Endothelin receptor antagonism improves glucose handling, dyslipidemia, and adipose tissue inflammation in obese mice

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ABSTRACT

Endothelin-1 (ET-1) is elevated in patients with obesity; however, its contribution to the pathophysiology related to obesity is not fully understood. We hypothesized that high ET-1 levels cause dyslipidemia, inflammation, and insulin resistance within the adipose tissue of obese mice. To test this hypothesis, male C57BL/6J mice were fed either normal diet (NMD) or high fat diet (HFD) for 8 weeks followed by 2 weeks of treatment with either vehicle, atrasentan (ET$_A$ receptor antagonist, 10mg/kg/day), or bosentan (ET$_A$/ET$_B$ receptor antagonist, 100mg/kg/day). Atrasentan and bosentan lowered circulating non-esterified free fatty acids and triglycerides seen in HFD mice, while atrasentan-treated mice had significantly lower liver triglycerides compared to non-treated HFD mice. ET-1 receptor blockade significantly improved insulin tolerance compared to insulin resistant HFD mice and lowered expression of genes in epididymal white adipose tissue (eWAT) associated with insulin resistance and inflammation. Flow cytometric analyses of eWAT indicated that HFD mice had significantly higher percentages of both CD4$^+$ and CD8$^+$ T cells compared to NMD mice, which was attenuated by treatment with atrasentan or bosentan. Atrasentan treatment also abolished the decrease in eosinophils seen in HFD mice. Taken together, these data indicate that ET$_A$ and ET$_A$/ET$_B$ receptor blockade improves peripheral glucose homeostasis, dyslipidemia, and liver triglycerides, and also attenuates the proinflammatory immune profile in eWAT of mice fed a HFD. These data suggest a potential use for ET$_A$ and ET$_A$/ET$_B$ receptor blockers in the treatment of obesity-associated dyslipidemia and insulin resistance.
Clinical Perspectives

1. Endothelin-1 is upregulated in patients with and animal models of obesity; however, its contribution to the pathophysiology related to obesity is unknown.

2. Blockade of ET-1 receptors improved insulin resistance, dyslipidemia, and adipose tissue inflammation in a mouse model of diet induced obesity.

3. ET-1 antagonists, which are currently FDA approved for the use in patients with pulmonary hypertension, could prove beneficial in reducing obesity related cardiovascular disease.

INTRODUCTION

Obesity is a disease that affects over 40 percent of the population of the United States. It dramatically increases the risk of mortality due to cardiovascular disease.\(^1\) Obesity is associated with alterations in lipid metabolism as well as the development of insulin resistance and metabolic syndrome. One potential contributor to the pathophysiology related to obesity is increased endothelin-1 (ET-1), both circulating and at the tissue level.\(^2\) ET-1 is a vasoactive peptide that is produced mainly by vascular endothelial cells but is also produced by several other cell types including adipocytes and immune cells. Two ET-1 receptor subtypes exist in mammalian species, ET\(_A\) and ET\(_B\).

The ET-1 gene and its cognate receptors arose during the development of vertebrates and play a key role in development of the jaw and enteric nervous system in vertebrates. Post-developmentally, ET-1 plays a crucial role in physiology, including regulation of blood pressure and vascular tone. Pathophysiologically, ET-1 promotes inflammation in
various diseases including hypertension, kidney disease, and diabetes.\textsuperscript{3} Data from our laboratory indicate that patients undergoing bariatric surgery have a 20% reduction in circulating ET-1 six months following vertical sleeve gastrectomy, thus implicating increased adiposity for the elevated serum ET-1 levels observed in patients with obesity. Furthermore, patients with higher circulating ET-1 had higher levels of macrophage chemoattractant protein-1, a marker of inflammation, in visceral adipose.\textsuperscript{4} Results from two clinical trials suggest that high levels of ET-1 promote dyslipidemia in patients with diabetic nephropathy\textsuperscript{5, 6}; however, the mechanisms are not yet understood. Therefore, ET-1 appears to play a major role in pathophysiology of obesity, and ET-1 receptors may be attractive targets to attenuate cardiovascular risk in patients with obesity.

Obesity causes a shift to a pro-inflammatory immune cell profile,\textsuperscript{2} especially observed in visceral adipose tissue.\textsuperscript{7-9} In addition, visceral adipose tissue abundance is highly correlated to cardiovascular disease risk, whereas other depots such as subcutaneous adipose have little or no correlation.\textsuperscript{10} In white adipose, obesity causes tissue to become hypoxic and immune cell populations become dysregulated, leading to an increase in macrophages, an increase in T lymphocytes and a decrease in eosinophils, among others.\textsuperscript{11} This shift in immune cell population promotes dysfunction with the adipose tissue, contributing to pathophysiology related to obesity. This includes insulin resistance at the level of the adipocyte as well as peripherally due to alterations in circulating adipokines, including insulin sensitizing adipokines such as adiponectin and adipisin. Given the importance of immune cells in modulating adipocyte function, more studies are needed to identify methods to target and improve inflammation within the adipose tissue.
A strong relationship between ET-1 and inflammation has been established in several models of disease including chronic kidney disease and sickle cell disease. ET-1 receptor antagonism has been shown to reduce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α). Given the relationship between ET-1, obesity, and inflammation, we hypothesize that upregulation of ET-1 within the eWAT of obese mice promotes an inflammatory immune cell profile which contributes to dyslipidemia and insulin resistance. The goal of the current study is to determine if ET-1 receptor antagonism attenuates dyslipidemia, insulin resistance and adipose tissue inflammation in the eWAT of mice chronically fed a Western diet.

MATERIALS AND METHODS

Animals

Male C57Bl/6J mice were purchased from Jackson Laboratories at 7 weeks of age and housed at the University of Mississippi Medical center Animal Facility under controlled light conditions (12-h light/12-h dark). Male mice were used for this study because in pilot experiments, female mice did not become insulin resistant after 8 weeks of high fat feeding (Supplemental Figure 2), which is consistent with other studies showing that female mice are protected from HFD-induce metabolic syndrome compared to males. Mice were habituated to the animal facility for one week upon arrival and fed ad libitum.

At 8 weeks of age, mice were randomized and individually housed into four groups; normal diet-fed (NMD; 12.6% kcal fat, 30% kcal carbohydrate, Envigo, TD.05230) (n=7), high fat diet-fed (HFD; 45% kcal fat, 42% kcal carbohydrate, Envigo TD.88137) (n=7), HFD atrasentan (HFD Atr) (n=5), and HFD bosentan (HFD Bos) (n=6) for 8 weeks. Mice
were then treated for two weeks with either vehicle (0.1% ethanol) atrasentan (10 mg/kg/day) (PepTech) or bosentan (100 mg/kg/day) (Alomone) administered through the drinking water. Atrasentan is a selective ET_{A} receptor antagonist, and bosentan is a non-selective antagonist of both ET_{A} and ET_{B} receptors. Doses were based on previous publications showing effectiveness.\textsuperscript{13, 14} Water intake was measured prior to experiments and during administration of treatment. Dosing was adjusted based on weekly water intake and growth. Food and water intake were measured every day until the end of treatment. Treatment was continued for a third week while insulin and glucose tolerance experiments were performed, with mice continued on their respective diets. Mice were euthanized after a 6 hour fast in clean cages to minimize coprophagia, and tissues were collected at the end of week 11. All assays were conducted on the same mice and on the same day. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

Cell Culture

3T3-L1 preadipocytes (ATCC) were plated at a density of 40,000/cm\(^{2}\) on 24-well plates and differentiation was induced at two days post-confluence by culturing cells in differentiation medium (33 \(\mu\)M biotin, 0.1 \(\mu\)M dexamethasone, 1 \(\mu\)M insulin, 200 \(\mu\)M indomethacin, 17 \(\mu\)M pantothenic acid, 10 \(\mu\)g/ml triiodothyronine, 1% FCS in DMEM/F12) for 3 days. Then, cells were cultured with DMEM/F12 for 6 days post-differentiation, after which they were treated in either normoxia (21% O\(_{2}\), 5% CO\(_{2}\)) or hypoxia (1% O\(_{2}\), 5% CO\(_{2}\)) for six hours with or without Hif1a inhibitor IDF-11774 (company) at concentrations
of 1µM, 25µM, and 50µM. Cells were then lysed in Tri Reagent (Zymo R2050) and RNA was extracted using Direct-Zol RNA microprep kit (Zymo R2052).

*Body composition analysis*

Lean mass, fat mass and total water composition was measured using Echo MRI (4-in-1 EchoMRI-900TM, Echo Medical System, Houston, TX) at weeks 0, 4, 8 and 10 while on diets.

*Liver triglyceride Content*

Livers were weighed and then homogenized in 5% NP-40 (Sigma-Aldrich). Hepatic triglyceride content was determined using commercially available colorimetric triglyceride quantification kit (BioVision, K622-100). Absorbance was read at a wavelength of 570nm (BioTek® Synergy H1) and triglyceride concentration was determined by standard curve and normalized to liver weight.

*Insulin and Glucose Tolerance.*

Mice were fasted for 6 hours prior to intraperitoneal insulin tolerance test (ITT) and oral glucose tolerance test (OGTT). Glucose was measured using a glucometer (Accu-Chek® Guide) via tail vein. For ITT, insulin (0.75 IU/kg of lean mass) was injected into the peritoneal cavity. For OGTT, 2 g/kg of dextrose was administered via oral gavage needle (22 gauge). Glucose was measured at baseline or time 0, 15, 30, 45, 60, and 120 minutes following insulin injection or glucose bolus. Tests were performed 3 days apart and similar to guidelines set forth by Benede-Ubieto et. al. Since only a small drop of blood was taken at each time point, 3 days rather than a week was allowed for recovery. Area under the curve was calculated using GraphPad Prism statistical software. Baseline was set at time=0 for each individual mouse.
Flow cytometric analyses of adipose tissue

Given the high correlation between visceral adipose tissue abundance, inflammation and cardiovascular disease risk in obesity, immune cell populations in epididymal white adipose tissue (eWAT) were assessed. eWAT is the most abundant visceral adipose tissue depot in mice. eWAT was homogenized in GentleMACS Octo Dissociator (Miltenyi Biotec). Briefly, ~1 g of adipose tissue was minced into ~5 mm pieces and placed in a GentleMACS C (Miltenyi Biotec) tube with 10 mL RPMI media containing 200 U/mL DNase and 10 mg/mL collagenase IV. The sample was homogenized using the manufacturer’s program designed for adipose tissue. The homogenate was then filtered through a 70 μm filter into a 50 mL tube and allowed to settle for 10 min to allow the adipocytes to separate from the stromal vascular fraction (SVF). The lower SVF was removed and transferred to a 15 mL tube and centrifuged for 10 min at 400 x g. The resulting cell pellet was resuspended in 3 mL of 1X PharmLyse (BD Biosciences) and incubated for 5 min at room temperature to lyse erythrocytes. Ten mL of 1X PBS, 2% FCS was added to the cells to wash followed by centrifugation at 350 x g for 5 min. Cells were then used for flow cytometry.

Briefly, cells were washed and resuspended in 1X PBS, 2% FCS, and 0.9% sodium azide at a concentration of 1 x 10^7 cells/mL. 1X10^6 cells (100 μL) were aliquoted into a flow cytometry tube and incubated with 0.25 μg of anti-mouse CD32/CD16 (FcR block, BD Biosciences) for 5 min. on ice. Cells were then stained with either isotype control antibodies or antibodies shown in Table 1 for 30 min on ice protected from light. All antibodies were diluted 1:200 in 1X PBS, 2% FCS, and 0.09% sodium azide. Samples were analyzed on an LSR II flow cytometer (BD Biosciences), and a total of 20,000
CD45+ cells were acquired for each sample. Data were analyzed using FCS Express 7 Software (De Novo Software).

**Digital droplet PCR gene expression**

Mouse tissue was collected, snap frozen in liquid nitrogen and stored at -80°C. Tissue was homogenized in TRI reagent® (Zymo, R2050-1-200) in 2ml lysing matrix D tubes (MP, 6913500). Total RNA was isolated from epidydimal white adipose tissue (eWAT) using Direct-zol™ RNA MiniPrep kit (Zymo, R2052). 1µg of RNA was converted to cDNA with iScript Reverse Transcription kit (BioRad Laboratories, Hercules, CA). Next, gene expression was carried out by droplet PCR. The PCR reaction was set up by manufacture’s recommendations using ddPCR probes Supermix (no dUTP) and one µL of TaqMan primer/probes (Applied Biosystems) and cDNA from 50 ng or RNA. To determine the effect of ET-1 receptor blockade on expression of several adipokines and PPARG (a major regulator of adiponectin expression), which are all altered in obesity, PCR was carried out using the following respective primer/probe sequences: Adiponectin (AdipoQ; Mm00456425_m1), Cfd/Adipsin (Mm01143935_g1), Leptin (Mm00434759_m1), Resistin (Mm00445641_m1) and Pparg (Mm00440940_m1). To characterize ET-1 and ET-1 receptor in adipose tissue of obese mice, the following primer/probes were used for PCR: Edn1 (Mm00438656_m1), EdnrA (Mm01243722_m1), EdnrB (Mm00432989_m1). In order to assess if adipose tissue is hypoxic in the current model of diet induced obesity, Hif1α (Mm00468869_m1) was measured. To determine the effect of ET-1 receptor blockade on cytokine expression, the following primers were used: Il12b (Mm01208835_m1), Il-6 (Mm00446190_m1), Tnf (Mm00443258_m1), Il-10 (Mm01288386_m1), Csf1 (Mm00432686_m1) from 50 ng of total RNA. The reaction mix
was separated into nanodroplets using the automated droplet generator (BioRad). PCR was carried out for 40 cycles per manufacturer’s instructions. Droplets were counted using the QX200 Droplet Reader and data was analyzed and copy count calculated using QuantaSoft software.

Biochemical analysis

Blood was collected under anesthesia via cardiac puncture using a 22g X 1 needle, placed in 1.5ml tubes coated with EDTA (0.5M), and immediately placed on ice. Blood was spun at 400 g for 15 minutes at 4° C to separate plasma. Blood chemistry (ALT, CHOL, HDL, LDL, NEFA, TRIG) was analyzed via Vet Axcel® chemistry analyzer (Alfa Wasserman). Plasma insulin and adiponectin concentrations were measured by mouse enzyme-linked immunoassay (ELISA) (Crystal Chem, 90080, 80569) according to the manufacturer’s protocols.

Randomisation and Statistics.

Although it is not possible to blind researchers from diet of mice, samples were blinded for all assays. All data are expressed as mean ± SEM. Data were tested for statistical significance by one-way ANOVA for one variable datasets or two-way repeated measure ANOVA (ITT and OGTT). Tukey’s post hoc test used to compare groups. P<0.05 was considered statistically significant. All graphs and statistical analyses were performed using GraphPad Prism.

RESULTS
ET-1 receptor blockade does not significantly affect body weight or fat mass of HFD-fed mice. C57BL/6J mice were fed a NMD or HFD for 10 weeks. As expected, male mice fed NMD had significantly lower body weight, fat mass, and lean mass compared to HFD fed mice. Female mice on HFD gained less body weight and fat mass over the course of the experiment compared to male mice (Supplemental Figure 2A and 2B). Further, there were no significant differences in fasting blood glucose, glucose tolerance, or insulin tolerance (Supplemental Figure 2D, 2E, and 2F) between NMD or HFD fed females; therefore, we proceeded with only male mice. There were no differences in body weight, lean mass, or fat mass between HFD, HFD+Atr and HFD+Bos mice throughout the experimental protocol (Fig. 1A-1C). In addition, there were no significant differences in total body water during treatment between any group (Fig. 1D). Finally, there were no detectable differences in caloric or water intake among all four treatment groups throughout the duration of the treatment, indicating that the differences in any endpoints were independent of caloric intake and hydration status (Fig. 1E and 1F).

ET-1 receptor blockade improves dyslipidemia in HFD-fed mice. Fasting plasma NEFA and triglyceride concentrations were increased in HFD-fed mice compared to NMD-fed mice (p=0.001 and p<0.0001 respectively). The increase in triglycerides and NEFA was attenuated in HFD-fed mice treated with atrasentan (p=0.005 and p=0.0003 respectively) or bosentan (p=0.06 and p=0.001 respectively; Fig. 2A and 2B). Similar to the circulating lipid profile, hepatic triglyceride content was significantly increased in HFD-fed mice compared to NMD (p<0.001). Atrasentan treated mice had lower hepatic triglyceride content compared to HFD-fed mice treated with vehicle
(p=0.009, Fig. 2C) while bosentan treatment had no significance difference in hepatic triglycerides (p=0.09). Total cholesterol was increased in HFD-fed mice compared to NMD-fed mice (p<0.0001), with no significant effect of treatment with either atrasentan or bosentan (Fig. 2D). There were no differences in HDL levels between NMD-fed mice and HFD-fed groups. There was, however, a significant increase in HDL in HFD+Bos (p=0.03) treated mice compared to HFD vehicle, but no statistical difference between HFD and HFD+Atr (p=0.08; Fig. 2E). LDL levels were higher all in all HFD-fed mice groups compared to NMD-fed mice (Fig. 2F).

**ET-1 receptor blockade reduces blood glucose and improves insulin tolerance in HFD-fed mice.**

We next determined whether ET-1 receptor blockade improved glucose and insulin tolerance in HFD-fed mice. After a 6 hour fast, HFD-fed mice exhibited significant hyperglycemia compared to NMD-fed mice (p<0.0001), an effect that was attenuated in mice treated with atrasentan or bosentan (p=0.07 and p=0.02 respectively; Fig. 3A). In addition, fasting plasma insulin concentration was increased in HFD fed mice compared to NMD (p=0.004), and there was no significant effect of treatment with atrasentan or bosentan (p=0.33 and p=0.81 respectively; Fig. 3B). As expected, HFD fed mice had significantly impaired glucose tolerance compared to NMD-fed mice evidenced by a significant increase in AUC (p=0.004; Fig. 3C and 3D). Treatment with atrasentan improved glucose tolerance with a significant reduction in AUC (p=0.04; Fig. 3C and 3D). Similarly, insulin tolerance was significantly impaired in HFD-fed mice compared to NMD (p=0.001; Fig. 3E and 3F). Treatment with atrasentan or bosentan improved insulin
tolerance compared to HFD mice indicated by a significant increase in AUC (p<0.0001 and p=0.01 respectively; Fig. 3E and 3F).

**ET-1 is upregulated in adipose tissue via Hif1α.**

ET-1 expression has been shown to increase in hypoxic environments, which occurs in the adipose tissue of patients with obesity and rodent models of obesity. In the current study, Hif1α mRNA, a marker of hypoxia, was significantly increased in eWAT of HFD fed mice compared to NMD fed mice (p<0.0001). Interestingly the increase in Hif1α mRNA was attenuated by 49% in mice treated with atrasentan (p=0.002; Fig. 4A).

In addition, ET-1 mRNA in eWAT was increased from 711 to 1086 copy counts/50 ng RNA in response to diet induced obesity (p=0.04; Fig. 4B). Atrasentan exacerbated the increase in ET-1 expression, although there was no detectable difference between vehicle and bosentan treated HFD fed mice. (p=0.95; Fig. 4B). Surprisingly, protein content had a negative correlation with mRNA expression, in that HFD fed mice had significantly lower eWAT ET-1 protein content compared to NMD mice, and atrasentan treatment reduced ET-1 content even more. This is likely due to increased ET-1 binding to ETB receptors. ETA receptor expression was significantly reduced in HFD-fed and atrasentan treated mice compared to NMD mice, while bosentan treated mice had no detectable difference in ETA mRNA expression compared to NMD mice. (Fig. 4D). There were no significant differences in ETB mRNA expression among all groups (Fig. 4E), although eWAT had higher gene expression levels of ETB mRNA compared to ETA mRNA in all groups.

To determine if ET-1 is elevated in response to hypoxia in adipocytes, 3T3-L1 fibroblast cells were differentiated into adipocytes and exposed to hypoxia. Hypoxia
induced a 3-fold increase in Hif1α mRNA (Fig. 4F) and this was associated with 4-fold increase in ET-1 mRNA. The increase in ET-1 was attenuated in a dose-dependent manner when cells were pretreated with the Hif1α inhibitor IDF-11774 (Fig. 4G) suggesting that Hif1α drives the increase in ET-1 expression in the adipose of obese mice.

**ET-1 receptor blockade improves hypoadiponectinemia in HFD-fed mice.**

Circulating plasma adiponectin was significantly decreased in HFD compared to NMD mice (p<0.0001), whereas treatment with atrasentan or bosentan attenuated the decrease in plasma adiponectin (p=0.02 for both treatments; Fig. 5A). Gene expression analysis of eWAT indicate a decrease in Pparg, a transcription factor that promotes adiponectin production, and AdipoQ mRNA. Further, Adipsin/Cfd and Retn, adipokines that are associated with improved insulin sensitivity and/or release, and an increase in Lep expression in HFD mice compared to NMD mice. Even though there was an increase in circulating adiponectin in atrasentan or bosentan treated mice compared to HFD mice, there was no significant differences in eWAT gene expression of any adipokines (Fig. 5B-5F).

**ET-1 receptor blockade attenuates increase in pro-inflammatory cells in epididymal white adipose tissue in HFD-fed mice.**

To determine the effect of ET-1 receptor blockade on adipose tissue inflammation, flow cytometry was performed on the stromal fraction following eWAT tissue dissociation. Representative gating strategies are available in Supplemental Figure 1. HFD-fed mice had a significant increase in both CD4+ T cells (p<0.0001) and CD8+ T cells (p=0.001) in eWAT compared to NMD fed mice. Bosentan treatment
significantly attenuated the increase in the CD4+ T cell population percentage (p=0.002; Fig. 6A), while atrasentan and bosentan treatment attenuated the increase in the CD8+ T cell population percentage (p=0.01 and p=0.02 respectively; Fig. 6B). In addition, the percentage of eosinophils was significantly reduced in obese mice (p=0.003). The reduction in eosinophils was attenuated in mice administered atrasentan and bosentan (p=0.004 and p=0.14 Fig. 6C). There were no differences in the percentages of NK1.1+ NK cells between NMD and HFD mice, but both atrasentan and bosentan treated mice had significantly lower levels of NK cells (p=0.006 and p=0.008 respectively vs. HFD; Fig. 6D). There were no differences in the percentages of CD45R+ B cells or CD11b+F4/80+ macrophages among the groups (Fig. 6E and 6F). HFD mice had increased gene expression levels of Tnfa (p=0.0006), IL-12b (p=0.001), IL-10 (p=0.001), IL-6 (p=0.04), and IL-1r (p=0.003) in eWAT compared to NMD mice. Treatment with atrasentan or bosentan attenuated the increase in expression of Tnfa (p=0.05 and p=0.0001 respectively) and IL-12b (p=0.04 for both treatments; Fig. 6G and 6H), while the increased expression for IL-10 was only significantly attenuated by atrasentan treatment (Fig. 6I). There were no significant differences in gene expression for IL-6 and IL-1r between treated and HFD-fed mice (Supplemental Figure 2).

**DISCUSSION**

Data from animal experiments and clinical trials suggest that ET-1 promotes several pathophysiological conditions related to obesity including dyslipidemia, insulin resistance, and inflammation.17, 18 In this study, male mice fed a HFD were treated with the ET<sub>A</sub> receptor blocker atrasentan or the nonselective ET<sub>A/ET<sub>B</sub> blocker bosentan for...
two weeks. The major findings of this study are that pharmacological blockade of either ET_A or both the ET_A and ET_B receptor improved glucose handling, insulin sensitivity, dyslipidemia, adipokine levels, and eWAT inflammation in HFD-fed mice, independent of changes in body weight or body composition. Overall, these results support the hypothesis that high ET-1, whether circulating or within the adipose tissue, promotes and exacerbates insulin resistance, dyslipidemia and eWAT inflammation in obesity.

Overweight and obese states are associated with higher circulating levels of ET-1 and increased ET-1 activity via ET_A receptor activation as evidenced by impairments in flow mediated dilation in a cohort of mixed male and female subjects. Sex differences in ET-1 production in obesity has not been extensively studied, although 17β-Estradiol administration reduces circulating ET-1 in postmenopausal women, suggestive of possible sex differences in obesity associated increases in ET-1. One potential source for elevated ET-1 in obesity is the adipose tissue which becomes hypoxic as adipose tissue expands, which could result in the upregulation of ET-1. Our group recently reported that circulating ET-1 concentration is reduced following drastic weight loss in patients who underwent vertical sleeve gastrectomy, suggesting the adipose tissue as the source of elevated ET-1 in obesity. In support of this concept, HFD-fed mice in the present study had a significant increase in Hif1-α mRNA in eWAT as compared to NMD-fed mice, and this was associated with a significant increase in ET-1 production. Further, hypoxia induced a 4-fold increase in ET-1 mRNA of 3T3-L1 adipocytes, and this was attenuated with pretreatment of a Hif1-α inhibitor. These data suggest that the most likely source of the increase in ET-1 in patients with obesity is the adipose tissue due to the relatively hypoxic environment. Although the adipocyte is likely partly responsible,
identifying the relative contribution of other cell types such as endothelial cells will require experiments using tissue-specific knockout animals.

A major finding of the current study is that $\text{ET}_A$ or dual $\text{ET}_A/\text{ET}_B$ receptor blockade significantly attenuated the increase in triglycerides and free fatty acids in plasma of HFD-fed mice. Two recent clinical trials aimed at determining if treatment with $\text{ET}_A$ receptor antagonist improves or delays the onset of diabetic nephropathy also showed efficacy in reducing circulating lipids, although this was not specifically done in subjects with obesity. Results from the Reducing Residual Albuminuria in Subjects with Diabetes and Nephropathy (RADAR) trial indicate that atrasentan significantly reduces circulating triglycerides and LDL cholesterol. This was followed up with similar results in a study published Farrah et. Al. showing sitaxentan, an $\text{ET}_A$ receptor antagonist significantly lowered circulating triglycerides and LDL cholesterol. Taken together, the data suggest an important role for ET-1 in promoting dyslipidemia in obesity and suggest that treatment with an $\text{ET}_A$ receptor antagonist may be beneficial to reduce cardiovascular risk in patients with obesity.

Hepatic steatosis and liver dysfunction are commonly associated with obesity. $\text{ET}_A$ receptor blockade significantly reduced HFD-induced hepatic triglyceride levels, whereas $\text{ET}_A/\text{ET}_B$ receptor blockade modestly reduced hepatic triglycerides by an average of 33%. It has been previously shown that $\text{ET}_A$ receptor blockade with ambrisentan has anti-fibrotic effects in the liver, and $\text{ET}_A/\text{ET}_B$ receptor blockade with bosentan (50mg/kg) has hepatoprotective effects in diabetic rats, to our knowledge, we are the first to show that endothelin receptor blockade is protective against hepatic triglyceride accumulation in a model of diet-induced obesity. Though not fully understood,
the beneficial effects of ET receptor blockade in HFD-induced dyslipidemia may be due to increased hepatic blood flow, which would allow more efficient transport of insulin and insulin sensitizing adipokines, such as adiponectin/adipsin, to the liver.\textsuperscript{31-34} This would in turn act to increase fatty acid oxidation and utilization of lipids, thereby decreasing hepatic lipid accumulation.

Numerous studies suggest a direct involvement of ET-1 signaling in the pathogenesis of hyperglycemia and insulin resistance;\textsuperscript{18, 35, 36} however, the contribution of ET-1 in promoting insulin resistance in obesity has yet to be tested. Our data indicate that pharmacological blockade with either an \(\text{ET}_A\) specific or dual \(\text{ET}_A/\text{ET}_B\) receptor antagonist improves insulin mediated glucose uptake and fasting blood glucose in rodents. Several potential receptor and tissue dependent mechanisms exist by which ET-1 may promote insulin resistance. One of the most likely mechanisms is through actions at the level of the adipose tissue. First, ET-1 mediates vascular function by acting as a potent vasoconstrictor via \(\text{ET}_A\) receptor activation.\textsuperscript{3} This contributes to hypoxia observed in the adipose tissue, because treatment with either atrasentan or bosentan partially attenuated the increase in Hif1-\(\alpha\) expression in eWAT of HFD-fed mice. A second potential mechanism by which ET-1 may promote insulin resistance is through activation of the \(\text{ET}_B\) receptor. Van Harmelen \textit{et. al} demonstrated that chronic activation of the \(\text{ET}_B\) receptor decreases insulin’s ability to inhibit lipolysis in human primary adipocytes, in addition to demonstrating a significantly higher \(\text{ET}_B\) to \(\text{ET}_A\) receptor ratio in human primary adipocytes.\textsuperscript{37} Gene expression data from this study also supports a higher \(\text{ET}_B\) to \(\text{ET}_A\) receptor ratio in the eWAT of mice, which suggests that ET-1 signaling in eWAT is primarily through the \(\text{ET}_B\) receptor, although protein expression analysis would be needed.

to confirm. Further, our lab recently showed that rats lacking functional ET$_B$ receptors have reduced fasting blood glucose and improved glucose and insulin tolerance.$^{18}$ Taken together, the current data are inconclusive on whether there is an improvement in insulin sensitivity at the level of the adipocyte following ET-1 receptor blockade, and more studies are warranted to definitively answer this question. A third potential mechanism is through a reduction in circulating FFA’s which are thought to cause insulin resistance in all insulin sensitive tissues, although there is still debate about whether levels seen in obesity cause insulin resistance.$^{38}$ Blockade of the ET$_A$ receptor decreased circulating FFA’s, possibly through a decrease in basal adipocyte lipolysis.$^{39, 40}$ In summary, activation of ET-1 receptors most likely causes insulin resistance through multiple mechanisms at several different insulin sensitive sites.

Individuals with metabolically-abnormal obesity exhibit deleterious changes in circulating adipokines including increased circulating leptin and reduced adiponectin and adipsin,$^{41}$ all of which are emulated in rodent models of diet induced obesity, including the current study. Adiponectin is an insulin-sensitizing adipokine whose circulating levels inversely correlate with obesity and insulin resistance.$^{42-44}$ In fact, hypoadiponectinemia is thought to be related to increased ET-1 production, although this has yet to be formally demonstrated.$^{45}$ Here we showed that treatment with both atrasentan and bosentan attenuated the hypoadiponectinemia induced by high fat feeding and improved adiponectin gene expression in eWAT, with ET$_A$/ET$_B$ receptor blockade showing the greatest improvements in gene expression (21% increase vs HFD). Studies in humans and rodents show that increasing either circulating or adipocyte specific levels of adiponectin are sufficient to ameliorate obesity induced insulin resistance$^{46, 47}$ suggesting
another contributing factor by which ET-1 receptor blockade improves in circulating lipids
and insulin sensitivity in the current model of diet induced obesity. In addition, Adipsin
gene expression in eWAT was increased with both ET<sub>A</sub> and ET<sub>A/ET<sub>B</sub></sub> receptor blockade.
Adipsin is an adipokine, also known as compliment factor D, that is highly expressed in
white adipose tissue. Circulating adipsin levels in humans correlate negatively with insulin
resistance, and in mice, adipsin improves β cell function in HFD-induced insulin
resistance.<sup>48, 49</sup> These data suggest that high circulating or adipose tissue levels of ET-1
during obesity, could impair adiponectin and adipsin production by adipocytes thereby
exacerbating peripheral insulin resistance in obesity.

Elevated visceral adipose in humans is major contributor to cardiovascular risk in
patients with obesity.<sup>10</sup> As adipose expands, the total number of immune cells increases
with the tissue, and the cells adopt a more proinflammatory phenotype. The immune cell
profile within adipose tissue plays an integral role in regulating lipid metabolism and
storage at the level of the adipocyte.<sup>50</sup> Briefly, lean adipose primarily is composed of M2-
like anti-inflammatory macrophages, eosinophils, regulatory T cells, and iNKT cells,
among others. In an obese state, however, the cells are markedly different, and are
primarily M1-like macrophages, CD4<sup>+</sup> Th1 and Th17 cells, and CD8<sup>+</sup> cytotoxic T
lymphocytes (CTL).<sup>51, 52</sup> In the present study, we measured relative percentages of
immune cell populations in the eWAT, an abundant visceral adipose depot in mice. We
observed increase percentages of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the eWAT in mice
administered HFD, which has been previously shown by other laboratories.<sup>45</sup> CD4<sup>+</sup> and
CD8<sup>+</sup> T cell percentages were significantly lower in mice that received bosentan, while
atrasentan caused a significant decrease CD8<sup>+</sup> T cell percentages. CD4<sup>+</sup>Th1 cells
secrete IFN-γ, which can disrupt insulin signaling in the adipose and could lead to or exacerbate insulin resistance systematically.^[53] TNF-α, which has been widely studied in obesity,^[54, 55] is also secreted by Th1 cells, among other cell types. Expression of TNF-α was elevated in eWAT of mice fed a HFD, but was decreased in mice that received atrasentan. Congruous with our results, previous studies have shown that blockade of the ET<sub>A</sub> receptor lowered TNF-α concentrations in humans and rats^[56, 57] although the exact mechanism has yet to be determined. Further studies are needed to characterize the Th subset dynamics in adipose tissue in response to endothelin antagonism. CD8<sup>+</sup> CTL infiltrate the adipose in obesity and accumulate, along with macrophages, in crown-like structures around dying adipocytes. These cells are important in the recruitment of macrophages, and their depletion decreases pro-inflammatory cytokine production and improves insulin sensitivity.^[58] We also detected increased IL-12 and IL-10 mRNA expression in eWAT of HFD-fed mice. IL-12 is a pro-inflammatory cytokine which is important for the polarization of CTL to an activated Tc1 phenotype.^[59] IL-10 is principally known as an anti-inflammatory cytokine that decreases the activity and/or expression of various proinflammatory cytokines and chemokines.^[60] IL-10 is upregulated in white adipose tissue during obesity, where it may have a deleterious role since knockdown of IL-10 in mouse adipose tissue protects from obesity and promotes thermogenesis.^[61]

A seeming contradiction observed in the current study is the apparent negative correlation between ET-1 mRNA and ET-1 peptide content in eWAT. Obesity increased ET-1 mRNA in eWAT, and ET<sub>A</sub> blockade increased ET-1 mRNA further, while protein content was proportionally lower. One potential reason is the increase in ET-1 receptor expression, especially that of the ET<sub>B</sub> receptor. ET<sub>B</sub> receptor content in the lungs is high
compared to most other tissues and is responsible for clearing ET-1 from the circulation by internalizing bound ET-1. This inverse relationship between ET-1 mRNA and protein levels has been observed previously in obese mice. In fact, obese mice fed a high fat diet had significantly lower peptide levels in the lung, even in mice that overexpress ET-1 in vascular endothelial cells. This is thought to be from upregulation of ET_B receptors in the lungs leading to increased clearance. We speculate the same phenomenon in the current study.

Eosinophils are necessary for proper adipocyte maturation and contribute to insulin sensitivity and glucose tolerance through the release of anti-inflammatory cytokines, such as interleukin-4 (IL-4), that promote macrophage polarization toward the M2 phenotype.

It has been previously shown that eosinophil cell numbers significantly decrease in adipose tissue in response to HFD feeding. Endothelin antagonism reversed the decrease in eosinophils in HFD-fed mice. Endothelin receptors are expressed on immune cells, including T cells and polymorphonuclear cells such as eosinophils, so it is unknown whether the alterations in immune cells in the adipose are an indirect result of changes in adipokine expression and glucose handling or due to direct effects of the antagonist on immune cells.

**Conclusions**

The current findings suggest an important role for ET-1 in promoting cardiometabolic risk in patients suffering from obesity. Currently, three ET-1 receptor antagonists have been approved for patients with pulmonary hypertension; however, there have been no clinical trials to determine their efficacy in reducing cardiovascular risk in obesity. It should be noted that these studies were carried out on male mice.
because of their earlier susceptibility to HFD-induced IR and dyslipidemia compared to females, and that further studies on the effectiveness of ET-1 receptor antagonists in ameliorating IR and dyslipidemia in obese female mice should be evaluated. Given their efficacy in reducing blood pressure, improving dyslipidemia and insulin sensitivity, and inflammation, ET-1 antagonists could prove beneficial in reducing obesity related cardiovascular disease.

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Data Availability:
All data will be made available upon request.

Figure Legends:
Table 1: Monoclonal antibodies used for flow cytometry.

Figure 1. ET-1 receptor blockade does not significantly affect body weight or fat mass of HFD-fed mice. Weight (A), percent fat mass (B) and lean mass (C) of NMD (n=7), HFD (n=7) HFD+Atr (n=5) and HFD+Bos (n=6) mice fed a NMD or HFD, measured on weeks 0, 4, 8 and 10. Total water was measured through weeks 8 and 10 (D). Food (kcal/day) (E) and water intake (ml/day) (F) was measured daily. A-D were analyzed by two-way ANOVA, and panels E and F were analyzed by one-way ANOVA with post-hoc Tukey’s test between individual groups. Data are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001.

Figure 2. ET-1 receptor blockade improves dyslipidemia in HFD-fed mice. Plasma analysis of Cholesterol (A), Triglycerides (B), HDL (C), LDL (D), and NEFA (E) of NMD (n=7), HFD (n=7), HFD+Atr (n=5), and HFD+Bos (n=6) mice after ten weeks on diets and two weeks of treatment with atrasentan or bosentan. Liver triglycerides were measured among all groups and normalized to liver weight (F). Data were analyzed by one-way ANOVA with Tukey’s post-hoc test for individual groups. E and F were analyzed by two-way ANOVA. Data are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 3. ET-1 receptor blockade reduces blood glucose and improves insulin tolerance in HFD-fed mice. *In vivo* glucose homeostasis was determined by OGTT (A) and IPITT (C) for of NMD (n=7), HFD (n=7), HFD+Atr (n=5), and HFD+Bos (n=6) mice after 10 weeks on diet and two weeks of treatment with atrasentan or bosentan. Area under the curve for GTT (B) and GTT (D). Fasting blood glucose was measured after a 6-hour fast (E). Plasma insulin was measured via ELISA (n=5 per group). Data in A, B, D, and F-H were analyzed by one-way ANOVA with Tukey's post-hoc test for individual groups.

Statistical analysis for panels A, B, D, F-H were done by one-way ANOVA with post-hoc Tukey's test between individual groups, and panels C and E were analyzed by two-way ANOVA with repeated measures. Data are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4. ET-1 is upregulated in adipose tissue via Hif1α. (A) Hif1α mRNA, (B) Pre-pro ET-1 mRNA, (C) ET-1 protein, (D) Ednra mRNA and (E) Ednrb mRNA from eWAT of mice after 10 weeks on diet and two weeks of treatment with atrasentan or bosentan were determined via ddPCR (n=5 per group). (F) Hif1α and (G) Pre-pro ET-1 gene expression from 3T3-L1 adipocytes exposed to 6 hours of normoxia or hypoxia in the presence of Hif1α inhibitor IDF-117744 (1µM, 25 µM, 50 µM) (n=6). Data from A-E were analyzed by one-way ANOVA with Tukey’s post-hoc test for individual groups. F and G were analyzed by two-way ANOVA. Data are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5. ET-1 receptor blockade improves hypoadiponectinemia in mice fed a HFD. Plasma adiponectin was analyzed via ELISA after 10 weeks on diet and two weeks of treatment with atrasentan or bosentan (A). Gene expression of AdipoQ, Pparγ, Adipsin/Cfd, Retn and Leptin from eWAT of mice after 10 weeks on diet and two weeks of treatment with atrasentan or bosentan were determined via ddPCR (B-F) (n=5 per group). Data were analyzed by one-way ANOVA with Tukey’s post-hoc test for individual groups. Data are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. ET-1 receptor blockade attenuates increase in pro-inflammatory cells in epididymal white adipose tissue in HFD-fed mice. Percent of total stromal fraction cells of CD4+, CD8+, eosinophils, NK, B and F480+ cells (A-F) (n=5 per group) obtained by flow cytometry from eWAT of mice after 10 weeks on diet and two weeks of treatment with atrasentan or bosentan. Gene expression of Tnfa(G), Il-12b(H) and Il-10 (I) from eWAT of mice after 10 weeks on diet and two weeks of treatment with atrasentan or bosentan were determined via ddPCR (n=5 per group). Data were analyzed by one-way ANOVA with Tukey’s post-hoc test for individual groups. Data are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
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| Antibody  | Supplier         | Clone   |
|----------|------------------|---------|
| CD32     | BD Biosciences   | 2.4G2   |
| CD45     | BD Biosciences   | 30-F11  |
| CD3      | BD Biosciences   | 145-2C11|
| CD4      | BD Biosciences   | GK1.5   |
| CD8α     | BD Biosciences   | 53-6.7  |
| CD45R    | BD Biosciences   | RA3-6B2 |
| NK1.1    | BD Biosciences   | PK136   |
| CD11b    | BD Biosciences   | M1/70   |
| F4/80    | BioLegend        | BM8     |
| Siglec-F | BD Biosciences   | E50-2440|
**Figure 1**

A-B: Weight (g) and % Fat Mass plots over 10 weeks on diet for NMD, HFD, HFD+Atr, and HFD+Bos.

C-D: Lean Mass (g) and Total Water plots over 10 weeks on diet for NMD, HFD, HFD+Atr, and HFD+Bos.

E-F: Calorie intake (kcal/day) and Water intake (ml/day) for NMD, HFD, HFD+Atr, and HFD+Bos.
Figure 2

A: Cholesterol (mg/dL)

B: Triglycerides (mg/dL)

C: HDL (mg/dL)

D: LDL (mg/dL)

E: NEFA (mEq/L)

F: Liver Triglycerides (mg/dL)
Figure 4

A

B

C

D

E

F

G

HIF1α mRNA (Copies/50ng RNA)

Pre-pro ET-1 mRNA (Copies/50ng RNA)

EdnA mRNA (Copies/50ng RNA)

EdnB mRNA (Copies/50ng RNA)

Hypoxia

IDF-11774

Control

1 µM

25 µM

50 µM

ET1 (pg/mg of fat)

Hypoxia

IDF-11774

Control

1 µM

25 µM

50 µM

IDF-11774

Control

1 µM

25 µM

50 µM

HIF1α mRNA (Copies/50ng RNA)

Pre-pro ET-1 mRNA (Copies/50ng RNA)
Figure 5

A
Plasma Adiponectin (ug/ml)

B
Adiponectin mRNA (Copies/50ng RNA)

C
Pparγ mRNA (Copies/50ng RNA)

D
Adipsin mRNA (Copies/50ng RNA)

E
Resistin mRNA (Copies/50ng RNA)

F
Leptin mRNA (Copies/50ng RNA)

NMD  HFD  HFD+  Atr  HFD+  Bos

0  5  10  15

0  50000  100000  150000

0  200000  300000  400000

0  30000  60000  90000  120000  150000

0  30000  60000  90000  120000  150000  180000  210000

0  2000  4000  6000  8000  10000

0  2000  4000  6000  8000  10000  12000  14000  16000  18000  20000

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****  ***  **  *  ****  ***  **  *
