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High Fat Consumption Exacerbates and Angiotensin Receptor Blockade Alleviates Antinatriuresis in a Model of Metabolic Syndrome

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in

Quantitative and Systems Biology

by

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The thesis of Jacqueline Norma Minas is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2014

To my family,

for your love, patience, and constant encouragement.

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List of Abbreviations

8-iso, 8-isoprostane
Agt, Angiotensinogen
AT1, Angiotensin Type 1 receptor
Ang II, Angiotensin II
ARB, Angiotensin Type 1 receptor blocker
ASDN, Amiloride sensitive distal nephron
BM, Body mass
CKD, Chronic kidney disease
Cl, Chloride
Cre, Creatinine
CVD, Cardiovascular disease
ECFV, Extracellular fluid volume
ENaC, Epithelial Sodium Channel
FE_{Na}, fractional excretion of sodium
HFD, High fat diet
IRI, Insulin resistance index
LETO, Long Evans Tokushima Otsuka
MetS, Metabolic syndrome
Na^+, sodium
NEFA, Non-esterified fatty acid
oGTT, Oral glucose tolerance test
OLETF, Otsuka Long Evans Tokushima Fatty
PRA, Plasma renin activity
RAAS, Renin angiotensin aldosterone system
SBP, Systolic blood pressure
TALH, Thick ascending loop of Henle
T2DM, Type II Diabetes Mellitus
UV, Urine volume

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Abstract

High Fat Consumption Exacerbates and Angiotensin Receptor Blockade Alleviates Impaired Glucose Tolerance and Antinatriuresis in a Model of Metabolic Syndrome

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High fat diet accelerates diabetic nephropathy while blockade of the renin angiotensin aldosterone system improves the proteinuria and antinatriuresis associated with diabetic nephropathy. However, whether renin angiotensin aldosterone system blockade can improve defects in sodium (Na^+) handling resulting from high fat diet induced insulin resistance remains unknown. The epithelial Na^+ channel (ENaC) is the rate-limiting step to Na^+ handling in the kidney and is sensitive to aldosterone. When ENaC is activated, as measured through ENaC cleavage, Na^+ is reabsorbed and causes Na^+-dependent hypertension. To assess the effects of HFD on Na^+ transport, we compared the changes in the epithelial Na^+ channel (ENaC) and urinary Na^+ excretion on the following groups (n=6-8) of 15 week old rats after 6 weeks of treatment and dietary intervention: 1) lean strain control, Long Evans Tokushima Otsuka (LETO; 23.5% fat chow), 2) LETO + high fat diet (HFD; 62% fat diet), 3) LETO HFD + Angiotensin type 1 (AT1) receptor blockade (ARB; olmesarten; 10mg/kg/day) 4) obese, insulin resistance, Otsuka Long Evans Tokushima Fatty (OLETF) 5) OLETF + HFD, 6) OLETF + ARB, and 7) OLETF + HFD + ARB.

High fat diet caused hyperinsulinaemia in LETOs and exacerbated hyperinsulinaemia and adiposity in OLETF rats. AT1 blockade reduced adiposity and plasma insulin levels in all treatment groups. Additionally, urine aldosterone excretion ($U_{aldo}V$) was elevated in all HFD groups and was significantly decreased by ARB treatment. Subsequently, γ-ENaC loop renal protein expression increased 39% in OLETF compared to LETO and mean γ-ENaC C-terminus (cleavage) expression increased 29% in OLETF HFD compared to OLETF normal chow. As a result of increased γ-ENaC protein expression, urine sodium excretion ($U_{Na}V$) decreased 50% in OLETFs compared to LETOs. Treatment with ARB increased $U_{Na}V$ in all treatment groups by 3 to 4 fold. High fat feeding decreased $U_{Na}V$ by 70 and 30% in LETO HFD and OLETF HFD groups compared LETO and OLETF, respectively. In addition to exacerbating RAAS activation, HFD augmented oxidative damage and podocyte injury in OLETF HFD as measured through increased 8-isoprostane excretion and albuminuria. AT1 blockade eliminated these increases, thus providing renoprotective effects during insulin resistance. Furthermore, these data suggest that HFD contributes to activation of AT1 in the kidney, resulting in impaired Na^+ regulation via ENaC in vivo and ultimately leads to renal damage but is independent of elevated blood pressure during insulin resistance. This

study shows that levels of cleaved γ-ENaC negatively correlated with $U_{Na}V$ indicating that cleavage *in vivo* is likely a key component in the regulation of $U_{Na}V$ during insulin resistance. Additionally, the changes in $U_{aldo}V$ correlate with changes in γ-ENaC subunit cleavage during HFD and insulin resistance suggesting that both insulin resistance and dietary fat affect renal Na^+ transport via aldosterone-induced changes in γ-ENaC subunit cleavage.

Introduction

Metabolic syndrome affects 47 million adults in the U.S. population and predisposes individuals to cardiovascular disease and Type 2 Diabetes Mellitus (T2DM) [1, 2]. Insulin resistance contributes to the development of metabolic syndrome and impaired glucose regulation [1]. Blockade of the renin-angiotensin-aldosterone system (RAAS) through the use of angiotensin receptor type 1 (AT_1) blockers (ARBs) improves glucose intolerance and insulin resistance, suggesting that angiotensin II (Ang II) contributes to the manifestation of these conditions [3-8]. T2DM often induces renal dysfunction, which is associated with a decrease in urine Na^+ excretion (antinatriuresis), an increase in protein excretion (proteinuria), and an increase in glucose excretion (glucosuria) [9-12]. Systemic RAAS and the kidney are primarily responsible for homeostatic fluid balance and therefore the volume-dependent regulation of arterial blood pressure [13]. Aldosterone reduces Na^+ excretion by stimulating its reabsorption primarily in the amiloride-sensitive distal nephron (ASDN), but also in the thick ascending loop of Henle (TALH) and the collecting duct [14, 15]. Additionally, intra-renal Ang II levels increase with the progression of diabetic nephropathy, and AT1 antagonism ameliorates the associated proteinuria and prevents renal injury in diabetic rats [5, 7, 11, 16].

The amiloride sensitive epithelial Na^+ channel (ENaC) is primarily responsible for Na^+ handling in the kidney and is stimulated by Ang II and aldosterone [17-22]. ENaC activity is the rate-limiting step to Na^+ reabsorption in the kidney before urine is excreted. Aldosterone enhances ENaC activity by increasing ENaC expression and increasing the expression of kinases that directly activate or increase ENaC. ENaC is comprised of three subunits, gamma (γ), alpha (α), and beta (β). The γ subunit of ENaC specifically is primarily responsible of maintaining ENaC activity and is more sensitive to amiloride [23]. Improper regulation of Na^+ due to renal injury leads to volume-dependent hypertension and causes further renal damage [9, 12, 24]. Therefore, the proper regulation of Na^+ is critically important to preventing diabetes-induced renal dysfunction. However, the contribution of impaired insulin signaling and inappropriate activation of AT1 to the regulation of urinary Na^+ excretion ($U_{Na}V$) during metabolic syndrome is not well defined.

Recent studies have demonstrated that a high fat diet (HFD) attenuates renal function in diabetic models [25, 26]. Additionally, excess dietary fat has been implicated in the development of T2DM and obesity through impaired glucose tolerance [27, 28]. However, the contribution of HF consumption to hypertension through improper Na^+ handling is unknown. Thus, we aimed to assess the contributions of AT1 activation and HFD on renal function and their effects on blood pressure and renal sodium handling in a model of metabolic syndrome. We employed the Otsuka Long-Evans Tokushima Fatty (OLETF) rat model to investigate the methods by which ENaC is regulated *in vivo* and its contribution to the manifestation of hypertension at 15 weeks of age. The OLETF rat develops diet-induced obesity, followed by hypertension and insulin resistance. The pathological conditions of OLETF rats closely resemble those of the progression of

human metabolic syndrome and type II diabetes [29-31]. Using the lean strain control Long Evans Tokushima Ostuka (LETO) and obese OLETF rats, we tested the hypothesis that HFD exacerbates AT1-mediated impaired Na+ regulation through increased ENaC expression and renal damage in a model of metabolic syndrome.

Methodology

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).

Animals

Male lean LETO and obese OLETF rats (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) were obtained at 9 weeks of age and were randomly assigned to one of the following groups (n = 7-8/group): 1) untreated LETO, 2) LETO + high fat diet (LETO HFD), 3) LETO HFD + angiotensin receptor blocker (10 mg olmesartan/kg/d; LETO HFD+ARB), 4) untreated OLETF, 5) OLETF + HFD (OLETF HFD), 6) OLETF + ARB (OLETF ARB), and 7) OLETF + HFD + ARB (OLETF HFD+ARB). All animals were given free access to water and fed ad libitum either a standard laboratory chow (23.5% fat, 18.6% protein, and 57.8% carbohydrate) or high fat diet rat chow (62.2% saturated fat, 18.2% protein, and 19.6% carbohydrate) (MF, Oriental Yeast Corp., Tokyo, Japan). Both standard and high fat chow contained 0.259% sodium chloride (NaCl). The ARB (olmesartan; Daiichi-Sankyo, Tokyo, Japan) was administered by gavage daily. All animals were maintained at the Kagawa Medical University vivarium, and housed three or four animals per cage in a specific pathogen-free facility under controlled temperature (23°C) and humidity (55%) with a 12-h light and dark cycle. By 15 weeks of age, OLETF rats develop insulin resistance, hypertension, and hyperlipidemia [32, 33].

Systolic Blood pressure and Metabolic Measurements

Systolic blood pressure (SBP) was monitored weekly by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan) to evaluate the progression of hypertension and the effectiveness of AT1 blockade. Animals were allowed to acclimate to the tail-cuff apparatus before measuring the blood pressure. Twenty-four hour urine samples were collected 0, 3, and 6 weeks (9, 12, and 15 weeks of age) during the study. Additionally, food consumption data was measured daily. Sodium chloride (NaCl) intake was estimated as the product of 0.259% and daily food consumption rates (g/d).

Sample Collection

After 6 weeks of treatment, 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 [34-36]. The kidneys and adrenals were rapidly removed, weighed, and snap-frozen in liquid nitrogen. Frozen samples were kept at −80°C until analyzed. Relative retroperitoneal fat mass was removed and weighed.

Analyses

Plasma and Urine Analyses

Plasma aldosterone (Siemens Healthcare Diagnostics, Los Angeles, CA), angiotensin II (Phoenix Pharmaceuticals, Burlingame, CA), renin activity (Diasorin, Stillwater, MN), and leptin (Millipore, Bedford, MA) were measured using a commercially available rat-

specific radioimmunoassay (RIA) kit. Plasma insulin was measured using a commercially available enzyme-linked immunosorbent assay (Shibayagi Co., Ltd., Japan). Plasma levels of nonesterified fatty acid (NEFA) were measured using a commercially available calorimetric assay (Wako Chemicals, Richmond, Va). Plasma sodium (Na^+) and creatinine were measured by autoanalyzer (7020-Automatic analyzer, Hitachi-high-Technologies Corporation, Tokyo, Japan).

Aldosterone was extracted from urine samples and measured by RIA similar to plasma (Siemens Healthcare Diagnostics). Urinary Na^+ was measured by autoanalyzer (7020-Automatic analyzer, Hitachi-high-Technologies Corporation). Urine 8-isoprostane was quantified by ELISA (Cayman Chemical, Ann Arbor, MI). Urine samples were diluted 1:25 or 1:40 prior to measurement of total angiotensinogen (Agt; IBL America, Minneapolis, MN) [37] and 1:8 or 1:10 prior to measurement of 8-isoprostane (Cayman Chemical), respectively. Urinary creatinine (Cre) was measured by calorimetric assay (Cayman Chemical). Urinary excretion of each variable was calculated as the product of 24-hour urine volume (UV) and urinary concentration. Fractional excretion of Na^+ (FE_{Na}) was calculated as

$$FE\ (\%) = 100 \times ([U_{Na}] \times [P_{Cre}]) / ([P_{Na}] \times [U_{cre}])$$

where [U] and [P] represent urinary and plasma concentrations of either Na^+ or Cre. All samples were analyzed in duplicate and run in a single assay with intra-assay percent coefficients of variability of <10% for all assays.

Oral glucose tolerance test (oGTT)

One week before the end of the experimental period, oGTTs 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 µl of lidocaine (Henry Schein, Melville, NY). Blood glucose was measured before gavage (0) and at 15, 30, 60, and 120 min after the glucose infusion. In addition, the 0-, 15-, 30-, and 120-min 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 ($AUC_{glucose}$ x $AUC_{insulin}$).

Western Blot

Frozen tissue samples were homogenized in 500µl RIPA buffer (Boston BioProducts, Inc., Ashland Massachusetts) containing 5% protease and phosphatase inhibitor cocktail (Sigma). Tissue homogenate was centrifuged (16,000 ×g, 15 min), and the aqueous layer was transferred to a separate tube and stored at −80 C for later analyses. Total protein content was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of total protein were resolved in 7.5% Tris-HCl SDS gradient gels. Proteins were electroblotted using the Bio-Rad Trans Blot SD semidry cell onto 0.45-µm nitrocellulose membranes or PVDF membranes. Membranes were blocked with 5% BSA

or LiCOR blocking buffer in PBS and incubated overnight with primary antibodies (diluted 1:1000) against subunit specific antibodies for each γ-ENaC and α-ENaC, which have been characterized previously [38]. Membranes were washed, incubated with horseradish peroxidase conjugated secondary antibodies against rabbit (Pierce, Rockford, IL), rewashed, and developed by using the Enhanced Chemiluminescence (ECL) kit (Bio-Rad Laboratories). Blots were visualized using a Kodak Image Station 440 or LiCOR Odyssey CLx and quantified using Image Studio (Li-Cor Biosciences, Lincoln, Nebraska). All primary antibodies were confirmed by performing secondary only tests to confirm specificity of the primary antibodies. In addition to consistently loading the same amount of total protein per well, the densitometry values were further normalized by correcting for the densitometry values of beta actin.

Statistical Analysis

Means (±SE) for plasma, urine, organ measurements, and Western Blot were compared by analysis of variance (ANOVA). Relationships between dependent and independent variables were evaluated by simple regression and correlations were evaluated using Pearson correlation coefficients. All samples were analyzed in duplicate and run in a single assay with intra-assay, percent coefficients of variability of < 10% for all assays. Means, regression, and correlations were considered significantly different at $p<0.05$. Statistical analyses were performed with the SYSTAT 11.0 software (SPSS, Richmond, CA).

Results

BM, Food Consumption, NaCl Consumption, Kidney Masses, and Adiposity

Mean body mass and food intake were measured to assess the effects of HFD and AT1 blockade on food intake and body mass. BM increased 40% in OLETF compared to LETO and ARB had no effect on OLETFs supplemented a normal chow (Table 1). Body mass increased in LETO HFD and OLETF HFD by 13 and 16% compared to LETO and OLETF, respectively. Combination of HFD and ARB decreased body mass in both strains compared to animals fed a HF chow. Mean relative retroperitoneal fat mass increased 54% in OLETF compared to LETO (Table 1). HFD increased relative retro fat in both lean and obese animal 45 and 40%, respectively. ARB reduced relative retroperitoneal fat 57 and 54%, respectively, in both lean and obese animals supplemented with a HFD (Table 1). Mean insulin resistance index (IRI) increased 3-fold in OLETF compared to LETO (665 ± 78 vs. 212 ± 55; relative units) and ARB treatment completely reduced IRI levels to LETO levels the increase (214 ± 72). HFD increased mean IRI levels in OLETF 3-fold (665 ± 78 vs. 1838 ± 246) and ARB treatment attenuated the increase (268 ± 92 vs. 1838 ± 246). Mean food intake was increased 58% in OLETFs compared to LETOs, but HFD and ARB did not have an effect. Consumption of NaCl was estimated to confirm that changes in $U_{Na}V$ were a function of altered renal handling and less so a consequence of changes in consumption. NaCl intake increased 37% in OLETF compared to LETO, but no changes were detected within each strain (Table 1). Absolute kidney mass increased 59% in OLETF compared to LETO (Table 1). ARB treatment decreased absolute kidney mass 19% in OLETF HFD+ARB compared to OLETF HFD. Relative kidney mass increased 15% in OLETF compared to LETO and ARB treatment attenuated this increase. ARB increased relative kidney mass in OLETF HFD+ARB and LETO HFD+ARB 40 and 26 percent compared to LETO HFD and OLETF HFD, respectively.

Lipid Metabolism

Fasting plasma leptin and NEFA were measured to assess the effects of HFD and RAAS activation on lipid metabolism. Fasting plasma leptin increased more than 3-fold in OLETF compared to LETO whereas ARB treatment decreased leptin by 50% (Table 2). HFD exacerbated the increase in plasma leptin an additional 2-fold in OLETF HFD, whereas ARB treatment decreased leptin by 88% (Table 2). Fasting plasma NEFA increased 40% in OLETF compared to LETO and ARB treatment reduced this increase by 16% (Table 2).

Effects of ARB and HFD on local intrarenal and systemic RAAS

Plasma renin activity, Ang II, and aldosterone were measured to assess the effects of HFD and AT1 blockade on circulating RAAS. Plasma renin activity (PRA) increased in LETO HFD and OLETF HFD by 2.3 and 1.8-fold compared to LETO and OLETF, respectively (Table 2). Additionally, AT1 blockade increased PRA levels 13 and 5-fold in OLETF ARB and OLETF HFD+ARB compared to OLETF and OLETF HFD, respectively. Plasma Ang II levels increased 25% in OLETF compared to LETO (Table 2). AT1 blockade increased plasma Ang II levels in LETO HFD+ARB, OLETF ARB,

and OLETF HFD+ARB by 2.5-, 2-, and 3-fold compared to LETO HFD, OLETF, and OLETF HFD+ARB, respectively. Plasma aldosterone decreased 33% in OLETF compared to LETO and ARB treatment increased plasma aldosterone levels nearly 2-fold in OLETF ARB compared to OLETF (Table 2).

To assess the temporal effects of AT1 blockade and HFD on systemic RAAS, urine aldosterone excretion was measured at 0, 3, and 6 weeks of diet and treatment. At the onset of the study (week 0), urine aldosterone excretion in LETO was nearly double that in OLETF (Figure 1). At 3 weeks, LETO HFD and OLETF urine aldosterone excretion increased 60 and 41%, respectively, compared to LETO (Figure 1). Additionally, HFD increased urine aldosterone excretion 35% at 3 weeks compared to OLETF. Additionally, AT1 blockade decreased urine aldosterone excretion levels in both strains supplemented a HFD by 36% and 58% (LETO HFD+ARB, OLETF HFD+ARB). After 6 weeks of treatment, urine aldosterone excretion increased 49% in OLETF compared to LETO (Figure 1). Additionally, both lean and obese animals fed a HFD showed a 43% and 36% increase compared to those fed a normal chow. Treatment with AT1 blockade decreased urine aldosterone excretion 35% and 31% in both OLETF ARB and OLETF HFD+ARB compared to OLETF and OLETF HFD, respectively.

ENaC Protein Expression

Protein expression of γ-ENaC was measured at 6 weeks to assess the contribution of AT1 activation and HFD to impaired sodium handling during insulin resistance. Mean γ-ENaC loop expression increased 39% in OLETF compared to LETO (Figure 2b). High fat consumption increased mean protein expression 31% in the lean strain LETOs compared to normal chow fed rats. However, HF consumption had no effect on ENaC expression in the obese OLETF. AT1 blockade decreased mean γ-ENaC loop protein expression by 21, 18, and 19% in LETO HFD+ARB, OLETF ARB, and OLETF HFD+ARB compared to LETO HFD, OLETF, and OLETF HFD, respectively (Figure 2b). Mean γ-ENaC c-terminus protein expression increased 29% in OLETF HFD compared to OLETF (Figure 2c). Additionally, ARB treatment reduced mean γ-ENaC c-terminus protein expression 27, 28, and 55% LETO HFD+ARB, OLETF ARB, and OLETF HFD+ARB compared to LETO HFD, OLETF, and OLETF HFD, respectively.

Sodium Excretion Response

To better assess the contributions of insulin resistance, AT1 activation, and high fat consumption to sodium handling, urine sodium excretion ($U_{Na}V$) was measured, corrected for creatinine ($U_{Na/cre}V$), and fractional sodium excretion (FE_{Na}) was calculated. At 0 weeks of ARB treatment and HFD (9 weeks of age), $U_{Na}V$ was unchanged in OLETF compared to LETO (Figure 3a). Urine sodium excretion decreased in OLETF at 3 and 6 weeks of treatment compared to LETO. Additionally, HFD decreased $U_{Na}V$ in LETO HFD at 3 and 6 weeks of consumption. High fat consumption also decreased $U_{Na}V$ in OLETF HFD at 6 weeks compared to normal chow OLETF. Treatment with AT1 receptor blockers increased $U_{Na}V$ in LETO HFD+ARB at 3 and 6 weeks compared to LETO HFD, however, it only increased $U_{Na}V$ in OLETF ARB at 3 weeks. At 0 weeks of treatment, $U_{Na/cre}V$ decreased in OLETF compared to LETO (Figure 3b). Urine sodium excretion corrected for creatinine decreased in both LETO HFD and OLETF HFD at 3 and 6 weeks compared to LETO. Additionally, $U_{Na/cre}V$ increased in LETO HFD+ARB

and OLETF ARB at 3 weeks compared to LETO HFD and OLETF, respectively. At 6 weeks, $U_{Na/cre}V$ increased in OLETF HFD+ARB compared to OLETF HFD. Fractional excretion of sodium was significantly decreased OLETF at 6 weeks compared to LETO (Figure 3c). Additionally, HFD decreased FE_{Na} in both LETO HFD and OLETF HFD compared to normal chow LETO and OLETF, respectively. AT1 receptor blockade increased FE_{Na} in LETO HFD+ARB and OLETF HFD+ARB compared to LETO HFD and OLETF HFD after 6 weeks of treatment.

Renal Injury Response

To assess the contribution of insulin resistance, AT1 activation, and HF consumption to renal damage, urinary angiotensinogen ($U_{Agt}V$), 8-isoprostane ($U_{8\text{-}iso}V$), and albumin excretion were measured. Because urine $U_{Agt}V$ is a reliable marker of intrarenal RAAS activation and levels of kidney Ang II [39, 40], levels of $U_{Agt}V$ were determined at 6 weeks to assess the effects of insulin resistance, AT1 activation, and HFD on intrarenal Ang II content. Angiotensinogen excretion increased 65% in OLETF at 6 weeks compared to LETO (Figure 4). Although HFD had no effect on $U_{Agt}V$ in either strain, At1 blockade reduced levels of $U_{Agt}V$ in both OLETF ARB and OLETF HFD+ARB compared to OLETF and OLETF HFD, respectively. Urine 8-isoprostane excretion was elevated at 0 weeks in OLETF compared to LETO (Figure 5a). Both OLETF and LETO maintained $U_{8\text{-}iso}V$ levels throughout the duration of the study. High fat consumption increased $U_{8\text{-}iso}V$ in OLETF HFD compared to OLETF at 3 and 6 weeks. AT1 blockade reduced levels of $U_{8\text{-}iso}V$ in OLETF HFD+ARB at 3 and 6 weeks, but only reduced $U_{8\text{-}iso}V$ in OLETF ARB at 6 weeks. At 0 weeks, urine albumin excretion was increased nearly 3-fold in OLETF compared to LETO (Figure 5b). At 3 and 6 weeks, OLETF maintained a significant increase compared to LETO. Additionally, HFD increased urine albumin excretion in OLETF HFD at 3 and 6 weeks compared to OLETF. Treatment with AT1 blockade substantially decreased urine albumin excretion at 3 and 6 weeks in both OLETF ARB and OLETF HFD+ARB.

Systolic Blood Pressure

To ascertain the effects of AT1 activation and HFD on Ang II-mediated hypertension, SBP measures were performed by tailcuff. During the initial baseline measurement (0 weeks), SBP was the same between OLETF and LETO (Figure 6) which is consistent with previous findings regarding the OLETF model [6]. SBP remained unaltered in LETO; however, OLETF and OLETF HFD both became hypertensive by week 1 and maintained this elevated blood pressure. HFD had no additional effect on SBP in LETO. However, ARB administration decreased blood pressure in LETO HFD+ARB, OLETF ARB, and OLETF HFD+ARB beyond normotensive levels.

Discussion

The chronic consumption of high fat (typically saturated fat) induces a number of pathophysiologic conditions that promote MetS; however, the additional consequences of HFD in an established condition of obesity and insulin resistance on renal Na^+ handling are not fully understood [9, 25, 41]. Although previous reports have shown the relationship between HFD-induced obesity and RAAS activation, we show the impact of HFD in a model already presenting with insulin resistance and up-regulation of RAAS. The novel and important findings of the present study are that: 1) HFD does not exacerbate SBP over the study period despite a decrease in urinary Na^+ excretion associated with an aldosterone-induced increase in γ-ENaC subunit cleavage, 2) HFD further augments systemic oxidative stress and renal injury during insulin resistance, and 3) AT1 blockade greatly improves the status of fat mass, leptin, and NEFA in the presence of HFD.

Renin Angiotensin Aldosterone System, Sodium Handling, and Systolic Blood Pressure

It is well known that Ang II is a vasoconstrictor that regulates fluid homeostasis while aldosterone regulates fluid and sodium retention. Although Ang II directly affects blood pressure and induces hypertension, patients with primary hyperaldosteronism compared to patients without hyperaldosteronism have more adverse cardiovascular events independent of elevated blood pressure, suggesting that elevated aldosterone is more detrimental than high blood pressure [42]. Our data show that in both LETO and OLETF, HFD increases urine aldosterone excretion, PRA levels, and plasma aldosterone levels while decreasing $U_{Na/cre}V$ and $FE_{Na.}$ Although SBP did not change over the course of our study period, the HFD-induced elevation in aldosterone may eventually promote sufficient renal injury that will ultimately lead to exacerbation of arterial pressure [43]. Interestingly, chronic treatment with ARB failed to reduce plasma aldosterone levels which is indicative of aldosterone breakthrough suggesting that increased aldosterone secretion and production is a response to blockade of adrenal AT1 receptors [44, 45]. However, a previous study conducted by our group shows the benefits of ARB treatment on the heart in OLETF rats, specifically, that AT1 blockade prevents cardiac oxidative damage in this model [34]. A discrepancy was observed between the dissection plasma aldosterone and 24-hour $U_{aldo}V$ levels measured the day before the dissections. Because the plasma levels were measured in samples collected after an overnight fast (8-12 hr), these plasma aldosterone levels most likely reflect the sensitivity of the adrenal to an acute fast and not necessarily the chronic effects of the treatments (HFD and ARB). Therefore, $U_{aldo}V$ was measured over time to better assess the effects of the treatments on RAAS and the contribution of aldosterone to renal damage in this model [46]. The data demonstrate that MetS and HFD increase $U_{aldo}V$, and ARB treatment reduces this increase as expected. Our data suggest that both insulin resistance and dietary fat affects renal Na^+ transport via aldosterone-induced changes in γ-ENaC. Correspondingly, the changes in γ-ENaC protein and cleavage expressions are correlated with the appropriate changes in $U_{aldo}V$ (Table 3) suggesting that the changes in urinary aldosterone provide a robust indication of the observed changes in ENaC over time [46].

These changes in $U_{aldo}V$ and γ-ENaC may subsequently induce changes in $U_{Na}V$, and ultimately extracellular volume and arterial blood pressure. However, the decreases in sodium excretion observed in HFD groups did not translate into increases in systolic blood pressure, which may be explained by the lack of an increase in plasma Na^+ levels. For the purposes of this study, we focused only on renal ENaC expression, but increases in fecal sodium excretion may be a possible reason why the changes seen in urine aldosterone excretion, γ-ENaC, and $U_{Na}V$, $U_{Na/cre}V$, and FE_{Na} did not induce a corresponding increase in systolic blood pressure. Alternatively, the decreases in $U_{Na}V$ were not sufficient to increase extracellular fluid volume (ECFV) to a point to induce an elevation in arterial pressure beyond that already induced by elevated Ang II. Notwithstanding, the positive relationships among urine aldosterone excretion, γ-ENaC, and sodium handling during HFD and MetS elucidate the complicated interrelationships of sodium handling, hypertension, IR, and diabetic nephropathy (Figure 7).

Intrarenal RAAS and Oxidative Damage

Because the changes in $U_{Agt}V$ are closely associated with changes in intrarenal Ang II levels, $U_{Agt}V$ has become an important surrogate measure of intrarenal RAAS, especially of Ang II [39, 40]. Additionally, Ang II infusion increases renal angiotensinogen mRNA and protein expression in addition to $U_{Agt}V$ [39]. Nagai *et al.* showed that intrarenal Ang II levels are increased in OLETF rats prior to the onset of frank diabetes and that these increases are correlated with angiotensinogen mRNA expression [6]. Furthermore, ARB treatment in OLETF rats attenuated the development of the diabetes-induced renal injury [6]. The present study demonstrates that renal injury precedes the establishment of frank diabetes in OLETF rats suggesting that the early development of insulin resistance is sufficiently detrimental to the kidney. Furthermore, HFD exacerbated the oxidative damage (lipid peroxidation; $U_{8\text{-}iso}V$) and podocyte injury ($U_{alb}V$). Signs of oxidative damage are already apparent in this model in as early as 9 weeks of age suggesting that oxidative stress may contribute to the early phenotypic characteristics of metabolic syndrome. Moreover, the data clearly elucidate the involvement of AT1 activation in the manifestation of the MetS variables measured here; however, HFD did not impede the renoprotective benefits of AT1 blockade suggesting that the mechanisms by which HFD induces renal complications are at least partially mediated by activation of AT1.

Insulin Resistance and Adiposity

This study was designed to elucidate the effects of poor dietary choices on both lean and obese models. Increased dietary fat is used to induce obesity and insulin resistance in lean strain [27, 28]. To our knowledge, this is the first study to implement a HF dietary intervention at the pre-diabetic stage that shows HFD exacerbates IRI and increases adiposity in a model already predisposed to MetS and IR (Table 3). Overall, there was no change in food intake suggesting that the increased adiposity and IRI in OLETF HFD compared to normal chow OLETF are induced by HFD. The obesity epidemic amongst adolescents has increased substantially during the past few decades and dietary and physical exercise intervention is necessary to prevent the development of insulin resistance, T2DM, cardiometabolic, and cardiorenal disease [47, 48]. Recent publications suggest that the prevalence of childhood obesity has plateaued, however, these studies indicate that childhood obesity is highly prevalent and the rate is not decreasing [49, 50]. A recent study in OLETF rats found that leanness or obesity early on in life may have

irreversible effects on whole body metabolism [26]. Our data show that although OLETF rats become insulin resistant and obese, HFD increases the severity of adiposity and IRI suggesting that poor diet increases the rate of the onset of T2DM. Additionally, increased mean retroperitoneal fat mass is associated with mean plasma leptin levels, but did not decrease mean food intake which is indicative of leptin resistance as we have reported previously [35]. Collectively, these data suggest that dietary intervention is necessary to prevent the development of insulin resistance, T2DM, and cardiorenal disease.

Summary

The focus of this thesis was to assess the contributions of HFD on RAAS activation in a model of MetS and hypertension. Additionally, we sought to elucidate the effects of HFD and AT1 activation on renal oxidative damage and Na^+ handling during insulin resistance. The present study shows that in both lean strain LETO and obese OLETF rats, HFD increases urine aldosterone excretion, PRA levels, and plasma aldosterone levels while decreasing $U_{Na/cre}V$ and $FE_{Na.}$ Our findings also demonstrate that renal injury precedes diabetes in obese OLETF rats, suggesting that the development of insulin resistance is sufficiently detrimental to the kidney. Also, renal oxidative damage occurs in this model as early as 9 weeks, before the onset of hypertension, suggesting that oxidative damage may contribute to the early phenotypic characteristics of MetS. Furthermore, we demonstrated that HFD exacerbates oxidative damage and podocyte injury in OLETF rats, but does not impede the renoprotective benefits of AT1 blockade. The data demonstrate that MetS and HFD increase $U_{aldo}V$, and AT1 blockade reduces this increase. Correspondingly, the changes in γ-ENaC protein and cleavage expressions are correlated with the appropriate changes in $U_{aldo}V$ suggesting that the changes in urinary aldosterone provide a robust indication of the observed changes in ENaC over time. Collectively, these data suggest that both insulin resistance and dietary fat affect renal Na^+ transport via aldosterone-induced changes in γ-ENaC subunit cleavage.

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Table 1. Mean ± SE BM, food intake, absolute kidney mass, relative kidney mass, and relative retroperitoneal (retro) fat mass, plasma

	LETO	LETO HFD	LETO HFD+ARB	OLETF	OLETF HFD	OLETF ARB	OLETF HFD+ARB
Body Mass (g)	350±13	394±14*	281±14†	487±14*	566±8#	453±8	344±19†
Food intake (g/100g BM)	4.8±0.03	4.1±0.17	4.5±0.63	6.6±0.22*	5.9±0.64	6.8±0.23	4.87±0.39
NaCl intake (mg/100g BM)	1.25±0.01	1.05±0.04	1.18±0.16	1.71±0.06*	1.51±0.17	1.75±0.06	1.26±0.1
Absolute kidney mass (g)	0.94±0.04	0.99±0.09	0.99±0.05	1.49±0.06*	1.53±0.03	1.45±0.03	1.25±0.04†
Relative kidney mass (g/100g BM)	0.27±0.01	0.25±0.01	0.35±0.01†	0.31±0.01*	0.27±0.01#	0.32±0.01	0.34±0.01†
Relative retro fat mass (g/100g BM)	1.65±0.2	3.0±0.18*	1.3±0.18†	3.57±0.11*	5.98±0.11#	3.24±0.24	2.75±0.23†

for LETO, LETO HFD, LETO HFD+ARB, OLETF, OLETF HFD, OLETF ARB, OLETF HFD+ARB. *Significant difference vs. LETO ($P<0.05$); #Significant difference vs. OLETF ($P<0.05$); †Significant difference vs. untreated ($P<0.05$)

Table 2: Mean ± SE fasting plasma renin activity, angiotensin II, aldosterone, insulin, leptin, nonesterified fatty acid (NEFA), and sodium (Na+)

	LETO	LETO HFD	LETO HFD+ARB	OLETF	OLETF HFD	OLETF ARB	OLETF HFD+ARB
Renin Activity (ng Ang I/mL/hr)	2.2±0.4	5.1±0.8*	10.9±1.65	3.48±0.43	6.39±0.9	46±4#	31±4.6†
Angiotensin II, (fmol/L)	67±4	99±11	251±26†	93±3*	116±7	192±15#	364±15†
Aldosterone (pmol/L)	586±46	793±46	903±10†	391±23*	435±38	702±87#	1175±161
Insulin, (ng/mL)	0.79±0.15	1.6±0.16*	1.35±0.21	3.03±0.26*	4.3±0.28#	1.97±0.26#	1.05±0.17†
Leptin, (ng/mL)	2.54±0.4	3.63±0.51	1.61±0.1†	9.38±1.03*	22±3.4#	4.76±0.8#	2.64±0.3†
NEFA (mEq/L)	1.04±0.05	1.26±0.07*	1.38±0.12†	1.45±0.07*	1.51±0.05	1.23±0.07#	1.15±0.05†
Na^+, (mg/mL)	134±1.01	135±1.24	135±1.15	135±1.67	136±0.7	137±0.9	135±3.2

levels for LETO, LETO HFD, LETO HFD+ARB, OLETF, OLETF HFD, OLETF ARB, and OLETF HFD+ARB. *Significant difference vs. LETO, #Significant difference from OLETF †Significant difference vs. untreated ($P<0.05$)

Table 3: Summary of findings. ↑ = significant (P<0.05) increase from LETO, ↑↑ = significant (P<0.001) increase from LETO or normal chow, = no change, ↓ significant (P<0.05) decrease from LETO, normal chow, or untreated, ↓↓ significant (P<0.001) decrease from LETO, normal chow, or untreated.

	Insulin Resistance	HFD	AT1 Blockade
Rel. fat mass	↑	↑↑	↓
IRI	↑	↑↑	↓
Urine aldo excretion	↑	↑↑	↓
ENaC	↑	↑	↓
Fractional excretion-Na^+	↓	↓↓	↑
Urine Agt Excretion	↑	=	↓
Urine 8-iso excretion	↑	↑↑	↓↓
Albuminuria	↑↑	↑↑	↓↓
SBP	↑↑	=	↓↓

Figures

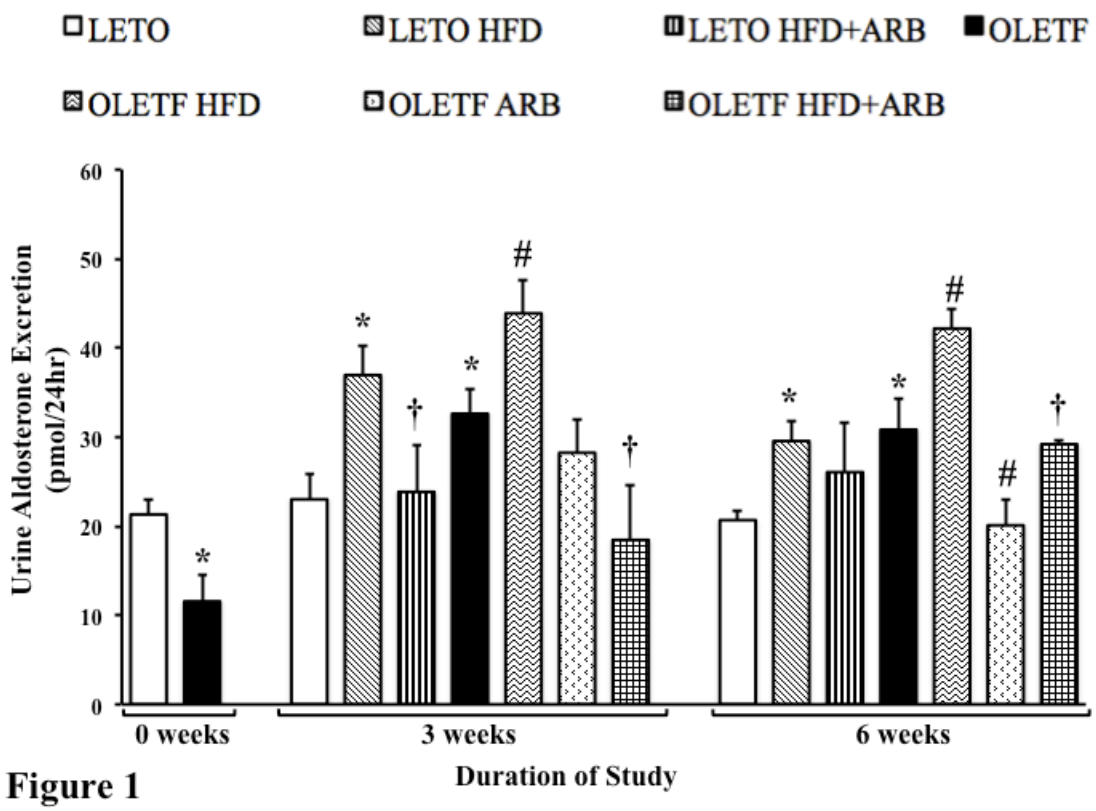

Figure 1. Mean (±SE) values for urine aldosterone excretion levels from Long Evans Tokushima Otsuka (LETO; *n* = 6), LETO + high fat diet (HFD); *n* = 7), LETO HFD+ angiotensin receptor type 1 blocker (ARB; *n* = 7), Otsuka Long Evans Tokushima Otsuka (OLETF; *n* = 8), OLETF HFD (*n* = 8), OLETF ARB (*n* = 8) and OLETF HFD+ARB (*n* = 7) rats after 0, 3, and 6 weeks of treatment and dietary intervention (9, 12, and 15 weeks of age). *Significant difference from LETO (P<0.05); †Significant difference from untreated (P<0.05); #Significant difference from normal chow (P<0.05).

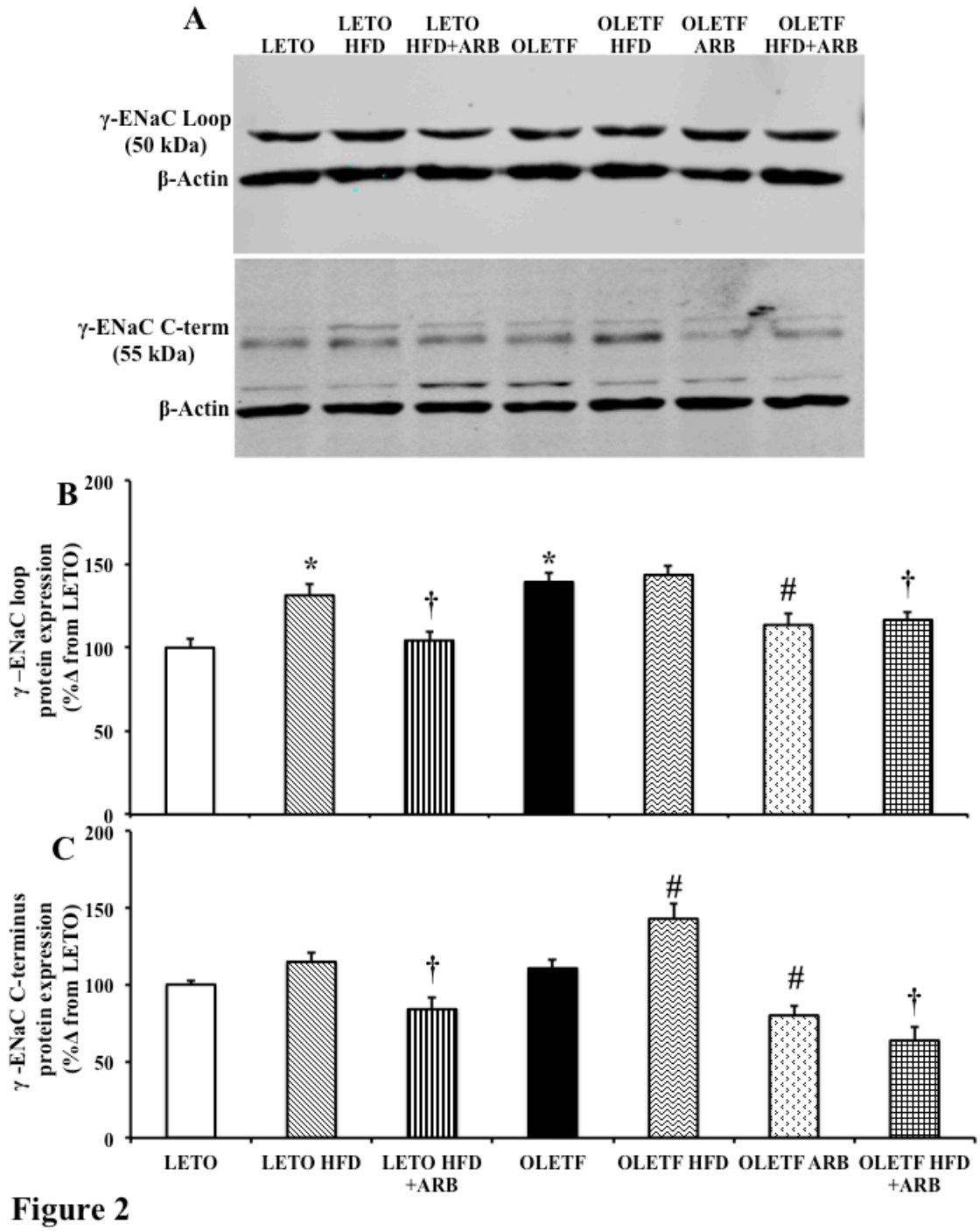

Figure 2

Figure 2. A: Representative Western blots for γ-ENaC loop and γ-ENaC C-terminus. Mean (±SE) values of γ-ENaC loop protein expression (*B*) and γ-ENaC C-terminus protein expression (*C*) levels from LETO, LETO HFD, LETO HFD+ARB, OLETF, OLETF HFD, OLETF ARB and OLETF HFD+ARB. *Significant difference from LETO ($P<0.05$); †Significant difference from untreated ($P<0.05$); #Significant difference from normal chow ($P<0.05$).

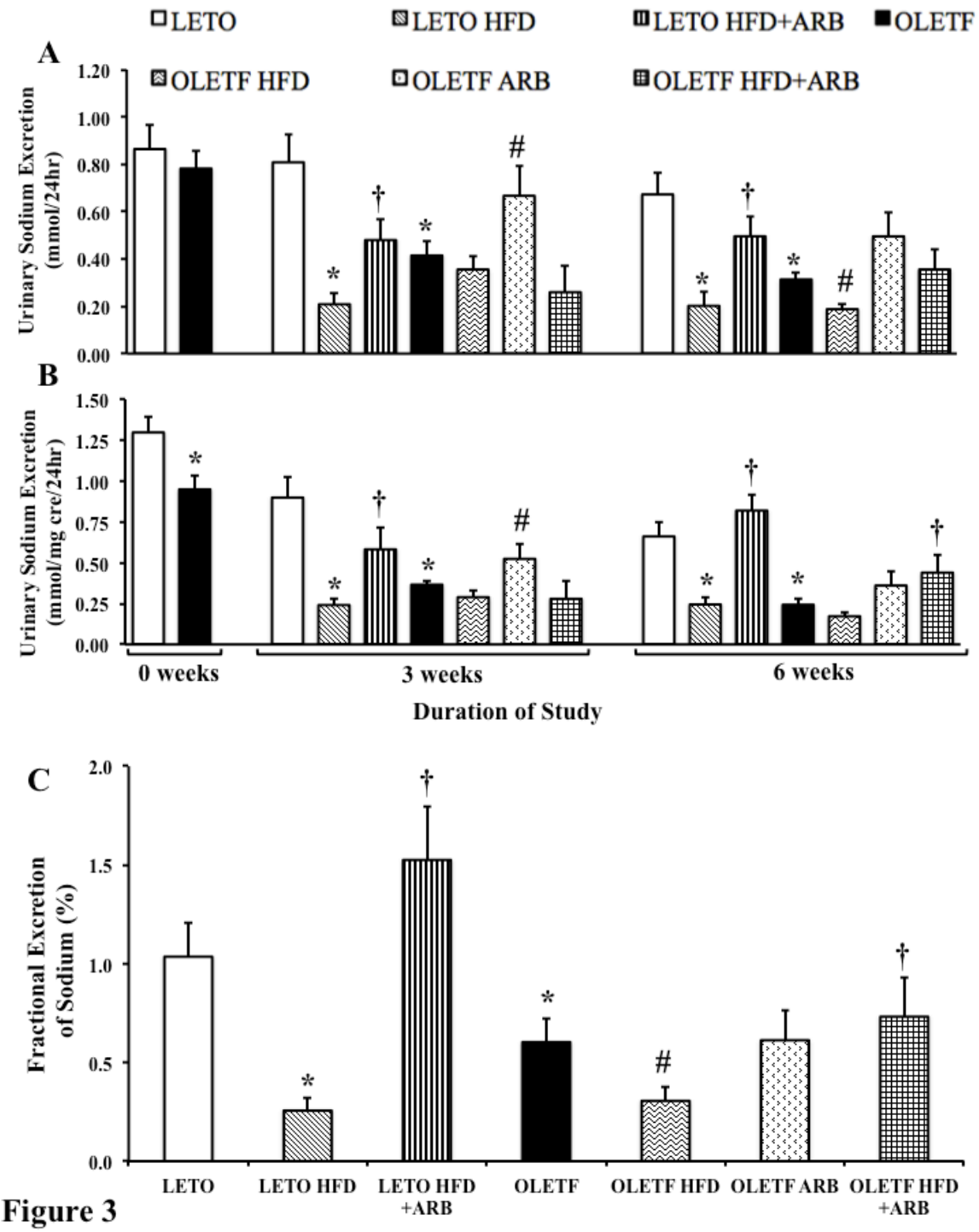

Figure 3

Figure 3. Mean (±SE) values of urine sodium excretion ($U_{Na}V$; 0, 3, and 6 weeks) levels (*A*), urine sodium to creatinine excretion ($U_{Na/cre}V$; 0, 3, and 6 weeks) levels (*B*) and fractional excretion of sodium (FE_{Na}; 6 weeks) percentage (*C*) from LETO, LETO HFD, LETO HFD+ARB, OLETF, OLETF HFD, OLETF ARB and OLETF HFD+ARB. *Significant difference from LETO ($P<0.05$); †Significant difference from untreated ($P<0.05$); #Significant difference from normal chow ($P<0.05$).

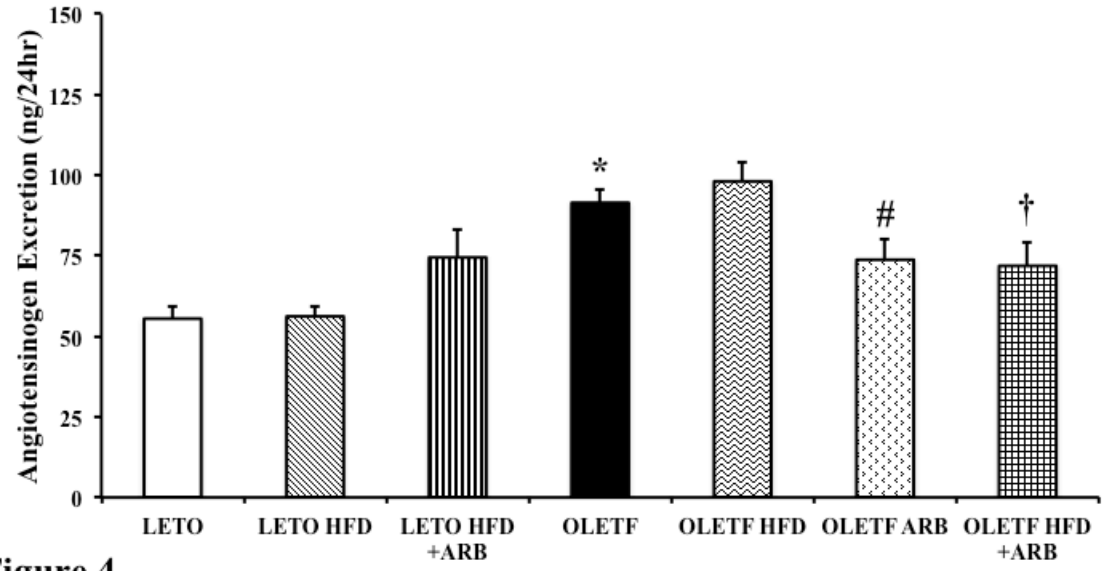

Figure 4

Figure 4. Mean (±SE) values of urine angiotensinogen excretion ($U_{Agt}V$) levels from LETO, LETO HFD, LETO HFD+ARB, OLETF, OLETF HFD, OLETF ARB and OLETF HFD+ARB. *Significant difference from LETO ($P<0.05$); †Significant difference from untreated ($P<0.05$); #Significant difference from normal chow ($P<0.05$).

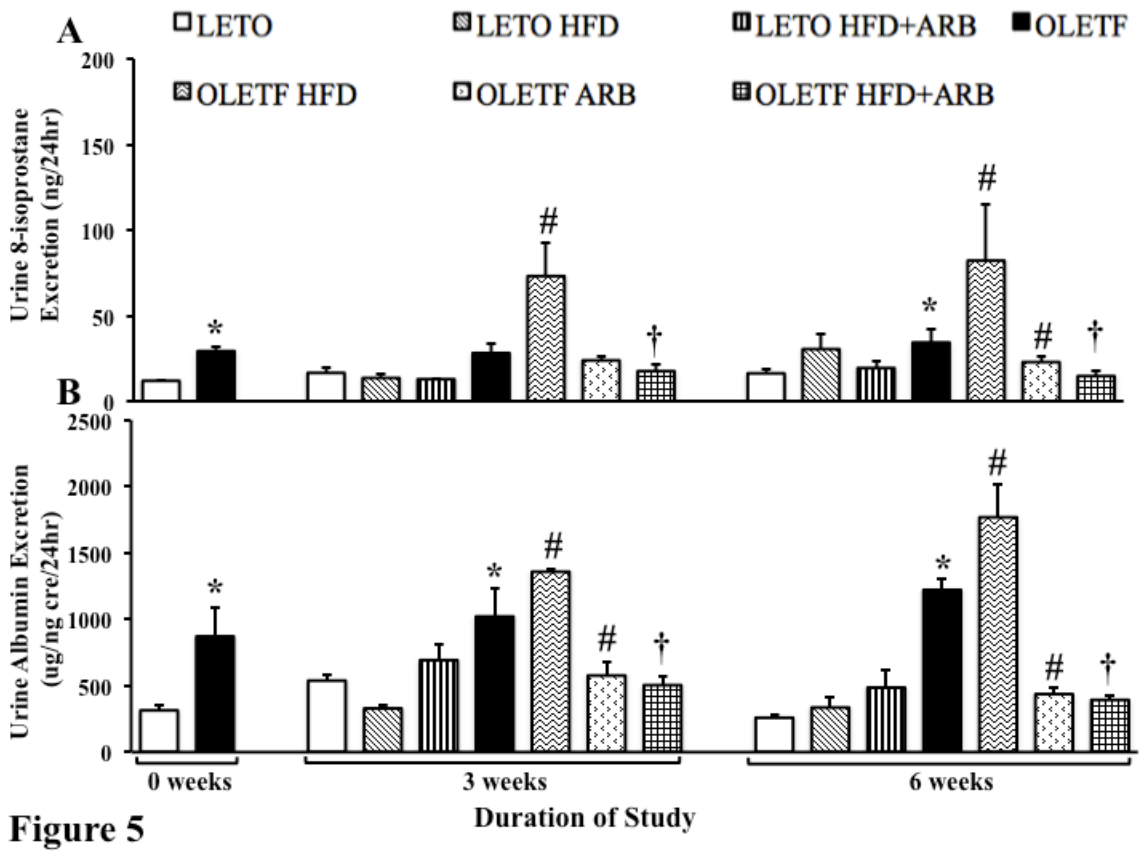

Figure 5

Figure 5. Mean (±SE) values urine 8-isoprostane excretion ($U_{8\text{-iso}}V$; 0, 3, and 6 weeks) levels (*A*) and urine albumin excretion (0, 3, and 6 weeks) levels (*B*) from LETO, LETO HFD, LETO HFD+ARB, OLETF, OLETF HFD, OLETF ARB and OLETF HFD+ARB. *Significant difference from LETO ($P<0.05$); †Significant difference from untreated ($P<0.05$); #Significant difference from normal chow ($P<0.05$).

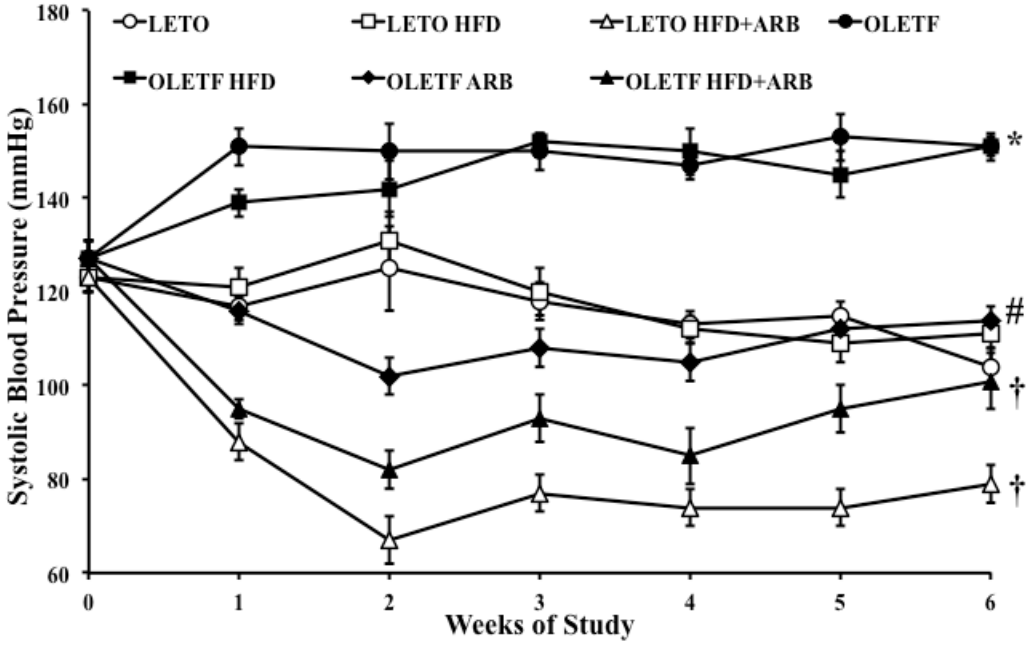

Figure 6

Figure 6. Mean (±SE) values of weekly systolic blood pressure from LETO, LETO HFD, LETO HFD+ARB, OLETF, OLETF HFD, OLETF ARB and OLETF HFD+ARB. *Significant difference from LETO ($P<0.05$); †Significant difference from untreated ($P<0.05$); #Significant difference from normal chow ($P<0.05$).

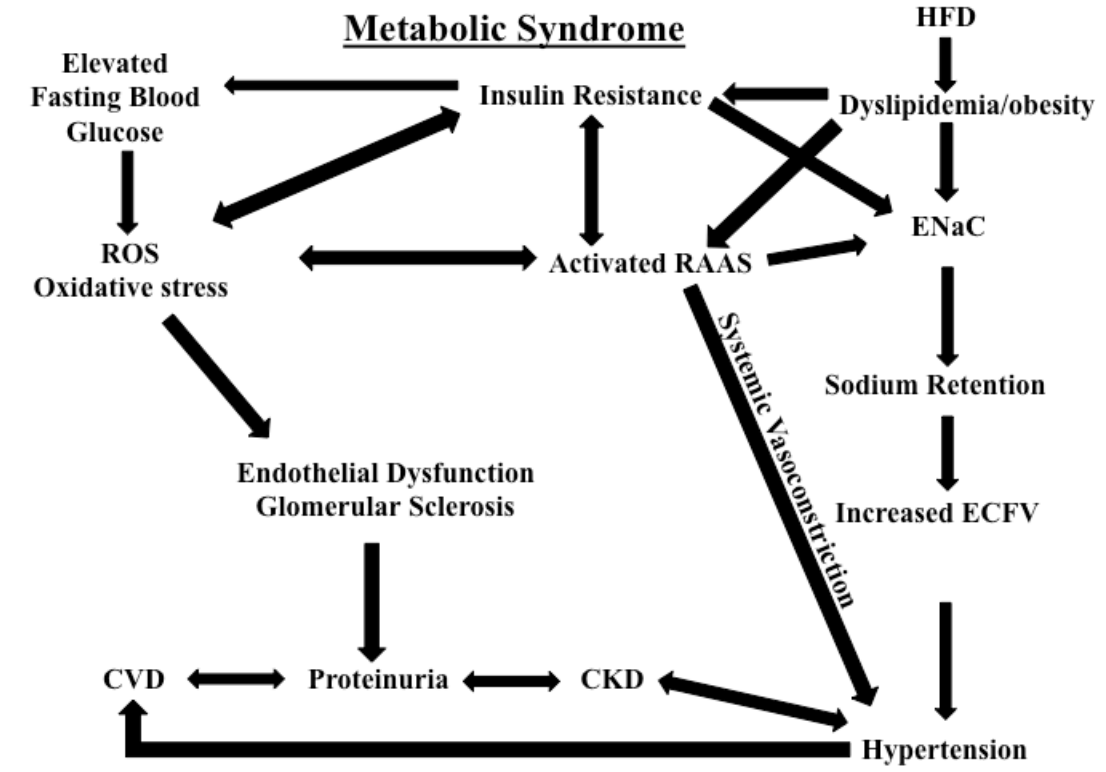

Figure 7

Figure 7. Schematic diagram of the relationship among Metabolic Syndrome (MetS), insulin resistance, hypertension, and cardiorenal disease. Abbreviations: HFD, high fat diet; ROS, reactive oxygen species; RAAS, renin-angiotensin aldosterone system; ENaC, epithelial sodium channel; ECFV, extracellular fluid volume; CVD, cardiovascular disease; CKD, chronic kidney disease.