Vascular dysfunction is a well-established cardiovascular risk factor and is involved in the development of atherosclerosis, which is expected to become the leading cause of death worldwide. During the last 2 decades, it has been shown that dietary salt intake is linked to the elevation of blood pressure (BP) and the impairment of vascular function. The vascular endothelium has been viewed to play a critical role in the maintenance of vascular tone. It has been accepted that endothelial function, especially of the nitric oxide (NO) system, is impaired in animals with chronic high-salt (HS) intake. However, not everybody is sensitive to salt, and the mechanisms of salt sensitivity are not fully understood. It is estimated that approximately 30% of the population will suffer from hypertension after exposure to an HS diet. A previous study demonstrated that ablation of uncoupling protein 2 (UCP2) enhanced HS intake-induced vascular dysfunction, indicating that UCP2 might play an important role in salt sensitivity. However, the role of upregulation of UCP2 protein in salt-induced vascular function has not been investigated.

UCP2 located in the inner membrane of mitochondria has been involved in the regulation of vascular biology. Moreover, UCP2 plays a crucial role in vascular dysfunction caused by several harmful factors. It has been reported that UCP2 preserves endothelial function in mice with diet-induced obesity. Additionally, UCP2 has also been linked to the survival of endothelial cells and vascular smooth muscle cells (SMCs). Thus, we hypothesized that overexpression of UCP2 might ameliorate salt-induced vascular dysfunction.

Accumulating evidence indicates that reactive oxygen species such as superoxide play a major role in the initiation and progression of salt-induced vascular dysfunction. NO produced by endothelial NO synthase (eNOS) is a classic mediator of vascular relaxation. Superoxide can quench the NO in vasculature and thereby cause vascular dysfunction. Depolarization of the inner mitochondrial membrane

Transgenic Overexpression of Uncoupling Protein 2 Attenuates Salt-Induced Vascular Dysfunction by Inhibition of Oxidative Stress

Shuangtao Ma, Qiang Wang, Yan Zhang, Dachun Yang, De Li, Bing Tang, and Yongjian Yang

BACKGROUND
Ablation of uncoupling protein 2 (UCP2) has been involved in the enhancement of salt sensitivity associated with increased superoxide level and decreased nitric oxide (NO) bioavailability. However, the role of overexpression of UCP2 in salt-induced vascular dysfunction remains elusive.

METHODS
UCP2 transgenic (TG) and wild-type (WT) mice were placed on either a normal-salt (NS, 0.5%) or a high-salt (HS, 8%) diet for 12 weeks. Blood pressure (BP) and hypotensive responses were measured, and the vascular tone, superoxide level, and NO bioavailability in aortas were measured in each group.

RESULTS
The TG mice had increased expression and function of UCP2 in vascular smooth muscle cells. The acetylcholine (ACh)– and nitroglycerin (NTG)–induced hypotensive responses and aortic relaxations were significantly blunted in WT mice fed with an HS diet compared with an NS diet. These harmful effects were prevented in UCP2 TG mice.

The impairments of ACh- and NTG-induced relaxation in aorta were inhibited by the endothelial NO synthase (eNOS) inhibitor L-NAME and mitochondrial antioxidant MitoQ, respectively. The HS intake led to a significant increase in superoxide production and a comparable decrease in NO bioavailability in aortas, and these effects were blunted in UCP2 TG mice. The expression of UCP2 was slightly increased in the HS group. However, the expression and phosphorylation of eNOS were not affected by an HS diet and overexpression of UCP2.

CONCLUSIONS
These findings suggest that overexpression of UCP2 can ameliorate salt-induced vascular dysfunction. This beneficial effect of UCP2 is mediated by decreased superoxide and reserved NO bioavailability.

Keywords: blood pressure; high salt intake; hypertension; hypotensive response; nitric oxide; superoxide; uncoupling protein 2; vascular dysfunction.

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potential can be induced by a proton leak from UCP2, which can reduce the production of superoxide and inhibit oxidative damage.16

In this study, we investigated the role of overexpression of UCP2 in salt-induced vascular dysfunction and explored the underlying mechanisms using transgenic mice specifically overexpressing human UCP2 in vascular SMCs.

METHODS

Animals

Transgenic mice expressing human UCP2 (UCP2 TG) in vascular SMCs were generated on an Friend virus B-type (FVB) background in a previous study.17 Wild-type (WT) littermates were used as controls. Genotyping was performed by polymerase chain reaction with the following primers: UCP2: forward 5′-GGA GAT ACC AAA GCA CCG TCA A and reverse 5′-CAT AGG TCA CCA GCT CAG CAC A, a 132-bp product; internal reference: forward 5′-TCT TAG GTC TGC TCT CCG GT and reverse 5′-CAC TGG CTG AGG AAG GAG AC, a 196-bp product. Mice aged 6–8 weeks were fed ad libitum and reared in normal lighting conditions (12/12 light/dark cycle). The mice were randomly divided into 2 groups that received a normal-salt (NS; 0.5% sodium chloride; n = 12) or an HS (8% sodium chloride; n=12) diet and were fed for 12 weeks. All procedures were performed in accordance with the Johns Hopkins University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Blood pressure

The systolic and diastolic BPs were measured in conscious, restrained mice by a noninvasive tail-cuff system (BP-98A; Softron, Tokyo, Japan). At the end of the experiment, mice were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). Then the right carotid artery was cannulated for measurements of mean arterial pressure (MAP) with a pressure transducer (model MLT 1030, Power Lab; AD Instruments, Sydney, Australia). The left jugular vein was cannulated for intravenous infusion of acetylcholine (ACh) and nitroglycerin (NTG). The baseline MAP values was cannulated for intravenous infusion of acetylcholine (ACh) and nitroglycerin (NTG). The baseline MAP values were obtained during a 1-hour control period. Responses to ACh (10−9–10−4 mol/L) and NTG (10−9–10−4 mol/L) were performed. Responses to ACh were repeated after pretreatment with an eNOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L), for 30 minutes. Some rings were incubated with mitochondrial antioxidant MitoQ (0.1 μmol/L) for 1 hour before NTG-induced relaxation was recorded.19

Superoxide assay

The aortas were cut into 30-μm-thick sections and incubated in the dark with dihydroethidium (Sigma-Aldrich, St. Louis, MO) at 5 μmol/L for 30 minutes. For dihydroethidium fluorescence, images were taken at excitation 515 nm and emission 585 nm.20

NO assay

Aortas were prepared in the manner described above for the superoxide assay and were loaded with diaminofluorescein-2 diacetate (Sigma-Aldrich) at 5 μmol/L in the dark for 45 minutes at 37 °C in Krebs solution (pH = 7.4). For diaminofluorescein-2 diacetate fluorescence, images were taken under the fluorescein isothiocyanate filter.21

Cell culture

Vascular SMCs were obtained from the thoracic aortas of UCP2 TG and WT mice and were cultured using a tissue explant method as described previously.22

Western blotting

Protein samples prepared from mouse aorta or vascular SMC homogenates were electrophoresed through a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Boehringer-Mannheim Corporation, Indianapolis, IN). The blots were incubated overnight at 4 °C with the primary antibodies: anti-UCP2 (1:1,000; Santa Cruz Biotechnology) and anti-eNOS (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-eNOS at Ser1177 (1:1,000; Santa Cruz Biotechnology), and anti-GAPDH (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-eNOS at Ser1177 (1:1,000; Santa Cruz Biotechnology), and anti-GAPDH (1:1,000; Santa Cruz Biotechnology). After washing, the membrane was incubated with a horseradish peroxidase–conjugated secondary antibody (1:1,000; Santa Cruz Biotechnology), and bound antibody was visualized using a colored reaction. Anti-GAPDH (1:1,000; Santa Cruz Biotechnology) was used as a housekeeping protein.23

Immunofluorescence

The vascular SMCs were fixed by 4% paraformaldehyde, washed in phosphate-buffered saline, permeabilized by 0.01% Triton X-100, and incubated with antibodies against UCP2
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(1:200, Santa Cruz Biotechnology) and α-smooth muscle actin (1:100; Santa Cruz Biotechnology,) overnight at 4 °C, followed by Cy3-conjugated donkey antigoat immunoglobulin G (1:1,000; Proteintech Group, Chicago, IL) and Alexa Fluor 647–conjugated goat antimouse immunoglobulin G (1:500; Byeotime, Shanghai, China) antibodies. Then, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes. Imaging was performed using a fluorescence microscope.

Figure 1. Uncoupling protein 2 (UCP2) expression and function in aortas. (a) Transgene of the human UCP2 gene was confirmed by genotyping. Polymerase chain reaction amplification of genomic DNA from wild type (WT) and human UCP2 transgenic (TG) mice was performed using specific primers for human UCP2 gene (132 bp) and internal reference (IR; 196 bp). M, marker. (b) Representative Western blots of UCP2 in vascular smooth muscle cells (SMCs) from WT and TG mice. (c) The protein expression level of UCP2/GAPDH is shown in the bar graph. (d) Representative immunofluorescence image of α-smooth muscle actin (α-SMA), UCP2, and 4′,6-diamidino-2-phenylindole (DAPI) in SMCs from WT and TG mice. (e) Representative immunohistochemical image of UCP2 in aortic tissue from WT and TG mice. (f) Relative mitochondrial membrane potential of vascular SMCs from WT and TG mice. Values are means ± SE of 3–6 independent experiments. *P < 0.05; **P < 0.01.
Immunohistochemistry

Paraffin-embedded arteries were cut into cross-sections (5 μm), dewaxed, and rehydrated. Incubation with primary antibodies against UCP2 (1:100; Santa Cruz Biotechnology) was performed in a humidified chamber overnight at 4 °C. Sections were washed and incubated with a goat biotinylated antirabbit secondary antibody (1:200 dilution; Boster, Wuhan, China), followed by incubation with a streptavidin-biotin-peroxidase complex reagents from an SABC kit (Boster). A dianaminobenzidine stain kit (Boster) was used to detect a positive reaction by producing a brown color.

Mitochondrial membrane potential

To monitor the mitochondrial membrane potential, cells were loaded with 5 μmol/L JC-1 dye (Beyeotime, Shanghai, China) at 37 °C for 30 minutes. Samples were imaged under the fluorescein isothiocyanate and rhodamine isothiocyanate filters. The ratio of rhodamine isothiocyanate (J-aggregate) to fluorescein isothiocyanate (J-monomer) staining was determined.24

Statistical analysis

Data are means ± SEs. The negative logarithm of the dilator concentration that caused 50% of the maximum response (pD2) and the maximum relaxation (Emax %) were calculated from individual agonist concentration–response curves using GraphPad Prism 6.0 (San Diego, CA). The statistical differences in mean values were assessed by the Student t test. Two-sided P values <0.05 were considered statistically significant.

RESULTS

UCP2 expression and function in aortas

We had established a TG mouse model specifically expressing human UCP2 in vascular SMCs. The TG mouse was constructed by pronuclear microinjection with a plasmid carrying 2.3-kb rabbit smooth muscle myosin heavy chain promoter and human UCP2 gene. The expression of human UCP2 gene in TG mice was confirmed by genotyping with polymerase chain reaction amplification of genomic DNA (Figure 1a). The transgenic overexpression of UCP2

![Figure 2](http://ajh.oxfordjournals.org/)

**Figure 2.** Blood pressure. (a and b) The tail-cuff systolic and diastolic blood pressure (BP) of wild-type (WT) and uncoupling protein 2 (UCP2) transgenic (TG) mice placed on a normal-salt (NS) or a high-salt (HS) diet. (c and d) The hypotensive response to intravenous bolus injection of acetylcholine (ACh) and nitroglycerin (NTG) in WT and UCP2 TG mice placed on an NS or an HS diet. Abbreviation: MAP, mean arterial pressure. Values are means ± SE of 6 mice per group. **P < 0.01.
resulted in significant increases in UCP2 protein levels in primarily cultured vascular SMCs from the TG mice according to Western blot analysis ($P < 0.01$) (Figure 1b, c) and immunofluorescence assay (Figure 1d). The upregulation of UCP2 was also observed in aortic tissues from TG mice (Figure 1e). To identify the function of UCP2, the cultured vascular SMCs were stained with TMRE to examine the effect of UCP2 expression on mitochondrial membrane potential. The vascular SMCs from the TG mice showed partial depolarization of mitochondrial membrane potential compared with WT cells ($P < 0.05$) (Figure 1f), verifying the functional overexpression of UCP2.

Figure 3. Vascular relaxation. (a and b) Acetylcholine (ACh)–induced relaxation in aortas of wild-type (WT) and uncoupling protein 2 (UCP2) transgenic (TG) mice placed on a normal-salt (NS) or a high-salt (HS) diet. (c) ACh-induced relaxation in the presence of endothelial nitric oxide synthase (eNOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME) in aortas of WT. (d and e) Nitroglycerin (NTG)–induced relaxation in aortas of WT and UCP2 TG mice placed on a nNS or an HS diet. (f) NTG-induced relaxation in the presence of mitochondrial antioxidant MitoQ in aortas of WT. Abbreviation: PE, phenylephrine.
Effect of overexpression of UCP2 on blood pressure

The baseline systolic BP and diastolic BP were similar between the TG mice and WT littermates (data not shown). After diet intervention for 12 weeks, there was no significant difference in systolic BP and diastolic BP between the TG mice and WT littermates (Figure 2a,b). The hypotensive responses to intravenous injection of ACh and NTG were measured at the end of diet intervention. The ACh–caused decrease in MAP was significantly blunted by HS intake in WT mice (P < 0.01) (Figure 2c). However, this effect was significantly prevented in UCP2 TG mice. In mice fed an HS diet, the ACh–caused decrease in MAP was significantly lower in UCP2 TG than in WT mice (P < 0.01) (Figure 2c). Similarly, the hypotensive response to NTG was significantly attenuated in UCP2 TG mice compared with WT mice after an HS diet (P < 0.01) (Figure 2d).

Effect of overexpression of UCP2 on vascular tone

In aortas from the WT mice, ACh-elicited concentration-dependent relaxations were significantly impaired in the HS group compared with the NS group (NS: E_max = 91.27 ± 2.35%; vs. HS: E_max = 73.46 ± 3.92%; P < 0.01) (Figure 3a; Table 1). However, in aortas from the UCP2 TG mice, ACh-induced vascular relaxation was not affected by the HS diet (Figure 3b; Table 1). L-NAME largely attenuated ACh-induced relaxation of aortas from the WT mice and eliminated the difference between the NS and HS groups (Figure 3c; Table 1). Similarly, NTG-induced relaxation was significantly impaired in WT mice fed with an HS diet compared with WT mice fed with an NS diet (NS: pD2 = 5.86 ± 0.13; vs. HS: pD2 = 5.21 ± 0.09; P < 0.01) (Figure 3d; Table 1). However, there was no difference in relaxation responses to NTG in UCP2 TG mice between the HS and NS group (Figure 3e; Table 1). Moreover, the HS diet–induced impairment in NTG-elicited relaxation was prevented in the presence of antioxidant MitoQ (Figure 3f; Table 1).

Table 1. Maximum response (E_max) and negative log of the half maximal effective agonist concentration (pD2) values for agonist-induced relaxation in aortas

<table>
<thead>
<tr>
<th>Group</th>
<th>WT</th>
<th>TG</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>E_max (%)</td>
<td>pD2</td>
</tr>
<tr>
<td>NS, Ach</td>
<td>91.27 ± 2.35</td>
<td>6.56 ± 0.12</td>
</tr>
<tr>
<td>HS, Ach</td>
<td>73.46 ± 3.92**</td>
<td>6.39 ± 0.25</td>
</tr>
<tr>
<td>NS, Ach + L-NAME</td>
<td>44.06 ± 6.00</td>
<td>5.05 ± 0.21</td>
</tr>
<tr>
<td>HS, Ach + L-NAME</td>
<td>42.70 ± 6.36</td>
<td>5.05 ± 0.29</td>
</tr>
<tr>
<td>NS, NTG</td>
<td>100.35 ± 2.27</td>
<td>5.86 ± 0.13</td>
</tr>
<tr>
<td>HS, NTG</td>
<td>101.54 ± 4.80</td>
<td>5.21 ± 0.09**</td>
</tr>
<tr>
<td>NS, NTG + MitoQ</td>
<td>100.25 ± 3.20</td>
<td>5.69 ± 0.05</td>
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Values are means ± SE for 6 rings from 6 mice per group. Relaxation induced by acetylcholine (ACh) and nitroglycerin (NTG) was calculated relative to the maximal changes from the contraction produced by phenylephrine and is represented as percentage of relaxation.

Abbreviations: HS, high-salt diet; L-NAME, NG-nitro-L-arginine methyl ester; NS, normal-salt diet; TG, uncoupling protein 2 transgenic mice; WT, wild-type littermates.

*P < 0.01 vs. NS group.

Effect of overexpression of UCP2 on superoxide production

Superoxide production in aortas was assessed by dihydroethidium staining. In aortas from the WT mice, the superoxide level was significantly increased by the HS diet (P < 0.01) (Figure 4a,b). Moreover, transgenic overexpression of UCP2 prevented the HS diet–induced increase in superoxide production (Figure 4a,b).

Effect of overexpression of UCP2 on NO bioavailability

NO bioavailability was assessed by diaminofluorescein-2 diacetate staining. In aortas from the WT mice, the NO level was significantly decreased by the HS diet (P < 0.01) (Figure 5a,b). Moreover, transgenic overexpression of UCP2 prevented the HS diet–induced decrease in NO level (Figure 5a,b).

Effect of overexpression of UCP2 on eNOS expression

In WT mice, the protein expression level of UCP2 in aortic tissue was slightly elevated by the HS diet (P < 0.01) (Figure 6a). However, the HS diet did not cause an additional increase in the expression of UCP2 in TG mice (Figure 6a). The protein expression and phosphorylation of eNOS were not affected by the HS diet in both WT and TG mice (Figure 6b). Moreover, transgenic overexpression of UCP2 also had no effect on the expression and phosphorylation of eNOS in the aorta (Figure 6b).

DISCUSSION

In this study, we showed that transgenic overexpression of UCP2 significantly ameliorated the HS diet–induced vascular dysfunction that was expressed as impaired hypotensive and relaxation response to ACh and NTG. This study also demonstrates that the beneficial effect of UCP2 result is likely due to an increase in mitochondrial NO bioavailability, which is supported by the finding that transgenic overexpression of UCP2 prevented the HS diet–induced increase in superoxide production.
overexpression on vascular expression was associated with the decreased production of superoxide and the increased bioavailability of NO.

Hypertension is the most important risk factor of cardiovascular disease, which is the leading cause of death and disability worldwide. There is strong evidence that salt intake is the major factor increasing BP and thereby target organ damage. Furthermore, an HS diet has direct harmful effects independent of its effect on BP. This study demonstrates that an HS diet caused a significant impairment of vascular function without elevating the BP in WT mice. The mechanism underlying the direct harmful effect of the HS intake on the vascular bed has not been fully understood. The gene polymorphism of UCP2 and the protein expression of UCP2 have been linked to the pathophysiologic process of salt sensitivity. A previous study indicated that deficiency of UCP2 may enhance salt-induced vascular dysfunction. However, whether overexpression of UCP2 could prevent the harmful effect of HS intake on vascular tone has not been determined. This study shows that the protein expression of UCP2 was slightly but significantly upregulated by the HS diet, indicating that the compensatory increase in UCP2 expression might play a protective role under conditions of the HS diet. However, this mild compensatory response cannot prevent the HS intake–caused damage to the vascular tone. Our study also showed that with a remarkable overexpression of UCP2 protein, HS-induced vascular dysfunction was significantly attenuated in UCP2 TG mice. These findings suggest that upregulation of UCP2 might be a potent strategy for the prevention of HS diet–induced vascular damage. It has been revealed that several drugs can transcriptionally upregulate UCP2, especially rosiglitazone, an activator of peroxisome proliferator-activated receptor γ. However, the protective roles of these potent drugs need to be investigated in future studies.

NO was originally discovered as an endothelium-derived relaxing factor and is recognized as a key determinant of vascular tone.
vascular function. The NO in vasculature is produced by eNOS. Moreover, the bioavailability of NO is dependent not only on the phosphorylation of eNOS but also on the elimination of NO, especially quenching by superoxide. This study demonstrates that the salt-induced impairment of relaxation was blunted by eNOS inhibitor L-NAME, indicating that salt-caused damage was NO dependent. We also show that the NO level in aortas was decreased by HS intake. However, the expression and phosphorylation of eNOS were not affected by the HS diet. These finding suggest that salt-induced, NO-dependent vascular dysfunction is attributed to the increased quenching of NO but not the decreased production of NO.

UCP2, which was found to be a potent regulator of energy metabolism and now is viewed as a critical physiological downregulator of oxidative stress, might play an important role in suppressing the production of superoxide. This study shows that salt-induced vascular dysfunction was ameliorated by antioxidant MitoQ or transgenic overexpression of UCP2, indicating that the action of UCP2 might be dependent on the suppression of superoxide production. Moreover, we found that overexpression of UCP2 can suppress the mitochondrial membrane potential and attenuate salt-induced superoxide production. These findings suggest that overexpression of UCP2 prevents salt-induced vascular dysfunction though suppressing superoxide production and thereby preserving NO bioavailability.

In summary, overexpression of UCP2 attenuated HS intake–induced superoxide production, preserved the bioavailability of NO, and consequently ameliorated salt-induced vascular dysfunction. The data collectively suggest that upregulation of UCP2 might be a potent strategy to prevent salt-induced vascular dysfunction.
UCP2 Attenuates Salt-induced Vascular Dysfunction

REFERENCES


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DISCLOSURE

The authors declared no conflict of interest.


