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Loss of endothelin type B receptor function improves insulin sensitivity in rats

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Abstract

High salt intake (HS) is associated with obesity and insulin resistance. ET-1, a peptide released in response to HS, inhibits the actions of insulin on cultured adipocytes through ET-1 type B (ETB) receptors; however, the in vivo implications of ETB receptor activation on lipid metabolism and insulin resistance is unknown. We hypothesized that activation of ETB receptors in response to HS intake promotes dyslipidemia and insulin resistance. In normal salt (NS) fed rats, no significant difference in body weight or epididymal fat mass was observed between control and ETB deficient rats. After 2 weeks of HS, ETB def rats had significantly lower body weight and epididymal fat mass compared to controls. Non-fasting plasma glucose was not different between genotypes, however plasma insulin concentration was significantly lower in ETB deficient rats compared to controls suggesting improved insulin sensitivity. In addition, ETB deficient rats had significantly higher circulating free fatty acids in both NS and HS groups, with no difference in plasma triglycerides between genotypes. In a separate experiment, ETB deficient rats had significantly lower fasting blood glucose and improved glucose and insulin tolerance compared to controls. These data suggest that ET-1 promotes adipose deposition and insulin resistance via the ETB receptor.

Introduction

Global dietary sodium intake as of 2010 has increased to ~3.95 g/day, nearly twice the recommended limit of 2 g/day established by the World Health Organization (Powles et al. 2013). Excess sodium intake raises blood pressure (BP) (Sacks et al. 2001), a major risk factor for cardiovascular disease (Aburto et al. 2013; Lim et al. 2012), stomach cancer (Ferlay et al. 2010), and renal disease. More recently, high dietary sodium has been associated with obesity and insulin resistance, a precursor for type 2 diabetes mellitus, independent of caloric intake (Grimes et al. 2016; Kang et al. 2016). Notably, mechanisms

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Disclosures

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by which high salt (HS) intake may promote obesity and insulin resistance are severely under-studied.

Factors associated with sodium homeostasis contribute to lipid metabolism and overall metabolic balance. Endothelin-1 (ET-1), a 21-amino acid peptide, is upregulated during chronic HS intake (Speed et al. 2015; Tsai et al. 2006). In response to chronic HS intake, ET-1 is thought to maintain vascular tone through vasoconstrictive and vasodilatory actions. This is mediated through endothelin type A and type B receptors (ET_A/ET_B) on vascular smooth muscle cells and endothelial cells respectively (Ogawa et al. 1991; Rubanyi and Polokoff 1994). In addition, renal ET-1 promotes natriuresis by inhibiting Na^+ transport via the ETB receptor. Although ET-1 is secreted mainly by endothelial cells (Kisanuki et al. 2010), it is also produced in a variety of cells, including vascular smooth muscle cells, cardiomyocytes, macrophages, leukocytes, fibroblasts, and adipocytes (Juan et al. 2007; Rubanyi and Polokoff 1994). ET-1 acts through two receptors, ETA and ETB , which are expressed throughout most organs and tissues within the body, including thoses associated with lipid metabolism and inuslin signling, therefore the potential that ET-1 contributes to metabolic function are high.

The endothelin system has recently been implicated in lipid metabolism. High levels of circulating ET-1 have been found in both obese and diabetic individuals (Ferri et al. 1997; Schneider et al. 2002). *In vitro* studies determined that ET-1 acting on the ET_A receptor causes lipolysis in differentiated adipocyte-like 3T3-L1 cells (Eriksson et al. 2009). In addition, ET-1 has been shown to inhibit insulin-stimulated glucose uptake in cultured adipocytes, mediated via the ET_B receptor (Chou et al. 1994; Juan et al. 2007; Ottosson-Seeberger et al. 1997). Therefore, we hypothesized that activation of ETB receptors in response to HS intake promotes dyslipidemia and insulin resistance, and the goal of the current study was to determine if loss of ET_B receptor function alters metabolic parameters, including body weight (BW), adiposity, plasma insulin, triglycerides, and glucose.

Materials and Methods

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved all protocols in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male endothelin B deficient (ET_B def) or transgenic control (control) littermates (11-12 weeks of age) were obtained from our in-house colony. This colony was produced from the spotting lethal rat, which has a naturally occurring mutation in the ET_B receptor gene rendering a non-functional protein. These animals did not survive long after birth due to abnormal formation of the enteric nervous system that lead to megacolon; however, Gariepy et al were able to “rescue” the model by inserting a human ET_B receptor transgene under the control of the dopamine β hydroxylase promoter. Therefore, expression of functional ET_B receptors is only present in sympathetic tissue (Gariepy et al. 2000). ET_B def rats have a 10-fold elevation in plasma ET-1 and a reduced ET_A receptor binding, at least in the kidney (Taylor et al. 2003). Animals were housed in temperature and humidity controlled rooms with 12:12 hour light:dark cycles and were allowed food and water *ad libitum*.

Protocol 1.

11-12 week old animals were placed on sodium adjusted Envigo diet containing either normal salt (NS; 0.4% NaCl) or high salt intake (HS; 4% NaCl) for 14 days. On the final day, animals were euthanized in 4-hour increments beginning at the time in which the lights turned on (ZT0). Plasma was collected in heparinized tubes and samples were collected and flash frozen in liquid nitrogen. In a subset of animals epididymal fat pads were excised and weighed to get an estimate of adiposity as a percentage of body weight. Plasma free fatty acids, glucose, and triglycerides were measured by the Metabolic Core at UAB.

Protocol 2.

12-week old ET_B def and control littermates that were maintained on normal chow from the animal facilities at UAB were used. Fasted animals were either subjected to an intraperitoneal insulin tolerance test (IPTT) in which 0.5 IU/kg insulin was injected IP at time 0 or an IP glucose tolerance test in which 2 mg/kg dextrose (50% solution) was injected IP at time 0. Blood glucose was measured by a glucometer (Abbott Laboratories) through a drop of blood from a tail prick.

Statistics.

For comparisons of two groups (i.e. fasting blood glucose), data were analyzed by Students t-test. For two variable data (i.e. genotype and diet), data were analyzed by two-way analysis of variance followed by Tukey's post hoc test. All data were analyzed using GraphPad Prism version 8. Statistical significance was set at α=0.05.

Results.

14-week old ET_B def rats maintained on NS had similar body weight compared to littermate controls (Figure 1A). When placed on HS diet for 2 weeks, ET_B def rats had significantly lower body weight than littermate controls (Figure 1A, 298 ± 6 vs. 253 ± 4 grams, control vs. ET_B def; $p<0.0001$ by post hoc analysis). In control animals maintained on NS, there was no detectable difference in epididymal fat mass relative to body weight (Figure 1B, 1.4 ± 0.06 vs. 1.5 ± 0.06 *%* of body weight, control vs. ET_B def, p=not significant). However, ET_B def rats fed HS for 2 weeks had significantly lower epididymal fat mass compared to ET_B def rats maintained on NS (Figure 1B, 1.5 ± 0.05 vs. 1.2± 0.06 % of body weight, control vs. ET_B def; $p<0.05$). These data suggest that high salt intake reduces lipid storage or increases lipid utilization in rats lacking ETB receptors.

To determine the effects of ETB receptor deficiency on glucose metabolism, plasma glucose and insulin were measured in 4-hour intervals (Figure 2). There were no detectable differences in plasma glucose between control and ET_B def rats whether maintained on NS or HS. As expected, NS fed animals had a circadian rhythm in plasma glucose with higher concentration occurring during the active phase at ZT20 (Figure 2A). Interestingly, there was no effect of time on plasma glucose in HS fed animals (Figure 2D). There was no significant effect of time on plasma insulin in NS fed control or ET_B def rats (Figure 2B; p=0.08). In contrast there was a significant effect of time on plasma insulin concentration in HS fed animals. In addition, HS fed ET_B rats had significantly lower plasma insulin

concentration compared to control (Figure 2E). When data was averaged from all time points, no significant difference was found in plasma glucose (Figure 2C); however, plasma insulin was significantly lower in ET_B def rats compared to control whether on NS or HS diet (Figure 2F). Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was significantly lower in ETB def rats and HS fed animals (Figure 2G). These data suggest that loss of ET_B function improves control of glucose.

We next sought to determine if ET_B def rats had alterations in plasma lipids. There were no detectable differences in plasma triglycerides between control or ETB def rats at any time point of NS fed animals (Figure 3A). There was a significant reduction in triglycerides at ZT12 (beginning of active phase) compared to other time points (Figure 3A). Interestingly, the only significant difference between genotypes in plasma triglyceride concentration of HS fed animals was at ZT12, where the dip in triglyceride concentration was attenuated in control rats but not ET_B def rats (Figure 3B; 93.8 ± 10.4 vs. 64.4± 6.2 mg/dl respectively). Average of all time points indicated that HS feeding significantly reduced plasma triglyceride concentration in both genotypes compared to NS fed animals (Figure 3C). Next, we determined the effect of ET_B deficiency on circulating free fatty acids. There were no detectable contribution of time on plasma free fatty acids (Figures 3D and 3E); however ET_B def rats, whether fed NS or HS, had a small increase in free fatty acid concentration, although not significant (Figure 3F; p=0.08).

The previous experiment suggests that ET_B deficient rats have an improvement in insulin sensitivity and glucose control; however, samples were collected under non fasting conditions and under anesthesia, thereby artificially inflating plasma glucose concentrations. Therefore, we next wanted to determine if ET_B deficiency improves glucose control using more direct measures of insulin sensitivity including glucose and insulin tolerance tests. Our results indicate that ET_B def rats have significantly lower fasting blood glucose (89.3±2.5 vs. 105.9±3.7 mg/dL respectively). In addition, ET_B def rats had improved glucose tolerance (33418±1891 vs 24715±3161 AUC; control vs. ET_B def rats) and insulin tolerance (3849±769 vs. 4669±976 AUC; control vs. ET_B def rats). These data indicate that even under normal salt feeding, ET_B deficiency improves insulin sensitivity.

Discussion

HS stimulation of ET-1 plays a major role in maintaining blood pressure by promoting the excretion of Na^+ by the kidney and extracellular Na^+; however, less is known about the contribution of ET-1/ET_B signaling to lipid metabolism and insulin signaling . The current study indicates that loss of ET_B receptor function improves insulin signaling and in HS fed animals, reduces adiposity. In rats lacking functional ET_B receptors, two weeks of HS intake significantly reduced body weight and epididymal fat mass compared to control rats. In addition, ET_B def rats have lower circulating insulin with no difference in plasma glucose (non-fasting) resulting in a lower HOMA-IR, suggesting that improved insulin sensitivity in ET_B def rats. Interestingly, ET_B def rats maintained on NS diet have lower fasting blood glucose and improved glucose tolerance independent of body weight differences. These data suggest that ET_B receptor activation may play a role in pathophysiology associated with obesity such as adiposity and insulin resistance.

HS intake is associated with dyslipidemia and insulin resistance in human populations independent of caloric intake;(Kang et al. 2016) however, this is not the case in rodent models, including the current study in which HS fed animals had lower circulating triglycerides and improved HOMA-IR compared to NS fed rats. Even more puzzling is that HS feeding had no effect on circulating free fatty acids, yet lowered circulating triglycerides. The reduction in triglycerides may be explained by a reduction in absorption of fats from the gut. It has been shown that HS feeding reduces the uptake of fatty acids by the intestine (Weidemann et al. 2015). One would expect compensation from the adipose tissue and liver to maintain free fatty acids for energy, possibly at the expense of lower triglycerides.

Several lines of evidence suggest that ETB receptor activation may promote insulin resistance and adiposity. A number of groups have shown a positive correlation between plasma ET-1 levels, obesity and insulin resistance (Cardillo et al. 2004; Ferri et al. 1995; Weil et al. 2011). In addition, visceral adipose from obese individuals produced 2.5 fold more ET-1 than adipose from lean counterparts. The same study showed that ET-1, via the ET_B receptor, blocks insulin mediated anti-lipolysis. This appears to be related to activation of phosphokinase C (PKC) activity (van Harmelen et al. 2008). Finally, a single nucleotide polymorphism in the *Ednrb* gene is associated with reduced risk of developing obesity in 2 separate populations in Spain (Martinez-Barquero et al. 2015). It is unclear if this SNP is associated with a gain or loss in function, but provides evidence that the ET_B receptor may be associated with obesity in humans. Our data indicates that loss of ETB receptor function can reduce adiposity when endogenous production of ET-1 is stimulated.

One of the major findings of this study is that loss of ET_B receptor function lowers circulating insulin and improves insulin sensitivity as measured by fasting blood glucose and glucose and insulin tolerance. One potential mechanism of reduced insulin is loss of ET_B mediated insulin release from pancreatic beta cells. It is well documented that ET-1 promotes insulin release via the ET_B receptor;(Brock et al. 1999; De Carlo et al. 2000) however, without improvement in insulin signaling in peripheral tissues, this would be expected to promote hyperglycemia. Therefore, whole body loss of ET_B receptor function appears to reduce insulin secretion, while also improving insulin signaling in insulin sensitive tissues. Polak et. al. recently showed that blocking ET_B receptors improves insulin sensitivity in a mouse model of sleep apnea (Polak et al. 2018). Several mechanisms by which activation of ET_B could promote insulin resistance exists. First, PKC is a known second messenger through which ETB receptors signal (Takuwa et al. 1990). PKC inhibits insulin receptor substrate-1 (IRS1) and is thought to be a major factor in the development of insulin resistance in muscle and liver of obese individuals (Li et al. 2015). Another potential mechanism by which ET_B receptor activation could cause insulin resistance is through activation of c-Jun N-terminal kinase, which leads to activation of MAPK (Aquilla et al. 1996). MAPK can also inhibits IRS-1 (Fujishiro et al. 2003). Another potential mechanism by which ET_B activation may promote insulin resistance occurs at the level of the adipocyte. ET_B receptor activation has been shown to inhibit peroxisome proliferator-activated receptor gamma (PPAR-λ) (Wolf et al. 2014). PPAR-λ is a transcription factor that promotes production of adipokines such as adiponectin by adipose. Adiponectin has profound insulin sensitizing effects on both liver and muscle, and adiponectin null mice have severe insulin

resistance (Nawrocki et al. 2006). More work is needed to uncover complete mechanisms by which loss of ET_B receptor function improves glucose control.

One potential confounder with the ET_B def rat is that loss of ET_B receptor function leaves the ETA receptor unopposed, therefore, it is possible that loss of adiposity is due to activation of the ET_A receptor. *In vitro* activation of the ET_A receptor causes increased lipolysis in differentiated 3T3-L1 adipocytes suggesting this as a potential mechanism for reduced adiposity (Eriksson et al. 2009). In addition, increased lipolysis via ET_A activation may also be the cause of elevated free fatty acids in ET_B def rats, although they were not elevated to a pathophysiological level. This would agree with a recent study published by Farrah et. al. indicating that blockade of ET_A receptors improves plasma lipids in patients with chronic kidney disease. Surprisingly, the increase in free fatty acids was in the absence of changes in circulating triglycerides in ET_B def animals. This may occur through increased lipoprotein lipase or hepatic lipase activity. More work, potentially with tissue specific knockout animals, in this area is needed to determine the role of and mechanisms by which ETA receptors may promote dyslipidemia in obesity.

Several limitations of the current study exist. First, plasma for protocol 1 was collected while animals were under anesthesia and the animals were not fasted. This artificially inflates plasma glucose concentrations. Insulin resistance was only estimated in HS fed animals under these circumstances. In addition, HS diet was only give for two weeks, and the difference in epididymal tissue weight was only about 15 percent. Longer duration of HS feeding may have created a larger effect size. This is somewhat supported by a study in which increased body weight was attenuated by HS in mice chronically fed high fat diet (Weidemann et al. 2015), although this was attributed to reduced digestive efficiency.

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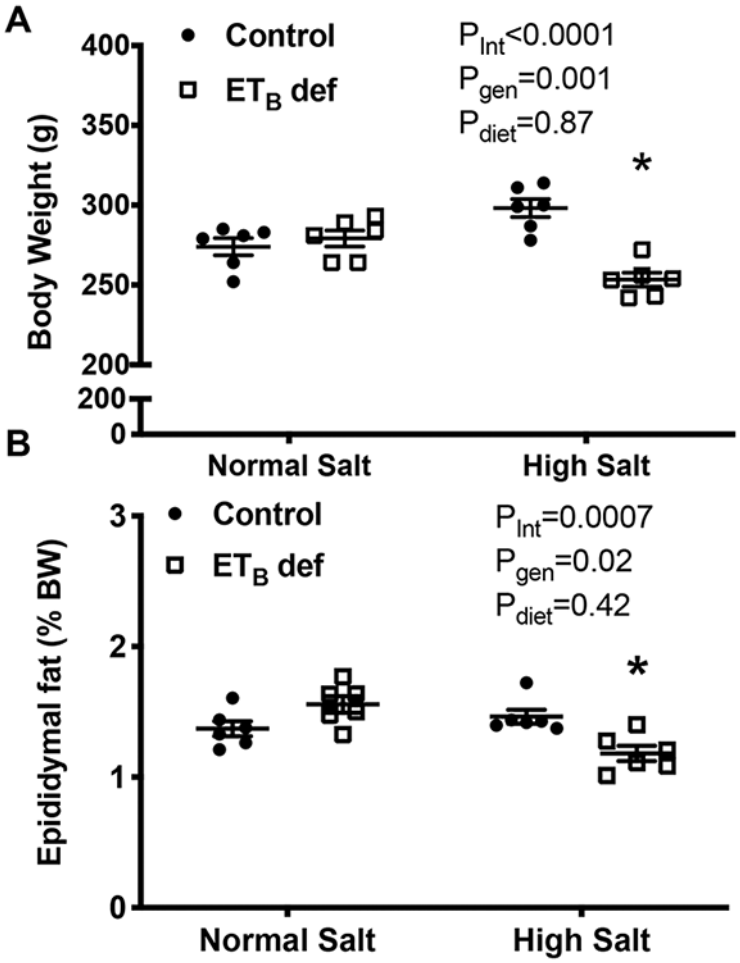

Figure 1:

Chronic high salt (HS) feeding reduces adiposity in endothelin B deficient (ET_B def) rats. A) Body weight and B) epidydimal fat weight to body weight ratio in transgenic control and ET_B def rats fed either a normal salt (NS) or high salt (HS) diet for two weeks. N=6/group; *$p<0.05$ vs. High Salt ET_B def. ANOVA table Key: interaction (int), genotype (gen)

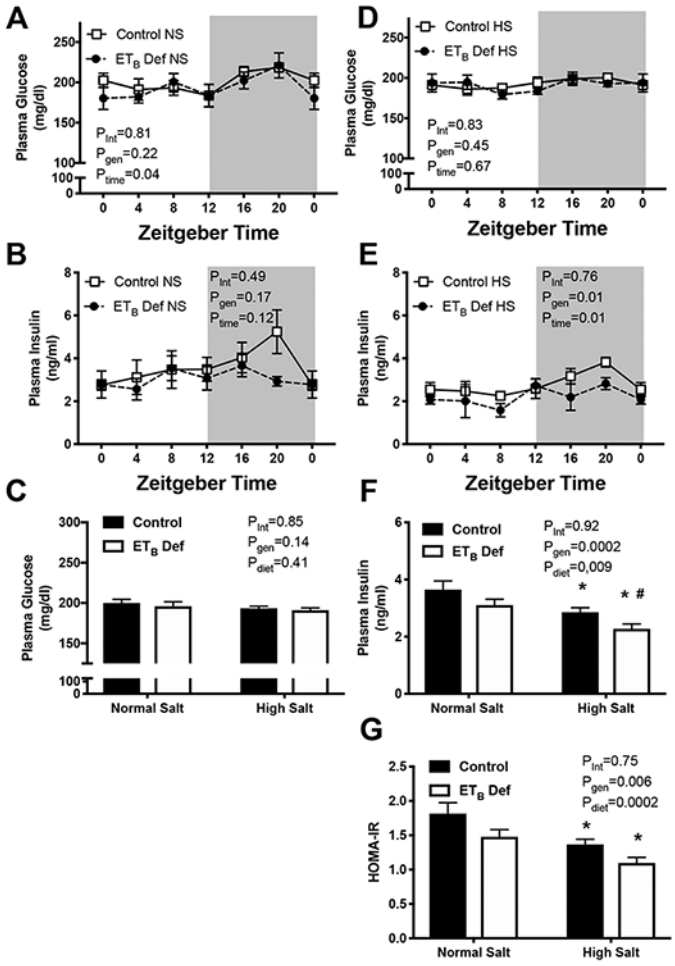

Figure 2:

Chronic high salt (HS) intake reduces circulating insulin in endothelin B deficient (ET_B def) rats. Transgenic control and ET_B def rats were fed either normal salt (NS) or HS diet for 2 weeks, and tissues were collected in non-fasted animals under anesthesia in 4 hour intervals. A & D) plasma glucose (mg/dL; n=5-7/group), B & E) circulating insulin (ng/ml; n=5-7/group), C) average plasma glucose of all time points (n=36-40/group), and F) average plasma insulin concentration of all time points (n=36-40/group) and and G) HOMA-IR caculated from non-fasting insulin and glucose. *p<0.05 vs. NS control; # p<0.05 vs NS ET_B def. ANOVA table Key: interaction (int), genotype (gen), time of day (time)

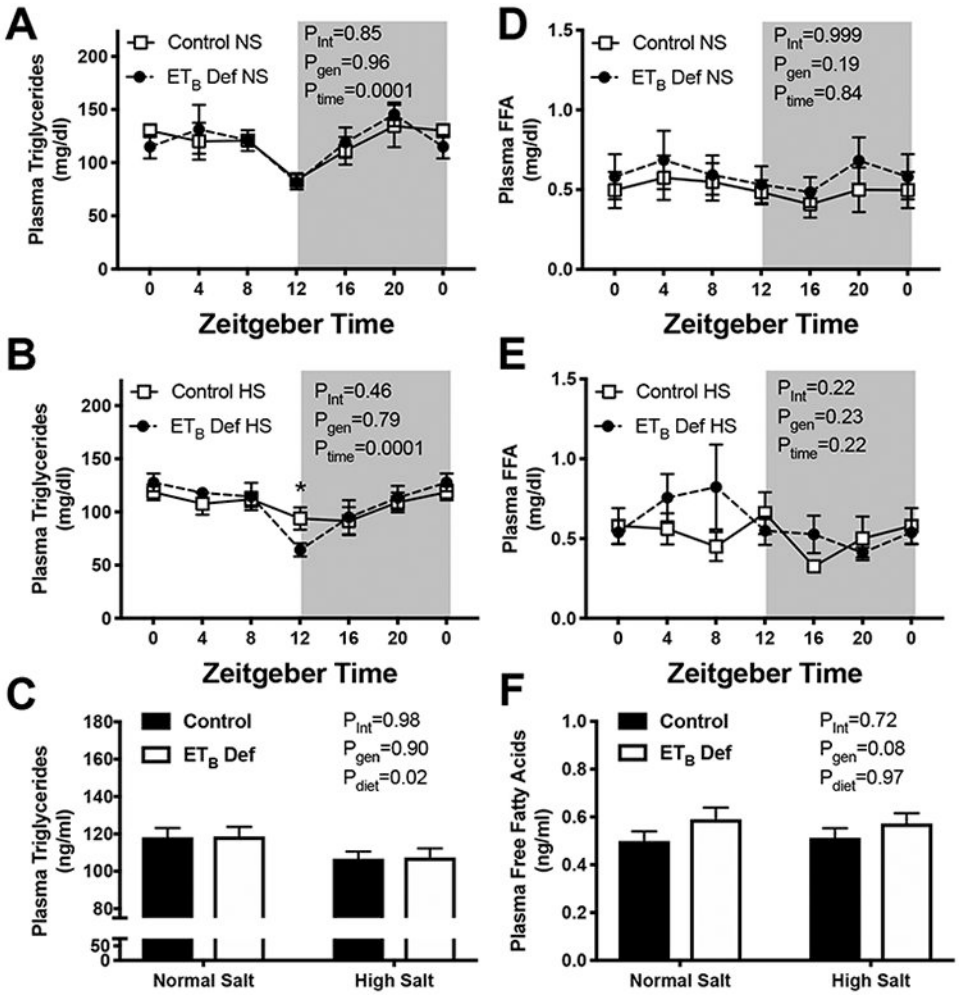

Figure 3:

Endothelin B deficient (ET$_B$ def) rats have increased circulating free fatty acids. Transgenic control and ET$_B$ def rats were fed either normal salt (NS) or high salt (HS) diet for 2 weeks, and tissues were collected in non-fasted animals under anesthesia in 4 hour intervals. A & B) plasma triglycerides (mg/dL; n=5-7/group), D & E) plasma free fatty acids (mg/dl; n=5-7/group), C) average plasma triglycerides of all time points (n=36-40/group), F) average plasma free fatty acid concentration of all time points (n=36-40/group). ANOVA table Key: interaction (int), genotype (gen), time of day (time)

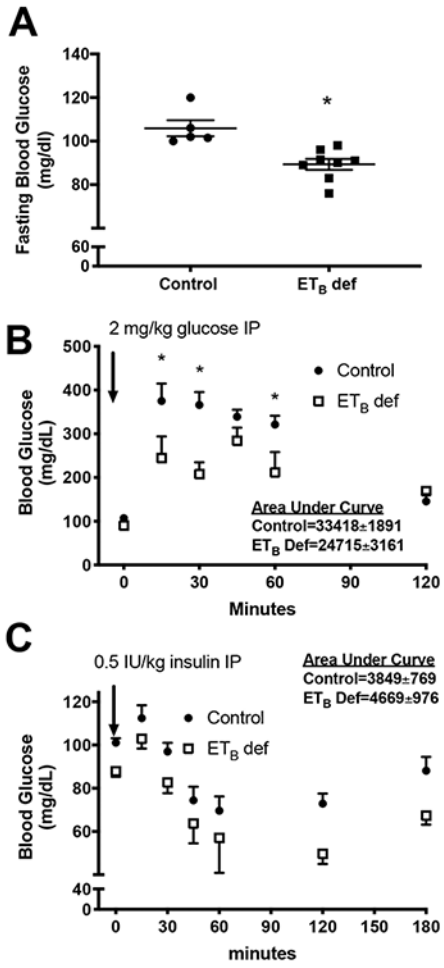

Figure 4:

Endothelin B deficient (ET_B def) rats have improved insulin sensitivity. A) Fasting plasma glucose (mg/dl; *p<0.05 vs. control), B) glucose tolerance test, and C) IP insulin tolerance test in transgenic control or ET_B def rats maintained on normal salt (NS) diet. *p<0.05 by post hoc analysis.