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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, 10 mg/kg/day) or bosentan (ET_A/ET_B receptor antagonist, 100 mg/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 with non-treated HFD mice. ET-1 receptor blockade significantly improved insulin tolerance compared with 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 with 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 pro-inflammatory immune profile in eWAT of mice fed 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.

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Data Availability

All data will be made available upon request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

CRediT Author Contribution

Osvaldo Rivera-Gonzalez: Conceptualization, Data curation, Formal analysis, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration. **Natalie A. Wilson:** Data curation, Formal analysis, Investigation, Writing—review and editing. **Laura E. Coats:** Data curation, Investigation, Writing—review and editing. **Erin B. Taylor:** Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing—review and editing. **Joshua S. Speed:** Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing.

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 of 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 [3]. Data from our laboratory indicate that patients undergoing bariatric surgery have a 20% reduction in circulating ET-1 six months following vertical T-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 [4]. Results from two clinical trials suggest that high levels of ET-1 promote dyslipidemia in patients with diabetic nephropathy [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 [2], especially observed in visceral adipose tissue [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 [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 [11]. This shift in immune cell population promotes dysfunction within 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 adipsin. 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 [12]. ET-1 receptor antagonism has been shown to reduce pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α). Given the relationship among ET-1, obesity and inflammation, we hypothesize that up-regulation of ET-1 within the epididymal white adipose tissue (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 the present study because in pilot experiments, female mice did not become insulin resistant after 8 weeks of high fat feeding (Supplementary Figure S2), which is consistent with other studies showing that female mice are protected from HFD-induced metabolic syndrome compared with males. Mice were habituated to the animal facility for 1 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 2 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 [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 everyday 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 killed after a 6-h 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 40000/cm^2 on 24-well plates and differentiation was induced at 2 days post-confluence by culturing cells in differentiation medium (33 μM biotin, 0.1 μM dexamethasone, 1 μM insulin, 200 μM indomethacin, 17 μM pantothenic acid, 10 μg/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 6 h with or without *Hif1a* inhibitor IDF-11774 (company) at concentrations of 1, 25, and 50 μM. 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-900™, 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 570 nm (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 h 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 min following insulin injection or glucose bolus. Tests were performed 3 days apart and similar to guidelines set forth by Benede-Ubieto et al. [15] 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 individual mouse.

Flow cytometric analyses of adipose tissue

Given the high correlation between visceral adipose tissue abundance, inflammation and cardiovascular disease risk in obesity [10], immune cell populations in eWAT were assessed. eWAT is the most abundant visceral adipose tissue depot in mice. eWAT was homogenized in GentleMACS Octo Dissociator (Miltenyi Biotec). Briefly, ~1g of adipose tissue was minced into ~5 mm pieces and placed in a GentleMACS C (Miltenyi Biotec) tube with 10 ml RPMI medium 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×***g***. The resulting cell pellet was resuspended in 3 ml of 1× PharmLyse (BD Biosciences) and incubated for 5 min at room temperature to lyse erythrocytes. Ten milliliters of 1× PBS, 2% FCS was added to the cells to wash followed by centrifugation at 350×***g*** for5 min. Cells were then used for flow cytometry.

Briefly, cells were washed and resuspended in 1× PBS, 2% FCS, and 0.9% sodium azide at a concentration of 1×10^7 cells/ml. A total of 1×10^6 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 1× PBS, 2% FCS, and 0.09% sodium azide. Samples were analyzed on an LSR II flow cytometer (BD Biosciences), and a total of 20000 $CD45^+$ 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 2 ml lysing matrix D tubes (MP, 6913500). Total RNA was isolated from eWAT using Direct-zol™ RNA MiniPrep Kit (Zymo, R2052). One microgram of RNA was converted into cDNA with iScript Reverse Transcription kit (Bio-Rad Laboratories, Hercules, CA). Next, gene expression was carried out by droplet PCR. The PCR was set up by manufacturer's recommendations using ddPCR probes Supermix (no dUTP) and 1 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 (Bio-Rad). PCR was carried out for 40 cycles per manufacturer's instructions. Droplets were counted using the QX200 Droplet Reader and data were analyzed and copy count calculated using QuantaSoft software.

Biochemical analysis

Blood was collected under anesthesia via cardiac puncture using a 22g × 1 needle, placed in 1.5-ml tubes coated with EDTA (0.5 M), and immediately placed on ice. Blood was spun at 400×***g*** for 15 min 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.

Randomization 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 NMD or HFD for 10 weeks. As expected, male mice fed NMD had significantly lower body weight, fat mass, and lean mass compared with HFD-fed mice. Female mice on HFD gained less body weight and fat mass over the course of the experiment compared with male mice (Supplementary Figure S2A,B). Further, there were no significant differences in fasting blood glucose, glucose tolerance, or insulin tolerance (Supplementary Figure S2D–F) between NMD- and 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 (Figure 1A–C). In addition, there were no significant differences in total body water during treatment between any group (Figure 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 (Figure 1E,F).

ET-1 receptor blockade improves dyslipidemia in HFD-fed mice

Fasting plasma NEFA and triglyceride concentrations were increased in HFD-fed mice compared with 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; Figure 2A,B). Similar to the circulating lipid profile, hepatic triglyceride content was significantly increased in HFD-fed mice compared with NMD (P<0.001). Atrasentan-treated mice had lower hepatic triglyceride content compared with HFD-fed mice treated with vehicle (P=0.009, Figure 2C), while bosentan treatment had no significance difference in hepatic triglycerides (P=0.09). Total cholesterol was increased in HFD-fed mice compared with NMD-fed mice (P<0.0001), with no significant effect of treatment with either atrasentan or bosentan (Figure 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 with HFD vehicle, but no statistical difference between HFD and HFD+Atr (P=0.08; Figure 2E). LDL levels were higher all in all HFD-fed mice groups compared with NMD-fed mice (Figure 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-h fast, HFD-fed mice exhibited significant hyperglycemia compared with 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; Figure 3A). In addition, fasting plasma insulin concentration was increased in HFD fed mice compared with NMD (P=0.004), and there was no significant effect of treatment with atrasentan or bosentan (P=0.33 and P=0.81, respectively; Figure 3B). As expected, HFD-fed mice had significantly impaired glucose tolerance compared with NMD-fed mice evidenced by a significant

increase in AUC (P=0.004; Figure 3C,D). Treatment with atrasentan improved glucose tolerance with a significant reduction in AUC (P=0.04; Figure 3C,D). Similarly, insulin tolerance was significantly impaired in HFD-fed mice compared with NMD (P=0.001; Figure 3E,F). 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; Figure 3E,F).

ET-1 is up-regulated 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 [16], was significantly increased in eWAT of HFD-fed mice compared with NMD-fed mice (P<0.0001). Interestingly, the increase in *Hif1α* mRNA was attenuated by 49% in mice treated with atrasentan (P=0.002; Figure 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; Figure 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; Figure 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 with NMD mice, and atrasentan treatment reduced ET-1 content even more. This is likely due to increased ET-1 binding to ET_B receptors. ET_A receptor expression was significantly reduced in HFD-fed and atrasentan-treated mice compared with NMD mice, while bosentan-treated mice had no detectable difference in ET_A mRNA expression compared with NMD mice (Figure 4D). There were no significant differences in ET_B mRNA expression among all groups (Figure 4E), although eWAT had higher gene expression levels of ET_B mRNA compared with ET_A 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 three-fold increase in *Hif1α* mRNA (Figure 4F) and this was associated with four-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 (Figure 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 with NMD mice (P<0.0001), whereas treatment with atrasentan or bosentan attenuated the decrease in plasma adiponectin (P=0.02 for both treatments; Figure 5A). Gene expression analysis of eWAT indicates a decrease in *Pparg*, a transcription factor that promotes adiponectin production, and *AdipoQ* mRNA. Further, there was a reduction in *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 with NMD mice. Even though there was an increase in circulating adiponectin in atrasentan or bosentan-treated mice compared with HFD mice, there was no significant differences in eWAT gene expression of any adipokines (Figure 5B–F).

ET-1 receptor blockade attenuates increase in pro-inflammatory cells in eWAT 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 dissociation. Representative gating strategies are available in Supplementary Figure S1. 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 with NMD-fed mice. Bosentan treatment significantly attenuated the increase in the $CD4^+$ T cell population percentage ($P=0.002$; Figure 6A), while atrasentan and bosentan treatment attenuated the increase in the $CD8^+$ T cell population percentage ($P=0.01$ and $P=0.02$, respectively; Figure 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 $P=0.14$; Figure 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; Figure 6D). There were no differences in the percentages of $CD45R^+$ B cells or $CD11b^+F4/80^+$ macrophages among the groups (Figure 6E,F). 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-1*r ($P=0.003$) in eWAT compared with 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; Figure 6G,H), while the increased expression for *Il-10* was only significantly attenuated by atrasentan treatment (Figure 6I). There were no significant differences in gene expression for *Il-6* and *Il-1r* between treated and HFD-fed mice (Supplementary Figure S2).

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_A receptor blocker atrasentan or the non-selective ET_A/ET_B blocker bosentan for 2 weeks. The major findings of the present 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 [19,20]. Sex differences in ET-1 production in obesity has not been extensively studied, although 17β-estradiol administration reduces circulating ET-1 in post-menopausal women, suggestive of possible sex differences in obesity associated increases in ET-1 [21]. 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 up-regulation of ET-1 [22,23]. 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 [24]. In support of this concept, HFD-fed mice in the present study had a significant increase in Hif1-α mRNA in eWAT as compared with NMD-fed mice, and this was associated with a significant increase in ET-1 production. Further, hypoxia induced a four-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 ET_A or dual ET_A/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 an ET_A receptor antagonist improves or delays the onset of diabetic nephropathy also showed efficacy in reducing circulating lipids [17], 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 [25]. This was followed up with similar results in a study published by Farrah et al. showing sitaxentan, an ET_A receptor antagonist, significantly lowered circulating triglycerides and LDL cholesterol [26]. Taken together, the data suggest an important role for ET-1 in promoting dyslipidemia in obesity and suggest that treatment with an 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 [27,28]. ET_A receptor blockade significantly reduced HFD-induced hepatic triglyceride levels, whereas ET_A/ET_B receptor blockade modestly reduced hepatic triglycerides by an average of 33%. It has been previously shown that ET_A receptor blockade with ambrisentan has antifibrotic effects in the liver [29], and ET_A/ET_B receptor blockade with bosentan (50 mg/kg) has hepatoprotective effects in diabetic rats [30]; however, 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 [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 [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 ET_A specific or dual ET_A/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 ET_A receptor activation [3]. This contributes to hypoxia observed in the adipose tissue, because treatment with either atrasentan or bosentan partially attenuated the increase in Hif1-α expression in eWAT of HFD-fed mice. A second potential mechanism by which ET-1 may promote insulin resistance is through activation of the ET_B receptor. van Harmelen et al. demonstrated that chronic activation of the ET_B receptor decreases insulin's ability to inhibit lipolysis in human primary adipocytes, in addition to demonstrating a significantly higher ET_B to ET_A receptor ratio in human primary adipocytes [37]. Gene expression data from this study also support a higher ET_B to ET_A receptor ratio in the eWAT of mice, which suggests that ET-1 signaling in eWAT is primarily through the 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 FFAs 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 FFAs, 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_A and ET_A/ET_B 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 [48,49]. 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 a major contributor to cardiovascular risk in patients with obesity [10]. As adipose expands, the total number of immune cells increases with the tissue, and the cells adopt a more pro-inflammatory phenotype. The immune cell profile within adipose tissue plays an integral role in regulating lipid metabolism and storage at the level of the adipocyte [50]. 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, $CD4^+$ Th1 and Th17 cells, and $CD8^+$cytotoxic T lymphocytes (CTLs) [51,52]. 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 $CD4^+$ and $CD8^+$ T cells in the eWAT in mice administered HFD, which has been previously shown by other laboratories [45]. $CD4^+$ and $CD8^+$ T cell percentages were significantly lower in mice that received bosentan, while atrasentan caused a significant decrease in $CD8^+$ T cell percentages. $CD4^+$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 HFD, but was decreased in mice that received atrasentan. Congruous with our results, previous studies have shown that blockade of the ET_A receptor lowered TNF-α concentrations in humans and rats [56,57], although the exact mechanism is yet to be determined. Further studies are needed to characterize the Th subset dynamics in adipose tissue in response to endothelin antagonism. $CD8^+$ CTLs 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 interleukin (IL) 12 (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 pro-inflammatory cytokines and chemokines [60]. IL-10 is up-regulated in white adipose tissue during obesity, where it may havea 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_A 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_B receptor. ET_B receptor content in the lungs is high compared with 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 HFD had significantly lower peptide levels in the lung, even in mice that overexpress ET-1 in vascular endothelial cells [62]. This is thought to be from up-regulation 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 IL-4, that promote macrophage polarization toward the M2 phenotype [63,64]. It has been previously shown that eosinophil cell numbers significantly decrease in adipose tissue in response to HFD feeding [65]. Endothelin antagonism reversed the decrease in eosinophils in HFD-fed mice. Endothelin receptors are expressed on immune cells [66], including T cells and polymorphonuclear cells such as eosinophils [67], 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 with 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CTL	cytotoxic T lymphocyte
ET-1	endothelin-1
eWAT	epididymal white adipose tissue
HFD	high-fat diet
IL	interleukin
ITT	insulin tolerance test
NMD	normal diet

OGTT	oral glucose tolerance test
SVF	stromal vascular fraction
TNF-α	tumor necrosis factor α

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Clinical perspectives

- ET-1 is up-regulated in patients with and animal models of obesity; however, its contribution to the pathophysiology related to obesity is unknown.
- Blockade of ET-1 receptors improved insulin resistance, dyslipidemia, and adipose tissue inflammation in a mouse model of diet-induced obesity.
- 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.

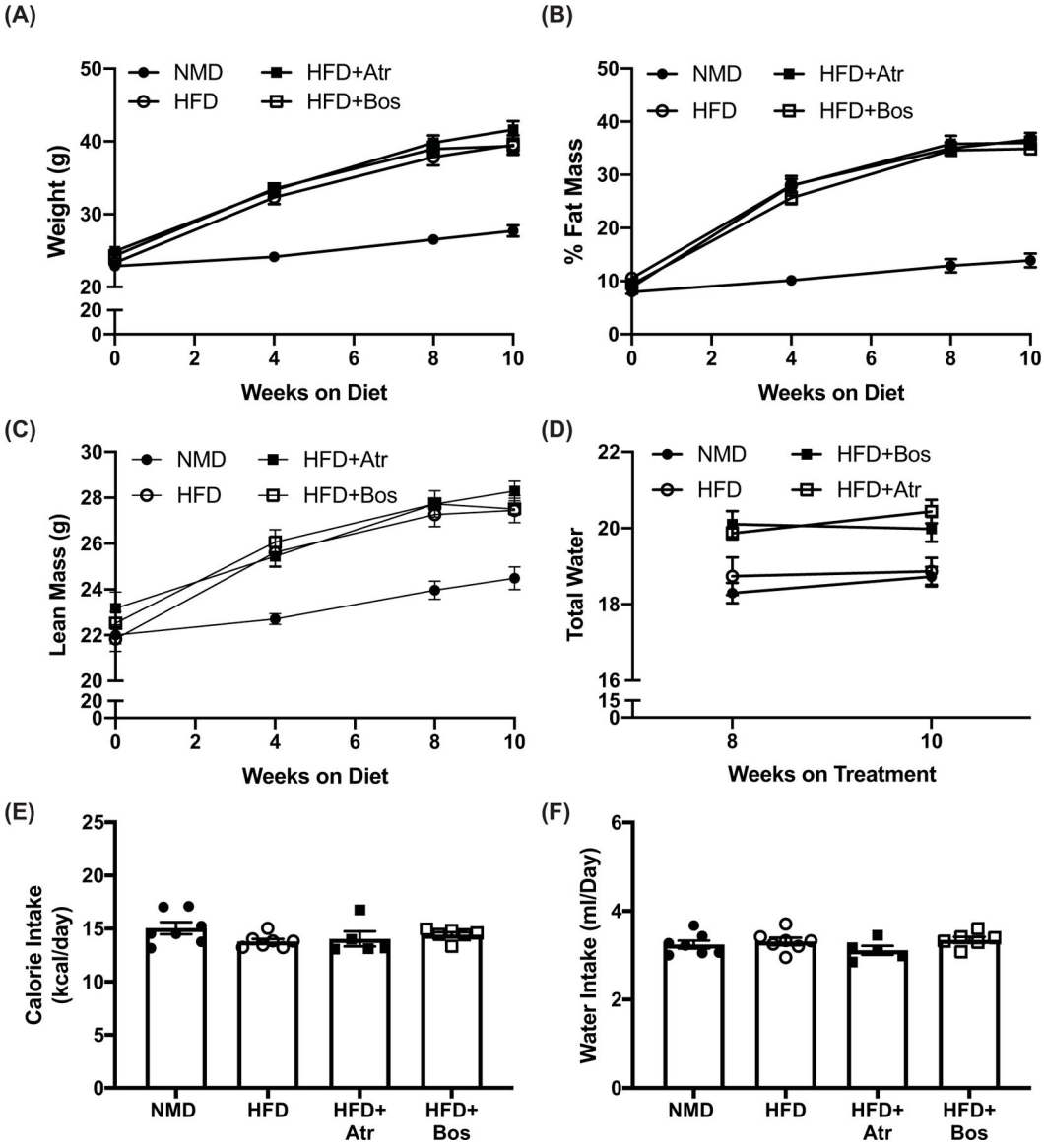

Figure 1. ET-1 receptor blockade does not significantly affect body weight or fat mass of HFD-fed mice

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 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,F) were analyzed by one-way ANOVA with post-hoc Tukey's test between individual groups. Data are expressed as mean ± SEM.

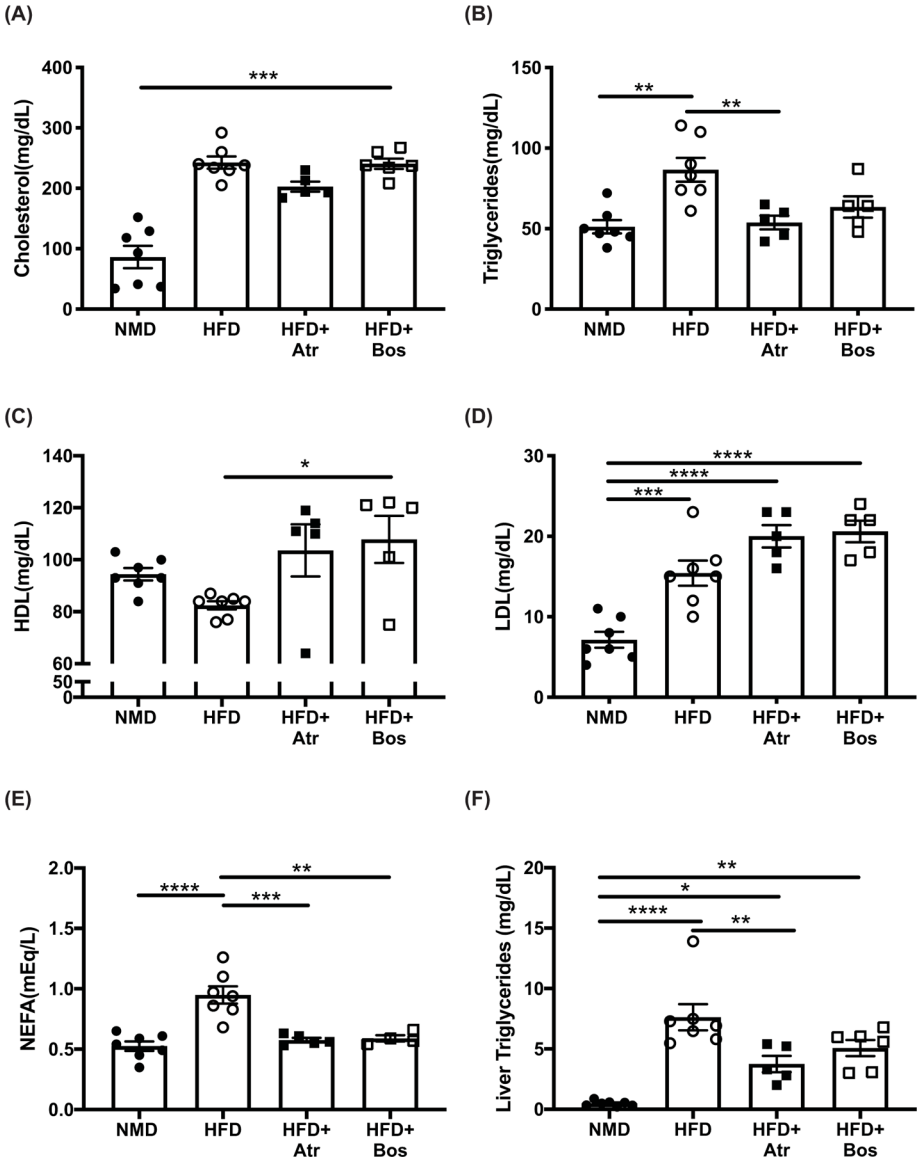

Figure 2. ET-1 receptor blockade improves dyslipidemia in HFD-fed mice

Plasma analysis of Cholesterol (**A**), Triglycerides (**B**), HDL (**C**), LDL (**D**), and NEFA (**E**) in NMD (n=7), HFD (n=7), HFD+Atr (n=5), and HFD+Bos (n=6) mice after 10 weeks on diets and 2 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,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.

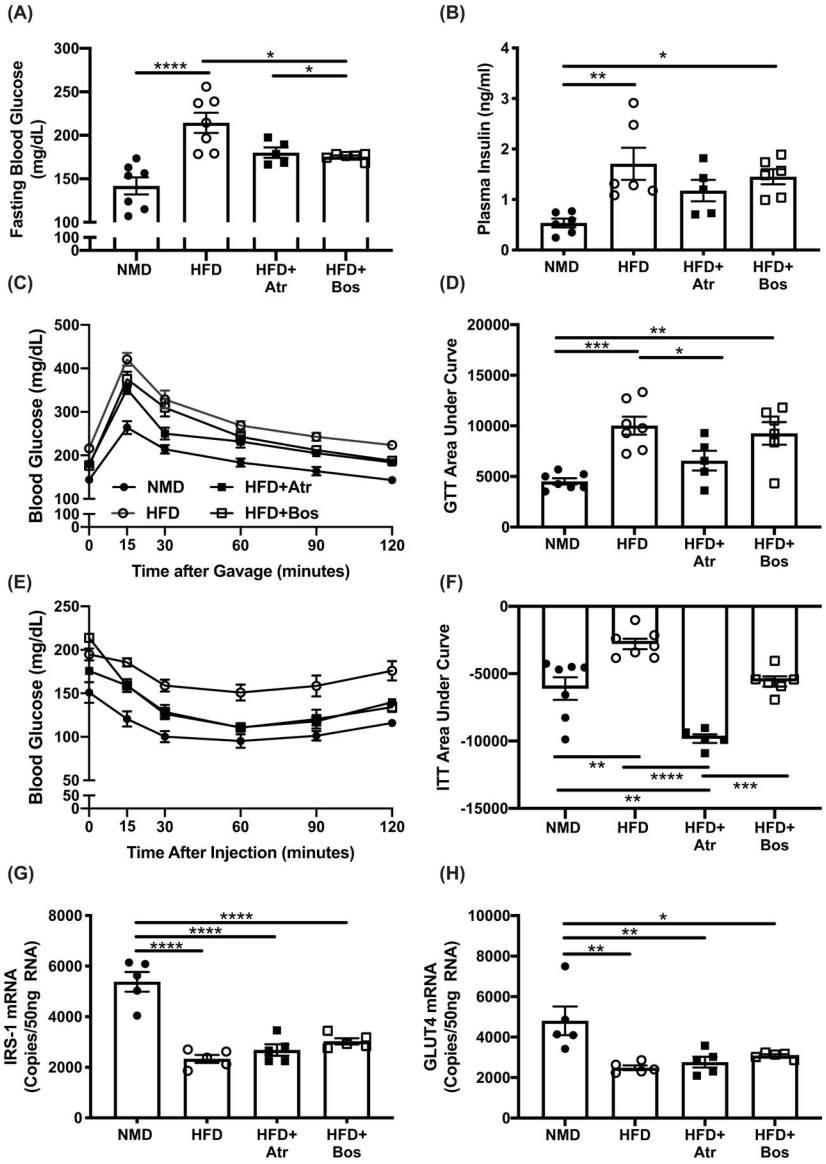

Figure 3. ET-1 receptor blockade reduces blood glucose and improves insulin tolerance in HFD-fed mice

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 2 weeks of treatment with atrasentan or bosentan. Area under the curve for plasma insulin (**B**) and GTT (**D**). Fasting blood glucose was measured after a 6-h fast (**E**). Plasma insulin was measured via ELISA (n=5 per group). Data in (A,B,D,**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,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.

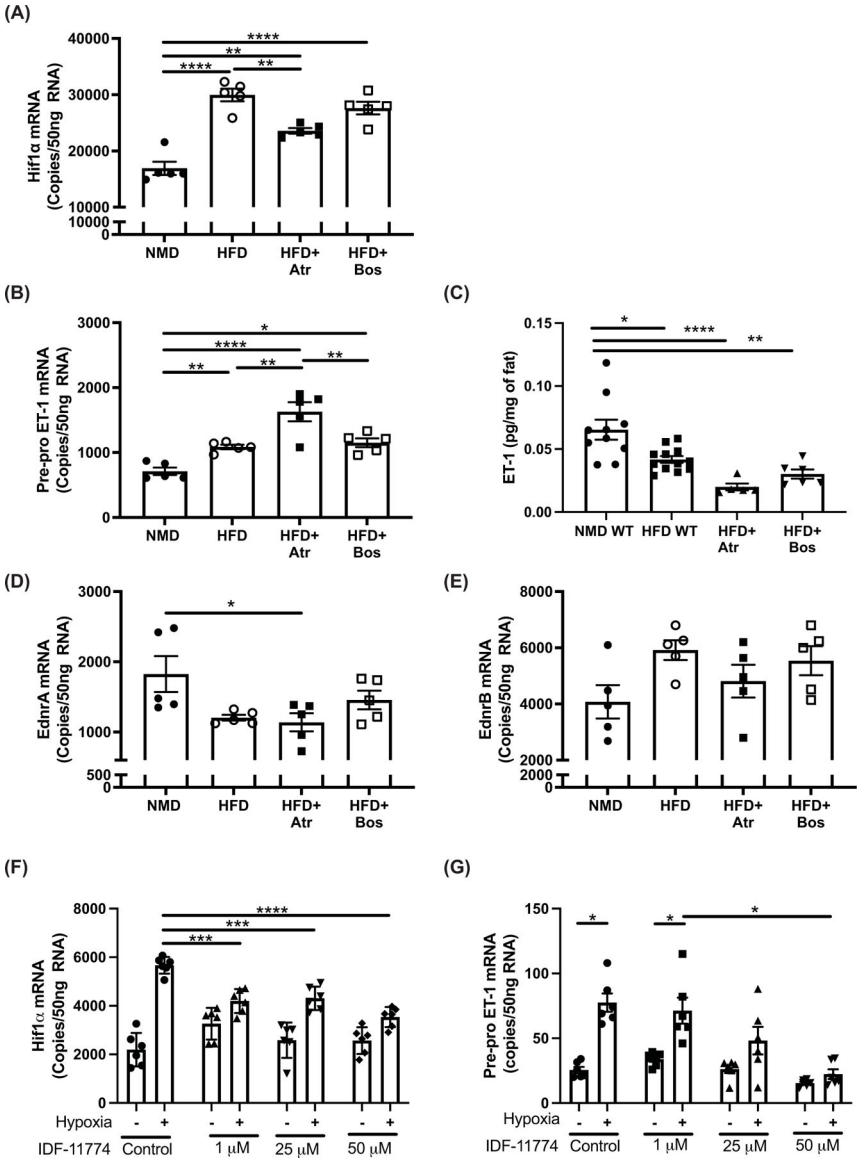

Figure 4. ET-1 is up-regulated in adipose tissue via *Hif1α*

(**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 2 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 h of normoxia or hypoxia in the presence of *Hif1α* inhibitor IDF-117744 (1, 25, 50 μM) (n=6). Data from (A–E) were analyzed by one-way ANOVA with Tukey's post-hoc test for individual groups. (F,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.

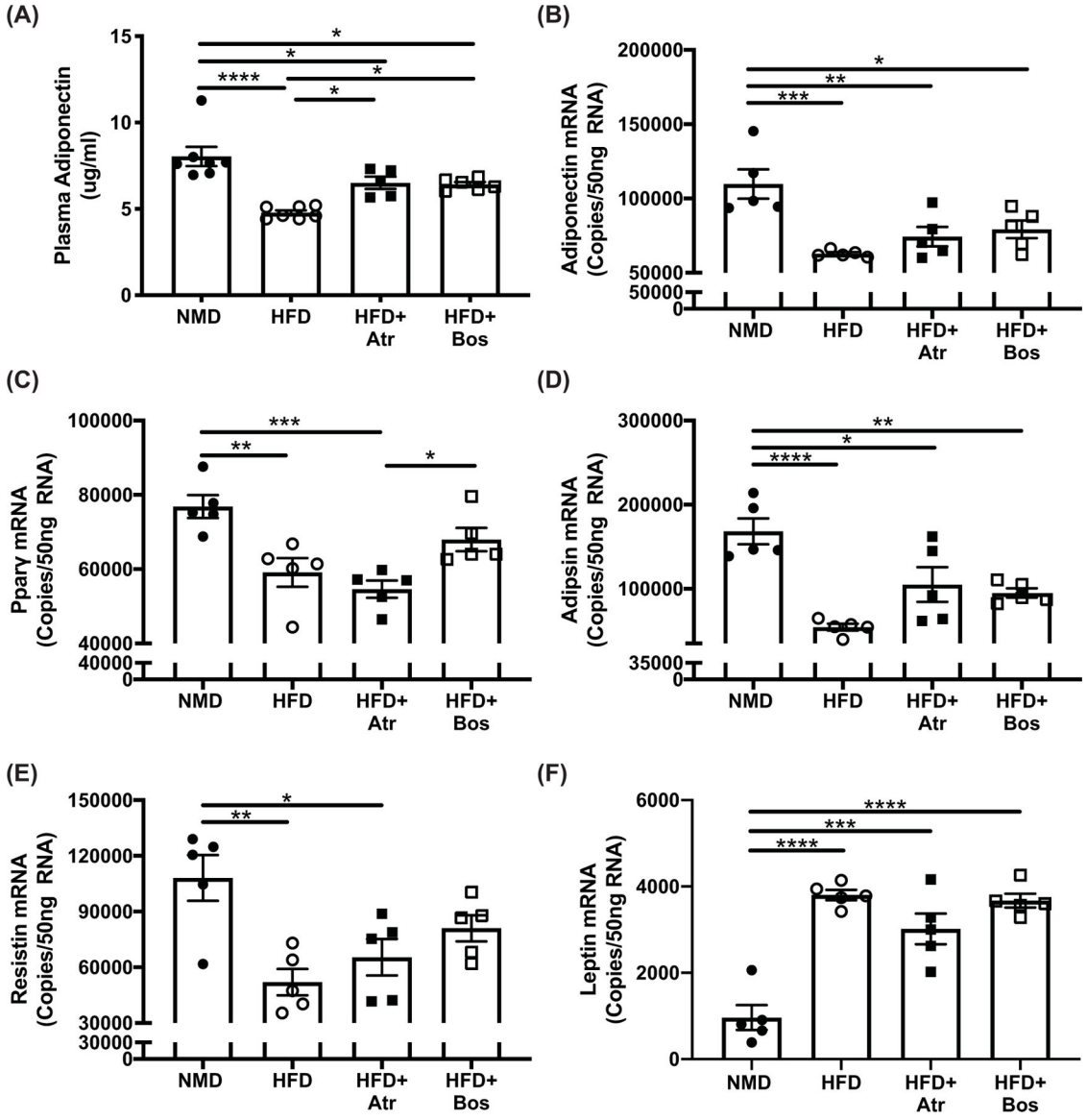

Figure 5. ET-1 receptor blockade improves hypoadiponectinemia in mice fed a HFD

Plasma adiponectin was analyzed via ELISA after 10 weeks on diet and 2 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 2 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.

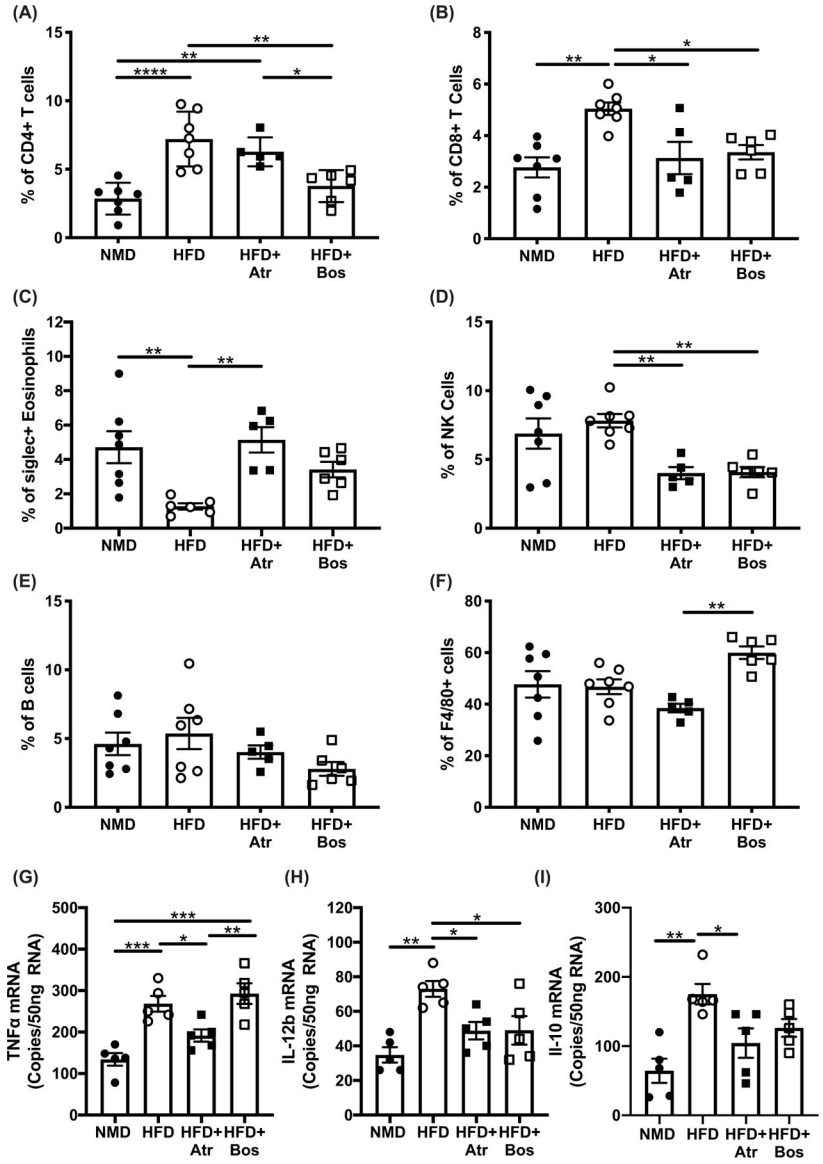

Figure 6. ET-1 receptor blockade attenuates increase in pro-inflammatory cells in eWAT in HFD-fed mice

Percent of total stromal fraction cells of $CD4^+$, $CD8^+$, eosinophils, NK, B, and $F480^+$ (**A–F**) (n=5 per group) obtained by flow cytometry from eWAT of mice after 10 weeks on diet and 2 weeks of treatment with atrasentan or bosentan. Gene expression of *Tnfα* (**G**), *Il-12b* (**H**), and *Il-10* (**I**) from eWAT of mice after 10 weeks on diet and 2 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.

Table 1

Monoclonal antibodies used for flow cytometry

Antibody	Supplier	Clone
CD32	BD Biosciences	2.4G2
CD45	BD Biosciences	30-F11
CD3	BD Biosciences	145–2C11
CD4	BD Biosciences	GK1.5
CD8a	BD Biosciences	53–6.7
CD45R	BD Biosciences	RA3–682
NK1.1	BD Biosciences	PK136
CD11b	BD Biosciences	M1/70
F4/80	BioLegend	BM8
Siglec-F	BD Biosciences	E50–2440