC_insulin and is considered to represent an indirect marker of whole-body insulin action. Data were considered significantly different at p ≤ 0.05. Statistical analyses were performed with the SYSTAT 13.0 software (SPSS, Richmond, CA).
Results

**Blood Pressure**
SBP was measured in order to confirm the development of hypertension and the effectiveness of each treatment. SBP was increased in OLETF rats compared to LETO (142 ± 2 vs. 114 ± 3 mmHg). Mean SBP in OLETF rats treated with ARB was similar to strain control LETO rats. In CCB treated rats, SBP was similar to LETO post-11 weeks. ETAN lowered blood pressure in OLETF to levels similar to LETO; however, the addition of ARB to ETAN did not reduce SBP further than ARB alone (Figure 1). ARB, ETAN, ETAN+ARB, and CCB treatments decreased mean SBP by 20%, 11%, 23%, and 16%, respectively, compared with OLETF by the end of the study (Figure 1).

**Ending Body and Tissue Masses and Food Consumption**
Mean body mass, relative retroperitoneal mass, relative epididymal mass, and mean food intake was measured in order to assess the impact of each treatment on body composition and food consumption. Body mass was 37% higher in OLETF compared with LETO, and ARB, ETAN, and ETAN+ARB treatments decreased body mass 4%, 9%, and 8%, respectively, while CCB treatment did not result in a significant change (Table 2). Mean relative epididymal and retroperitoneal fat masses were 81% and 169% greater, respectively, in OLETF compared with LETO. ARB, ETAN, ETAN+ARB, and CCB treatments reduced mean relative retroperitoneal fat mass 13%, 32%, 31%, and 24%, respectively, and relative epididymal fat mass by 12%, 20%, 25%, and 19%, respectively (Table 2). Mean kidney mass was 36% greater in OLETF compared to LETO but was unaffected by any treatment (Table 2). Mean liver mass was 19% greater in OLETF compared to LETO. ARB, ETAN, ETAN+ARB, and CCB treatments reduced mean liver mass by 6%, 16%, 13%, and 16% respectively (Table 2). Relative mean daily food intake was similar among all groups at cumulative mean 6.2 ± 0.1 (g/100 g body mass) (Table 2). This is expected since the starting weights of the animals were different resulting in a larger amount of food being consumed per day.

**Glucose Tolerance and Insulin Resistance Index (IRI)**
OGTT were performed to assess the impairment of glucose and insulin clearance and to calculate the degree of insulin resistance found with each of the treatments (Figure 2A, 2B). Areas under the curve were calculated for both glucose and insulin (AUC<sub>glucose</sub> and AUC<sub>insulin</sub>) (Figure 2C, 2D) and were observed to increase 33% and 94%, respectively, in OLETF compared with LETO (Figure 2). ARB, ETAN, ETAN+ARB treatment decreased AUC<sub>glucose</sub> by 13%, 28%, and 29%, respectively compared with OLETF while CCB did not reduce AUC<sub>glucose</sub>. ARB treatment decreased AUC<sub>insulin</sub> by 23%, while the other treatments did not significantly reduce AUC<sub>insulin</sub>. These results suggest that ARB improved glucose and insulin responses, ETAN improved only glucose response, and CCB did not affect either. In line with this, IRI was 330% greater in OLETF compared with LETO, and ARB, ETAN, and ETAN+ARB treatment lowered IRI by 29%, 36%, and 35%, respectively, while CCB had no significant effect on IRI (Figure 2E).
**Plasma Glucose, TNF-α, Triglyceride, and Hepatic NEFA**
Fasting plasma glucose, TNF-α, TG, and hepatic NEFA content were measured in order to assess the effects of each treatment on glucose handling, inflammation, and lipid metabolism respectively. Fasting plasma glucose increased 31% in OLETF compared with LETO, and all treatments decreased levels relative to OLETF (Table 3). Mean plasma TNF-α was not different between LETO and OLETF, and ARB and ETAN+ARB treatments did not affect circulating TNF-α levels. However, ETAN increased TNF-α 34% (Table 3) suggesting that the drug dosage effectively blocked the soluble receptors. Fasting plasma TG was 97% greater in OLETF compared with LETO, whereas ARB and CCB treatment lowered levels 24% and 31%, respectively. ETAN and ETAN+ARB did not significantly decrease levels (Table 3). Hepatic NEFA increased 107% in OLETF compared with LETO, and was decreased in ETAN, ETAN+ARB, and CCB by 58%, 58%, 32% respectively, relative to OLETF (Table 3).

**Renal TNF-α Protein Expression**
Renal TNF-α was measured in order to determine the impact of each treatment on the local levels of TNF-α in order to assess if local levels of TNF-α were influencing the progression of the derangements observed. Renal cortical TNF-α protein expression was not different between LETO and OLETF; however, TNF-α was lower in ARB or ETAN+ARB treated animals by 57% and 62%, respectively, compared to OLETF (Figure 3).

**Renal Injury and Antioxidant Enzyme Activities**
Urine albumin excretion was measured as a marker of renal function at the end of the six weeks of treatment. Urine albumin excretion was 52% greater in OLETF compared to LETO, and was reduced 36% in ETAN treated animals compared to OLETF (Figure 4A). Renal GPx, catalase, and SOD activities were measured to determine the levels of antioxidant activities. Renal GPx activity decreased 18% in OLETF compared to LETO, and increased 22% in ETAN compared to OLETF (Figure 4B). Renal catalase activity was increased by 48% in OLETF compared to LETO and was unchanged among all treatment groups (Figure 4C). Renal SOD activity was unchanged among all groups (Figure 4D). Renal 4HNE and nitrotyrosine content were measure to assess the oxidative and nitrosylative damage respectively. Renal 4HNE content increased 21% in OLETF compared to LETO, and decreased 11% and 15% in ETAN and ETAN+ARB, respectively, compared to OLETF (Figure 4E). Renal nitrotyrosine content was unchanged among all groups (Figure 4F).

**Hepatic Antioxidant Enzyme Activities and Damage**
Hepatic GPx and catalase activities were measured to determine the levels of antioxidant activities present in the liver. Both hepatic GPx and catalase activities were found to be unchanged among all groups (Figure 5A, B). Hepatic 4HNE and nitrotyrosine content were measure to assess the oxidative and nitrosylative damage respectively. Both hepatic nitrotyrosine and 4HNE contents were found to be unchanged among all groups (Figure 5C, D).
**Hepatic PPar-α and Acox1 Protein Expression**

Hepatic PPar-α and Acox1 were measured in order to determine the impact of each treatment had on lipid metabolism. Hepatic PPar-α was unchanged OLETF compared to LETO but was reduced in ETAN, ETAN+ARB, and CCB by 66%, 45%, 63% respectively, relative to OLETF. (Figure 6A) There were no changes in Acox1 protein expression among all groups. (Figure 6B)
Discussion

In the present study, we hypothesized that decreasing systolic blood pressure would improve metabolic outcomes associated with metabolic syndrome as well as improve kidney and liver health by reducing damage in a model of diet induced obesity. Using the OLETF rat, a model of diet-induced obesity, we made several new findings. These included: 1) chronic activation of TNF-α receptor is a contributing factor in MetS-associated hypertension, 2) activation of AT1 and TNF-α receptors independent of elevated arterial pressure are significant factors in the manifestation of insulin resistance, 3) activation of renal TNF-α receptors is a critical contributing factor in the development of renal oxidative stress independent of elevated arterial pressure, and 4) adiposity may not be directly related to hepatic health.

Activation of TNF-α Increases Systolic Blood Pressure

Chronic inflammation contributes to the development of hypertension in both humans and animal models. 70-72 Similarly, chronic inhibition of TNF-α receptor decreases SBP in both humans and animal models.57, 73 However, elevated plasma TNF-α levels or the activation of its receptors are not consistent events in the development of insulin resistance and MetS suggesting that TNFα-associated inflammation may not be a principal factor in all patients with metabolic syndrome. Here, we demonstrated that chronic inhibition of TNF-α biological activity with etanercept reduced SBP in insulin resistant rats (Figure 1A), and that the reduction in SBP is responsive to removal of the treatment. Correspondingly, there was no change observed with renal TNF-α in OLETF compared to LETO suggesting no real inflammation present in the kidneys during this age (Figure 3). Furthermore, because these levels were not significantly altered with etanercept treatment, suggesting that the primary mode of action by which inhibition of the TNF-α receptor reduces SBP during insulin resistance may not be renal specific at this stage of the condition. While no strain effect in circulating TNF-α levels was detected, the significant increase in plasma TNF-α in the etanercept-treated animals is similar to that observed in etanercept-treated humans74 and an indication of the drug’s effectiveness (Table 3).

Reduction in Blood Pressure Independent of RAS does not Reverse Insulin Resistance

In animal models of MetS RAS inhibition improves glucose tolerance by enhancing insulin secretion and ameliorating the elevated arterial pressure.33, 75 However, the direct impact of increased SBP on the progression of glucose intolerance independent of activated RAS remains unclear. We demonstrated that reducing SBP, independent of RAS (CCB group), did not improve the insulin resistance index in insulin resistant rats (Figure 1B). The reduction in SBP with CCB treatment was similar in magnitude to that in ETAN group; however, only ETAN was able to recover the IRI (Figure 2E). The amelioration of the insulin resistant status appears to be a result of improved glucose tolerance (response) suggesting that this improvement in tolerance may by impaired by increased inflammation during the manifestation of MetS in OLETF rats (Figure 2A, C). Conversely, improvement in the insulin resistant status due to restored insulin response to the oGTT was only observed in the ARB-treated group suggesting that ARB-mediated improvements targeted peripheral insulin sensitivity (Figure 2B, D). However, neither treatment (CCB nor ETAN) completely ameliorated the SBP (to LETO levels) suggesting that improper activation of RAS may be a more dominant contributing factor to the elevated SBP as
both ARB and ETAN+ARB treatments completely restored SBP. Lastly, the lack of an additive effect of the combination therapy (ETAN+ARB) on both IRI status and SBP suggests that the benefits of RAS inhibition achieved with ARB treatment represents the maximum possible regardless of the inhibition of TNF-α biological activity during the manifestation of insulin resistance.

Renal Oxidative Stress

The kidneys contribute significantly to the maintenance of glucose homeostasis.76, 77 Furthermore, the progression of MetS is strongly associated with CKD, which is linked with the progression of cardiovascular complications.20, 78 The derangements of CKD are also influenced by the onset of the symptoms of MetS, and are also associated with the final development of MetS itself23, 79 Among these symptoms is impaired oxidant-antioxidant balance resulting in increased oxidative stress. Oxidative stress is also associated with the progression of CKD through inappropriate activation of RAS and with the promotion of increased (TNFα-mediated80) inflammation.81, 82 Nitrotyrosine and 4HNE are products of tyrosine nitration and lipid peroxidation, respectively, and are measured to assess the extent of the oxidative injury (stress).83, 84 Antioxidant enzymes and processes (i.e., glutathione) are necessary to maintain oxidant-antioxidant balance because the overproduction of reactive oxygen species damages cellular components leading to impaired physiological function.85 However, the progression of renal impairments relative to the improvements in the conditions of MetS are not well defined. The only improvements in renal antioxidant enzyme activity detected were with chronic TNF-α inhibition on GPx activity suggesting that TNF-α contributes to the regulation of the glutathione cycle in the kidney during insulin resistant conditions (Figure 3B). This improvement in GPx was associated with reductions in albuminuria and renal 4HNE content suggesting that TNFα-mediated mechanisms in the kidney have an impact on renal derangements during the insulin resistant conditions (Figure 3A). Therefore, the renal damage, assessed by albuminuria, was the least in the ETAN treated rats suggesting that TNFα-associated inflammation and/or activation may be the primary component affiliated with increased oxidative stress in the development of CKD associated with insulin resistance and likely diabetic nephropathy.

Progression of Hepatic Damage and Health

NAFLD is being viewed as the hepatic component of the metabolic syndrome.86-88 A feature of the pathogenesis of NAFLD, is the accumulation of triacylglycerol in the liver which is attributed to the plasma NEFAs that flow into the liver.89 Insulin resistance has also been shown to be associated with dysregulation of adipose-derived fatty acids, however, NAFLD patients have shown that insulin does not suppress adipose tissue lipolysis like in healthy individuals.90-92 The origin of the lipids that accumulate in liver remains unknown, and identifying the origin of the accumulated lipids in the livers of patients with NAFLD may be important in the amelioration of this condition. Acox1 is the first and rate-limiting enzyme of the peroxisomal β-oxidation pathway involved with lipid metabolism.93 PPar-α is a nuclear receptor activated by fatty acids and promotes fatty acid oxidation in the liver.94 Due to the progression of adiposity in the O LETF model and the improvements observed with each treatment it could be implied that this was due to a reduction in the rat’s ability to metabolize lipids which was improved with each treatment. Our experiments show no changes in Acox1 protein expression.
from any of the groups, indication of no real changes in the promotion of fatty acid oxidation in the liver. However, decreases in expression of PPar-α with ETAN, ETAN + ARB, and CCB treatments was observed (Figure 6A). This same trend in reduction can be observed with the hepatic NEFA content as well (Table 3). This may indicate that the free fatty acids are no longer being oxidized in the liver and may be instead shuttled out of the liver. A decrease in retroperitoneal accumulation and absolute liver mass was observed in all of the treatment groups (Table 2). Hepatic antioxidant activity of GPx and catalase was unchanged among all groups as well as the 4HNE and nitrotyrosine content (Figure 5). Implying that the reduction in adiposity may not be due to improvement of liver health since no significant changes were observed in the antioxidant/oxidant balance. This may be due to the animals still being in the early stages of the development of MetS, but there is clearly another pathway which is influencing the progression of the adiposity found in the OLETF model.

Summary

A common practice in treating patients with MetS is primarily managing the increase in SBP; however this method is only addressing one of the five diagnosing symptoms of MetS. This also does not address the other derangements derived from the manifestation of MetS itself. In the present study, using a rat model of diet-induced obesity, we assessed the effects of ARB, ETAN, ARB + ETAN and CCB treatments on the development of the derangements of MetS (Table 4) and the impact they play on hepatic and renal impairment. Our data suggest that reducing the elevated arterial pressure associated with MetS does not directly lead to improvements in glucose tolerance and/or insulin resistance. The chronic inhibition of the TNF-α receptor had the greatest decrease in adiposity suggesting that inflammation may contribute a greater extent in the development of obesity than over-activation of RAS. The data presented also provide evidence of additional antihypertensive actions and improvements in renal oxidative stress exerted with chronic inhibition of TNF-α. Hepatic function was also observed to not have any significant improvements or damage but adiposity was found to be reduced in all treatment. Implying that other pathways may need to be investigated in order to determine the mechanism behind improper lipid regulation in the early development of MetS. Thus, the study highlights the complexity of the factors that contribute to the development of insulin resistance and the associated pathologies and suggests that elevated arterial pressure is a consequence of the initial events; while other metabolic complications, especially those associated with the activation of RAS and TNF-α, appear to be significant contributing factors. Suggesting that elevated arterial pressure should not be a primary marker for detecting early metabolic derangements and that more robust measurements should be used in future diagnoses and that other obesity-associated factors contribute to impaired glucose tolerance since none of the treatments completely ameliorated all the symptoms of MetS.
Future Directions

In the present study we observed changes in adiposity but no changes were observed in hepatic lipid metabolism. This leads us to hypothesize that the changes in adiposity may be due to improvements in the lipid mobilization. The liver secretes fatty acids in the form of very-low-density lipoprotein, from which fatty acids may be taken up for esterification in adipose tissue and for oxidation or esterification in muscle via the lipoprotein lipase (LPL) pathway.\textsuperscript{95} Previous studies have shown that LPL is associated in the prevention of diet-induced obesity and insulin resistance.\textsuperscript{96, 97} Therefore, measuring the lipase activity and protein expression of the components of this pathway, such as CD36 or LPL, may elucidate the mechanisms underlying the changed we observed occurring during early onset of MetS in the OLETF rats. Similarly, there were no significant changes observed in renal or hepatic damage. This is most likely due to the young 16 week age of the animals. As seen with humans there is a higher prevalence of MetS in older individuals which implies that age influences the body’s ability to maintain metabolic homeostasis.\textsuperscript{8} To better understand how AT1 and TNF-α impact the progression of MetS older animals or extended treatment duration should be used in a future study. Previous studies have shown that elevated levels of angiotensinogen and inflammatory cytokines do eventually develop in this model from 20 to 30 weeks of age.\textsuperscript{60, 98} Therefore, the use of older animals might be able to elucidate the impact each treatment has on the further manifestation of MetS.
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### Tables

**Table 1.** Short description of inhibitors used in the study

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<thead>
<tr>
<th>Inhibitor</th>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>Amlodipine</td>
<td>CCB</td>
<td>Calcium channel blocking agent used for treatment of hypertension</td>
</tr>
<tr>
<td>Etanercept</td>
<td>ETAN</td>
<td>Soluble tumor necrosis factor (TNF)-α blocker used to neutralize TNF bioactivity</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>ARB</td>
<td>AT1 receptor blocker used for treatment of hypertension</td>
</tr>
</tbody>
</table>
Table 2. Mean (±SE) values of body mass, relative fat masses, absolute kidney mass, absolute liver mass, and food consumption after 6 weeks of treatment in LETO, OLETF, OLETF ARB, OLETF ETAN, OLETF ETAN+ARB, and OLETF CCB.

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF</th>
<th>OLETF ARB</th>
<th>OLETF ETAN</th>
<th>OLETF ETAN+ARB</th>
<th>OLETF CCB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Mass (g)</td>
<td>366 ± 32</td>
<td>503 ± 24#</td>
<td>481 ± 11*</td>
<td>456 ± 24*</td>
<td>465 ± 8*</td>
<td>484 ± 20</td>
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<tr>
<td>Relative Retroperitoneal Fat (g/100 g BM)</td>
<td>1.5 ± 0.15</td>
<td>3.7 ± 0.41#</td>
<td>3.2 ± 0.45*</td>
<td>2.7 ± 0.28*</td>
<td>2.8 ± 0.28*</td>
<td>3.1 ± 0.30*</td>
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<tr>
<td>Relative Epididymal Fat (g/100 g BM)</td>
<td>1.2 ± 0.09</td>
<td>2.1 ± 0.30#</td>
<td>1.9 ± 0.17*</td>
<td>1.8 ± 0.21*</td>
<td>1.6 ± 0.04*</td>
<td>1.8 ± 0.16*</td>
</tr>
<tr>
<td>Kidney mass (g)</td>
<td>1.1 ± 0.02</td>
<td>1.5 ± 0.03#</td>
<td>1.5 ± 0.03</td>
<td>1.5 ± 0.06</td>
<td>1.5 ± 0.01</td>
<td>1.5 ± 0.05</td>
</tr>
<tr>
<td>Liver mass (g)</td>
<td>2.7 ± 0.1</td>
<td>3.2 ± 0.1#</td>
<td>3.0 ± 0.1*</td>
<td>2.7 ± 0.1*</td>
<td>2.8 ± 0.1*</td>
<td>2.7 ± 0.1*</td>
</tr>
<tr>
<td>Mean Daily Food Consumption (g/100 g BM)</td>
<td>6.1 ± 0.01</td>
<td>6.3 ± 0.8</td>
<td>6.2 ± 0.04</td>
<td>6.3 ± 0.03</td>
<td>6.2 ± 0.05</td>
<td>6.3 ± 0.13</td>
</tr>
</tbody>
</table>

# p<0.05 vs. LETO, * p<0.05 vs. OLETF.
Table 3. Mean (±SE) plasma biochemical measurements and hepatic NEFA and triglyceride concentration after 6 weeks of treatment in LETO, OLETF, OLETF ARB, OLETF ETAN, OLETF ETAN+ARB, and OLETF CCB.

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF</th>
<th>OLETF ARB</th>
<th>OLETF ETAN</th>
<th>OLETF ETAN+ARB</th>
<th>OLETF CCB</th>
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<tr>
<td>Plasma Triglyceride (mg/dL)</td>
<td>59 ± 22</td>
<td>117 ± 16 #</td>
<td>88 ± 31 *</td>
<td>101 ± 32</td>
<td>117 ± 23</td>
<td>89 ± 14 *</td>
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<tr>
<td>Plasma Glucose (mg/dL)</td>
<td>106 ± 7</td>
<td>139 ± 14 #</td>
<td>120 ± 5 *</td>
<td>123 ± 6 *</td>
<td>121 ± 17 *</td>
<td>125 ± 7 *</td>
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<tr>
<td>Plasma Tnf-α (ng/dl)</td>
<td>184 ± 34</td>
<td>200 ± 9</td>
<td>205 ± 30</td>
<td>269 ± 10 *</td>
<td>220 ± 52</td>
<td>ND</td>
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<tr>
<td>Hepatic NEFA (mM)</td>
<td>7.6 ± 1.8</td>
<td>15.8 ± 2.2 #</td>
<td>13.0 ± 1.7</td>
<td>6.7 ± 0.3 *</td>
<td>6.7 ± 0.3 *</td>
<td>10.8 ± 1.8 *</td>
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</table>

# p<0.05 vs. LETO, * p<0.05 vs. OLETF.
Table 4. Summary of changes in components of metabolic syndrome with respect to each treatment

<table>
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<tr>
<th>Components of Metabolic Syndrome</th>
<th>LETO</th>
<th>OLETF</th>
<th>ARB</th>
<th>ETAN</th>
<th>ETAN/ARB</th>
<th>AMLO</th>
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<td>=</td>
<td>↑</td>
<td>=</td>
<td>↓</td>
<td>=</td>
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<tr>
<td>Adiposity</td>
<td>=</td>
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<td>↓</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓</td>
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<tr>
<td>Fasting blood glucose</td>
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<td>↓</td>
<td>↓</td>
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<td>↓</td>
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<tr>
<td>Insulin resistance index</td>
<td>=</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>--</td>
</tr>
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</table>

= LETO levels  
↑ Increase relative to LETO  
↓ Decrease relative to OLETF  
-- No change relative to OLETF
Figures

**Figure 1.** Weekly progression systolic blood pressure obtained by tail cuff method of (A) LETO, OLETF, OLETF ETAN, OLETF ETAN+ARB (↓ Etanercept removed and reintroduced) (B) LETO, OLETF, OLETF ARB, and OLETF CCB (↓ CCB dose increased) during the 6 weeks of treatment and are presented as means (±SE). # p<0.05 OLETF vs LETO, * p<0.05 ARB vs OLETF, † p<0.05 ETAN vs OLETF, $ p<0.05 ETAN+ARB vs OLETF, ‡ p<0.05 CCB vs OLETF.
Figure 2. The response of blood glucose (A) and plasma insulin (B) levels to an oGTT and the mean (±SE) area under the curve (AUC) for glucose (C) and insulin (D) and the calculated (E) insulin resistance index (IRI) of fasted LETO, OLETF, OLETF ARB, OLETF ETAN, OLETF ETAN+ARB, and OLETF CCB after 6 weeks of treatment. # p<0.05 vs. LETO, * p<0.05 vs. OLETF.
Figure 3. Mean (±SE) percentage change from LETO of renal TNF-α expression obtained by Western blot of LETO, OLETF, OLETF ARB, OLETF ETAN, OLETF ETAN+ARB, and OLETF CCB after the 6 weeks of treatment.
### Raw data for renal TNF-α expression in Figure 3

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<th>Group</th>
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Figure 4. Mean (±SE) of (A) urinary albumin excretion, renal (B) glutathione peroxidase, (C) catalase, (D) superoxide dismutase renal activity, and (E) 4-hydroxynonenal (4HNE) (F) nitrotyrosine content after 6 weeks of treatment in LETO, OLET, OLETF ARB, OLETF ETAN, OLET ETAN+ARB, and OLETF CCB. # p<0.05 vs. LETO, * p<0.05 vs. OLETF.
Raw data for renal 4HNE content in Figure 4

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Figure 5. Mean (±SE) of hepatic (A) glutathione peroxidase (GPx), (B) catalase activity and (C) nitrotyrosine, (D) 4-hydroxynonenal (4HNE) content after 6 weeks of treatment in LETO, OLETF, OLETF ARB, OLETF ETAN, OLETF ETAN+ARB, and OLETF CCB.
Figure 6. Mean (±SE) percentage change from LETO of hepatic (A) Peroxisome proliferator-activated receptor alpha (PPαr-α) and (B) Peroxisomal acyl-coenzyme A oxidase 1 (Acox1) expression obtained by Western blot of LETO, OLETF, OLETF ARB, OLETF ETAN, OLETF ETAN+ARB, and OLETF CCB after the 6 weeks of treatment. # p<0.05 vs. LETO, * p<0.05 vs. OLETF.