

## Knockout of Endothelin-1 in Vascular Endothelial Cells Protects Against Insulin Resistance Induced by High-Salt Diet in Mice

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**The increased plasma Endothelin-1 (ET-1) level has been associated with development of insulin resistance in obese and hypertensive patients. However, the underlying mechanism remains elusive. Here we investigate the potential role of endothelial cell-derived ET-1 in mediating insulin resistance induced by high-salt diet. To address this issue, we used vascular endothelial cell-specific ET-1 knockout (VEETKO) mice and its littermates fed with a high-salt diet containing 8% NaCl for 3 weeks, and evaluated the metabolic parameters. High-salt diet increased systolic blood pressure similarly in both genotypes. We observed impairment of glucose tolerance in control mice despite comparable increase of serum insulin concentration with VEETKO mice. We further found that VEETKO mice showed preservation of circulating adiponectin level - an adipokine with insulin-sensitizing property - and prevention of the upregulation of the pro-inflammatory adipokine TNF- $\alpha$ , which lead towards better insulin sensitivity. These results provide evidence that blockade of endothelin signaling may be proven beneficial in preventing high-salt induced insulin resistance.**

Insulin resistance has been thought to play a causative role in development of metabolic syndrome, a cluster of phenotypes which is important to identify individuals at high risk of both type 2 diabetes and cardiovascular disease (3). Although several mechanisms such as dysregulation of adipocyte signaling and altered lipid homeostasis have been strongly reported to promote insulin resistance (13, 5); understanding the interactions among these mediators and identification of their upstream regulators that possess potential therapeutic property are needed.

Endothelin-1 (ET-1) is a 21-amino acid peptide produced mainly by vascular endothelial cells, which has a potent vasoconstrictive activity in vascular smooth muscle cells (20). ET-1 and its receptors, termed ET<sub>A</sub> and ET<sub>B</sub>, are also expressed in other organs including the stromal vascular fraction of adipose tissue, suggesting that ET-1 has various biological effects (16). Previously, plasma ET-1 level has been reported to increase in obese, diabetes and hypertension patients (2, 17). ET-1 was shown to have an insulin-like effect that is mediated through ET<sub>A</sub> receptor in 3T3-L1 adipocytes *in vitro*, and treatment with exogenous ET-1 also caused heterologous desensitization of insulin signaling in the same cells (10, 19).

Wilkes *et al* further showed that chronic *in vivo* ET-1 administration leads to whole-body insulin resistance, with decreased skeletal muscle glucose transport and impaired insulin signaling (18). However, these studies failed to show the roles of endogenous ET-1 in the development of insulin resistance.

Excessive salt intake has been reported to induce insulin resistance and hypertension in rodents' animal model (14, 15), and also in normal human subjects (9). We therefore investigate the potential contribution of ET-1 in mediating high-salt diet induces insulin resistance. In the present study, we observed that deletion of ET-1 in vascular endothelial cells in mice is sufficient to prevent high-salt diet-induced insulin resistance.

## MATERIALS AND METHODS

### Generation of mice and dietary intake

In this study, we used 19 week-old heterozygous ET-1<sup>fl/fl</sup>;Tie2-Cre (+) (VEETKO) mice and their littermates [ET-1<sup>fl/fl</sup>;Tie2-Cre (-)] as controls. Detailed description about generation of conditional knockout mice will be published elsewhere (Yaz Y. Kisanuki, Masashi Yanagisawa *et al.*, submitted manuscript, 2009).

Mice were fed with a high-sodium diet (8% NaCl: HSD) for 3 weeks to induce insulin resistance, and normal chow diet (NCD) was given to the control groups. All animal experimental protocols were conducted in accordance with the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine.

### Metabolic measurements

Nineteen week-old mice fed on normal chow or a HSD for 3 weeks were fasted overnight, and blood was collected from tail vein. Blood glucose was measured with OneTouch Ultra<sup>TM</sup> glucometer (Lifescan). The serum insulin and ET-1 concentration was determined with enzyme-linked immunosorbent assay (ELISA) kits (Morinaga and R&D System). Serum triglyceride (TG) and free fatty acid (FFA) concentration was measured by determination kit (Wako). Blood pressure and heart rate were measured by tail-cuff method (Muromachi, Japan).

### Glucose and insulin tolerance test

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) were performed before and after HSD. For a glucose tolerance test, VEETKO mice or littermates were fasted overnight and glucose was administered intraperitoneally (1g/kg body weight). For insulin tolerance test, VEETKO mice or littermates were fasted for 4 hours and 0.8U/kg body weight of human insulin was injected intraperitoneally. The glucose levels were measured in blood withdrawn from tail in subsequent time course.

### Statistical analysis

Data are expressed as the mean  $\pm$  s.e.m. Statistical significance was tested with an unpaired two-tailed Student's t-test and analysis of variance (ANOVA). The difference was considered to be significant if  $p < 0.05$ .

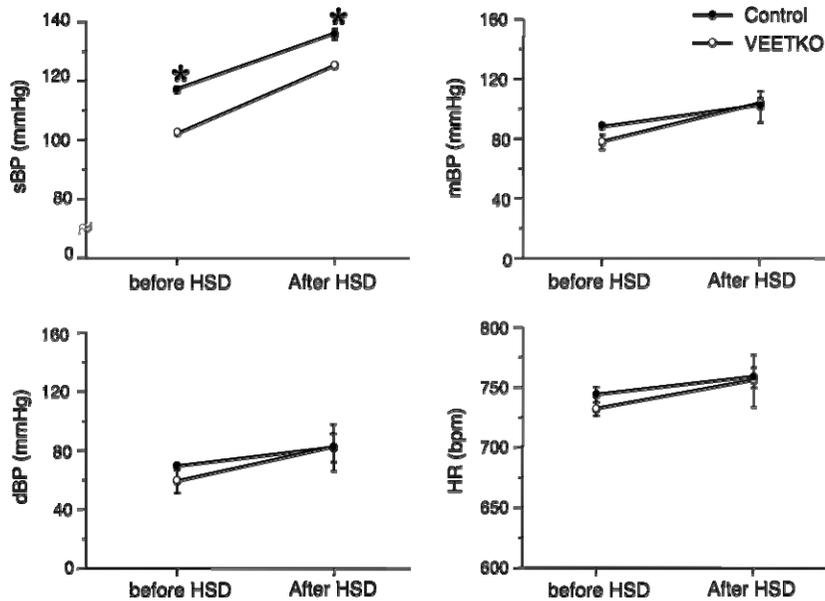
## RESULTS

### Effects of high-salt diet on blood pressure and heart rate

In baseline condition, systolic blood pressure (sBP) of VEETKO mice is slightly but significantly lower than that of control mice as previously reported (4). Two weeks of high-salt diet increased sBP in both genotypes similarly with no difference in the degree of the

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changes, while diastolic blood pressure (dBp), mean blood pressure (mBP) and also heart rate (HR) were remain unchanged (Fig. 1).

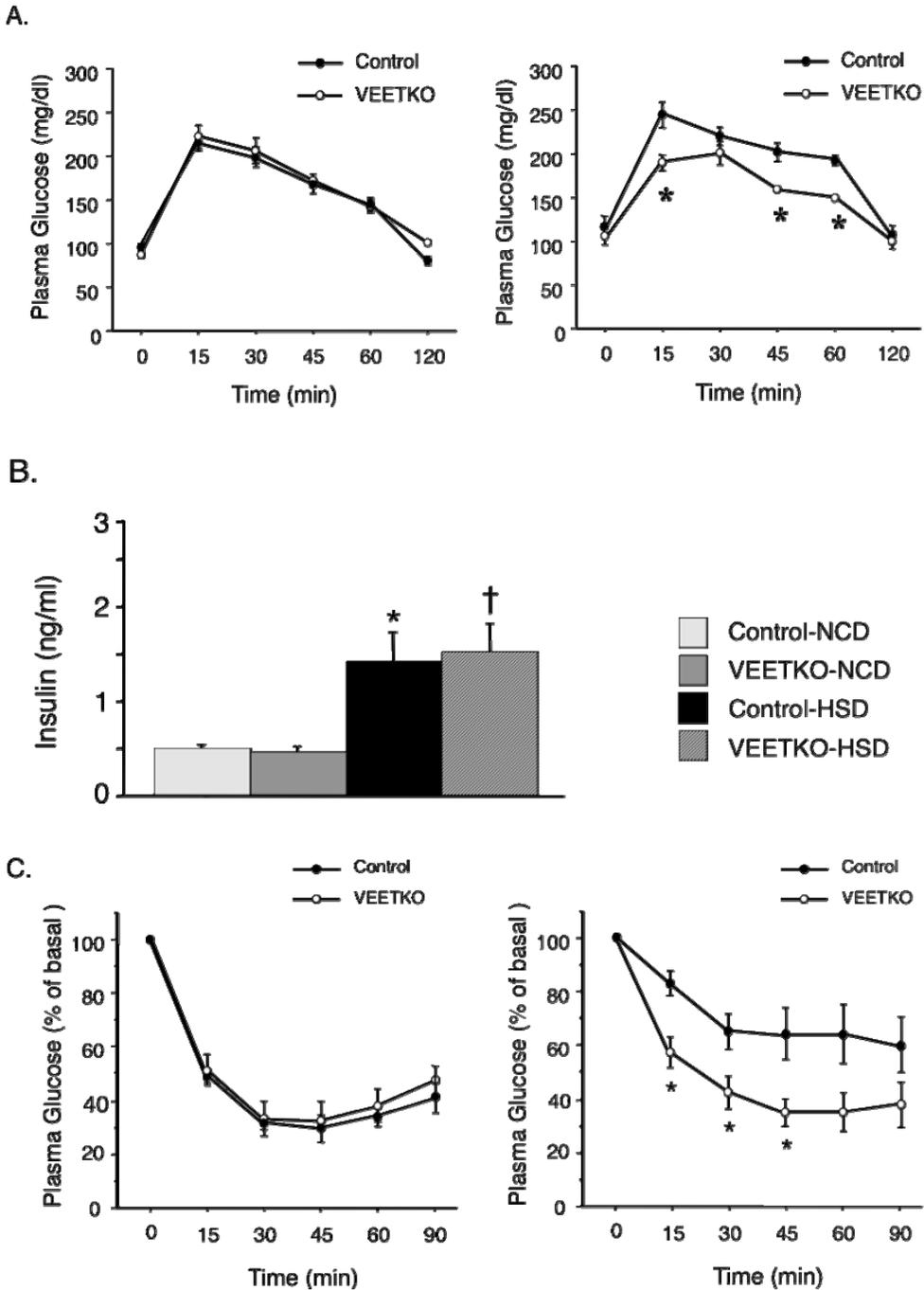


**Figure 1. Blood pressure and heart rate before and after high-salt diet.** Comparable increase of sBP observed in both genotypes after 2 weeks of HSD (left upper panel).  $n=8$  each group,  $*p<0.01$  vs. VEETKO. sBP, systolic blood pressure; dBp, diastolic blood pressure; mBP, mean blood pressure; HR, heart rate.

### High-salt diet induces glucose tolerance impairment

We performed glucose tolerance test to assess dynamics of the response to glucose. HSD-treated control mice showed glycemic excursion after intraperitoneal glucose challenge, while HSD-treated VEETKO mice interestingly showed significant improvement of glucose clearance (Fig. 2A). To determine the underlying cause of this enhanced glycemic control, we measured serum insulin level and found similar increased levels of serum insulin level in both genotypes (Fig. 2B). This result indicates that enhanced insulin secretion did not contribute to the improvement of glucose tolerance in VEETKO mice.

We further examined insulin sensitivity by insulin tolerance test and observed significantly better insulin-mediated glucose disposal in HSD-treated VEETKO mice as compared to control mice at 15, 30 and 45 min (Fig. 2C). Thus, we suggest that better insulin sensitivity may associate with improvement of glucose clearance after HSD in VEETKO mice.



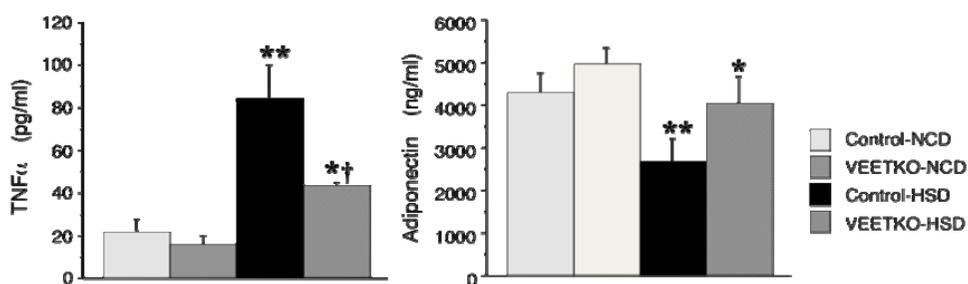
**Figure 2.** IPGTTs and ITTs at baseline and after high-salt diet. A, IPGTT at baseline (left) and after HSD (right) in VEETKO (open circle) and control mice (filled circle).  $n=12$  each group;  $*p<0.01$  for difference between VEETKO and control mice. B, Fasting serum insulin concentration.  $n=8$  each group;  $*p<0.01$  vs. control mice-NCD;  $\dagger p,0.01$  vs. VEETKO-NCD. C, ITT at baseline (left) and after HSD (right) in VEETKO and control mice.  $n=12$  each group;  $*p<0.01$  for difference between VEETKO and control mice.

**High-salt diet had limited effects on lipid profile**

We measured several metabolic parameters and found that HSD had no effect on body weight and plasma free fatty acid (FFA), while plasma triglyceride (TG) was slightly decreased with comparable level in both genotypes (data not shown).

**ET-1 mediates high-salt diet-induced dysregulation of adipokines**

We next examined the levels of TNF- $\alpha$  and adiponectin as representative adipokines that play important roles in insulin resistance syndrome. There was no difference in baseline serum TNF- $\alpha$  and adiponectin level between both genotypes. However, high-salt diet significantly increased serum TNF- $\alpha$  level in both VEETKO and control mice, but the concentration is much lower in VEETKO mice (Fig. 3, left panel). In contrast, circulating adiponectin - an adipokine with insulin-sensitizing property - was decreased in HSD-treated control mice, but not in VEETKO mice (Fig. 3, right panel).



**Figure 3. Effect of ET-1 in mediating adipokines secretion.** Left panel, Serum TNF- $\alpha$  concentration. n=8 each group. \*p<0.05 vs. control mice-HSD; \*\*p<0.05 vs. control mice-NCD; †p<0.05 vs. VEETKO-NCD. Right panel, Serum Adiponectin concentration. n=8 each group. \*p<0.1 vs. control mice-HSD; \*\*p<0.05 vs. control mice-NCD.

**DISCUSSION**

Here we reported that genetic inactivation of endothelin-1 in vascular endothelial cell in mice is protective against high-salt diet-induced insulin resistance. After 3 weeks of high-salt loading, mice with lack of ET-1 in endothelial cell showed improvement of glucose tolerance that is associated with better insulin sensitivity and inhibition of high-salt induced-alteration in secretion of TNF- $\alpha$  and adiponectin.

While lower baseline sBP in VEETKO mice as compared to its littermates suggested the role of endothelial cell-derived ET-1 in regulating basal vascular tone; the similar increase of sBP in both genotypes after high-salt diet in present study revealed that blood pressure regulation in response to salt loading is not solely determine by endothelial cell-derived ET-1. This result is in agreement to previous study that showed the importance of collecting duct-derived ET-1 in blood pressure regulation through the control of sodium excretion. Mice with knockout of ET-1 exclusively in collecting duct were reported to be hypertensive, and further had exacerbated hypertension (~ 35 mmHg in sBP greater than controls) when exposed to high-salt intake, which is associated with a reduced ability to excrete the sodium load (1).

We observed better insulin sensitivity in VEETKO mice after high-salt diet. These observation may explain the improvement of glucose clearance in VEETKO mice as compared to control mice. Several lines of evidence showed the contribution of ET-1 to the

development of insulin resistance, mainly through the impairment of insulin receptor and glucose transport signaling and also dysregulation of adipose tissue signaling (6, 7, 11).

Recently, adipose tissue has left its traditional identity as an energy storage depot and was highly recognized as an endocrine organ. It secreted various peptides and proteins named “adipokines”, such as leptin, adiponectin, TNF- $\alpha$ , MCP-1, IL-6 and PAI-1. Some of these peptides, including adiponectin, has an anti-inflammatory or insulin-sensitizing properties, while others like TNF- $\alpha$  and IL-6 are reported to be pro-inflammatory and had their circulating levels elevated in obese and metabolic syndrome patients (8, 12). The complex balance between adipokines is crucial in determination of insulin sensitivity. In this study, protective effect of ET-1 deficiency to high-salt induced insulin resistance may be explained by preservation of homeostasis in adipose tissue signaling towards insulin sensitive condition. Lack of ET-1 prevents overproduction of TNF- $\alpha$  by salt loading, and preserves the concentration of circulating adiponectin. Nevertheless, the detail molecular mechanism linking regulation of TNF- $\alpha$  and adiponectin by ET-1 in our model remains to be elucidated further.

In conclusion, we observed the important contribution of endogenous ET-1 produced by vascular endothelial cell in mediating high-salt induced insulin resistance, in part through stimulating dysregulation of adipose tissue signaling. Blockade of ET-1 signaling thus may be proven beneficial in prevention of insulin resistance induced by high salt intake.

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