Original Article

Troglitazone Improves Endothelial Function and Augments Renal klotho mRNA Expression in Otsuka Long-Evans Tokushima Fatty (OLETF) Rats with Multiple Atherogenic Risk Factors

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Targeted disruption of the klotho gene induces multiple phenotypes characteristic of human aging, including arteriosclerosis, pulmonary emphysema and osteoporosis. Moreover, we previously observed that insufficient klotho expression in mice leads to endothelial dysfunction. In the present study, we used Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which exhibit hypertension, obesity, severe hyperglycemia and hypertriglyceridemia, and are thus considered an animal model of atherogenic disease, to test the effects of oral administration of troglitazone (200 mg/kg) on renal klotho mRNA expression and endothelial function. Systolic blood pressure, body weight, plasma glucose and triglyceride levels were all significantly higher in 30-week-old OLETF rats than in controls (LETO; Long-Evans Tokushima Otsuka) (p<0.05, n=7). In addition, endothelium-dependent relaxation of the aorta in response to 10−5 M acetylcholine was significantly attenuated in OLETF rats (p<0.05, n=7), as was renal expression of klotho mRNA. Administration of troglitazone for 10 weeks significantly reduced systolic blood pressure, plasma glucose and triglyceride levels in OLETF rats, while augmenting endothelium-dependent aortic relaxation and renal klotho mRNA expression. These findings suggest that troglitazone protects the vascular endothelium against damage caused by the presence of multiple atherogenic factors. (Hypertens Res 2001; 24: 705–709)

Key Words: klotho, endothelium, hypertension, diabetes mellitus, hyperlipidemia

Introduction

The klotho gene was originally identified as a suppressor of various phenotypes characteristic of human aging, including arteriosclerosis, pulmonary emphysema, osteoporosis, infertility and a shortened lifespan (1). We previously showed that, upon parabiosis with wild-type mice, mice heterozy-

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gous for a defect in the klotho gene showed improved endothelial function, suggesting that the klotho gene product protects against endothelial dysfunction via a humoral pathway (2–4). We further demonstrated that klotho mRNA and protein are both abundantly expressed in the kidney, but are downregulated under conditions of sustained metabolic stress, such as hypertension, diabetes mellitus and hyperlipidemia, all of which promote atherosclerosis (5). It remains unclear, however, whether treatment of these common diseases increases the renal expression of klotho mRNA.

The aim of the present study was to determine whether troglitazone ameliorates vascular endothelial dysfunction and increases renal klotho mRNA expression in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which manifest various atherogenic risk factors, including hypertension, diabetes mellitus, hyperlipidemia and obesity. We found that troglitazone treatment improved their metabolic function and increased klotho mRNA expression in the kidney, resulting in the restoration of endothelial function. Thus, administration of troglitazone may be a useful strategy for protecting the vascular endothelium and preventing atherosclerosis in individuals presenting with multiple atherogenic risk factors.

Materials and Methods

Animals

All procedures were carried out within the institutional guidelines for animal research and the Law (No. 105) and Notification (No. 6) of the Japanese Government. Male OLETF and LETO rats (6 weeks-old, n = 7 in each group) were kind gifts from Otsuka Pharmaceuticals, Tokushima, Japan (6) and were bred at the laboratory animal facilities of Gunma University. The rats were fed standard laboratory chow and given tap water ad libitum. Troglitazone was given orally (200 mg/kg, n = 7) for 10 weeks, beginning at 20 weeks. This dose of troglitazone has been shown to lower plasma glucose levels in obese, insulin-resistant diabetic animal models (7, 8).

Blood Pressure and Blood Analysis

Systolic blood pressure was measured using the photoelectric volume oscillometric method with an automated tail cuff sphygmomanometer (Ueda, Nagano, Japan) without anesthesia. Blood samples were drawn from the abdominal aorta under sodium pentobarbital anesthesia, after which plasma samples were stored at −30°C. Biochemical analysis was performed using an autoanalyzer (MBC, Gunma, Japan).

Endothelial Function in the Aorta

The chest wall was opened and the thoracic aorta was carefully removed to avoid damaging the endothelium. After blood and connective tissue were removed, an aortic ring segment (3 mm long) was mounted between two stainless steel wires in an organ bath containing Krebs’ bicarbonate solution (120 mM NaCl, 5.2 mM KCl, 2.4 mM CaCl2, 1.2 mM MgSO4·7H2O, 25 mM NaHCO3, 0.03 mM Na2-EDTA, and 11 mM dextrose, pH 7.4) bubbled with a mixture of 95% O2 and 5% CO2, which enabled rapid mixing of drugs. One wire was attached to a fixed support, and the other was connected to a force-displacement transducer (model UR-50G; Minebea Co., Ltd., Nagano, Japan) (9). The preparation was allowed to equilibrate for 90 min, after which it was precontracted with 10−7 M phenylephrine. Dose–response curves for acetylcholine (10−5 to 10−3 M) and sodium nitroprusside (10−10 to 10−7 M) were obtained by cumulatively adding aliquots of stock solutions of the drugs to the organ bath. In some experiments, moreover, acetylcholine-induced vasorelaxation was similarly assessed after pretreating the preparation with the nitric oxide synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) for 30 min. Data were expressed as the percent relaxation of the phenylephrine-induced contraction.

Renal Expression of klotho mRNA

RNA extraction and Northern blot analysis were carried out as described previously (5). After the thoracic aorta was carefully removed to avoid damaging the endothelium, the kidneys were dissected from OLETF or LETO rats, immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction was performed. Total RNA was extracted from the kidneys using the acid guanidium thiocyanate-phenol-chloroform method (ISOGEN; Nippon Gene, Tokyo, Japan). Poly A+ RNA was then prepared with oligo (dT)-La
tex (oligotex dT30 <SUPER>; Japan Synthetic Rubber Co. and Roche, Tokyo, Japan), after which 2 µg samples were separated by electrophoresis on 1% agarose-formaldehyde denaturing gels and transferred to nylon membranes (Hy
bond N+; Amersham Co., Ltd., Tokyo, Japan) (3) prehy
bridized for 120 min at 42°C in a solution of 0.5% sodium dodecyl sulfate (SDS), 5 × SSPE = 3.6 M NaCl, 0.2 M NaH2PO4, 0.02 M Na2-EDTA, 10 × Denhardt’s reagent, 50% formamide, and 100 µg/ml denatured salmon sperm DNA. The membranes were then hybridized with rat klotho cDNA probes (305 bp, Sall-KpnI fragment) labeled with 32P-dCTP by random oligonucleotide priming (Boehringer Mannheim Biochemica, Mannheim, Germany), washed and exposed to X-ray film. As a control, membranes were washed and hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The radioactivity of the corresponding bands was quantitated using a FUJI BIO-Imaging Analyzer BAS 2000 Fuji Film Co., Tokyo, Japan. Klotho mRNA levels in the kidney were normalized to the level of GAPDH mRNA in each sample by calculating the klotho/GAPDH mRNAs ratios. The mean klotho mRNA levels in the kidneys of LETO (control) rats were assigned a
Table 1. Effects of Troglitazone on OLETF Rats

<table>
<thead>
<tr>
<th></th>
<th>OLETF</th>
<th>LETO</th>
<th>OLETF + TRO</th>
<th>LETO + TRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td>156 ± 3</td>
<td>146 ± 3*</td>
<td>141 ± 1*</td>
<td>148 ± 4*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>681 ± 14</td>
<td>502 ± 11*</td>
<td>646 ± 11</td>
<td>467 ± 19*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>354 ± 34</td>
<td>156 ± 14*</td>
<td>259 ± 12*</td>
<td>171 ± 13*</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>351 ± 34</td>
<td>39 ± 6*</td>
<td>148 ± 19*</td>
<td>31 ± 6*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>133 ± 7</td>
<td>90 ± 5*</td>
<td>107 ± 6*</td>
<td>83 ± 2*</td>
</tr>
</tbody>
</table>

OLETF, Osaka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka; OLETF + TRO, OLETF rats treated with troglitazone; LETO + TRO, OLETF rats treated with troglitazone. Values are mean ± SE. *p < 0.05 vs. OLETF rats.

![Fig. 1. Acetylcholine-induced, endothelium-dependent relaxation of the aorta is significantly attenuated in OLETF rats. Troglitazone administration restores acetylcholine-induced aortic relaxation in OLETF rats. *p < 0.05 vs. untreated OLETF rats.](image)

value of 1.0.

Drugs

All drugs except troglitazone were purchased from Sigma Chemicals (St. Louis, USA). Troglitazone was a kind gift from Sankyo Co., Ltd., Tokyo, Japan. Drugs were prepared fresh daily in saline.

Statistical Analysis

The data were presented as the means ± SEM. Measurements made in OLETF and LETO rats were compared using unpaired Student’s t-tests or analysis of variance (ANOVA) for repeated measures. Values of p < 0.05 were considered to indicate statistical significance.

Results

Hypertension and Metabolic Disorders in OLETF Rats

OLETF rats served as our animal model of atherogenic disease manifesting multiple risk factors. As compared to LETO (control) rats, by 30 weeks of age, OLETF rats had developed mild hypertension (156 ± 3 mmHg vs. 146 ± 3 mmHg, p < 0.05), obesity (681 ± 14 g vs. 502 ± 11 g, p < 0.05), severe hyperglycemia (354 ± 34 mg/dl vs. 156 ± 14 mg/dl, p < 0.05) and hypertriglyceridemia (351 ± 46 mg/dl vs. 39 ± 11 mg/dl, p < 0.05) (Table 1).

Endothelial Function and Renal klotho mRNA Expression in OLETF Rats

Acetylcholine (10^-5 M)-induced relaxation of precontracted aortic rings was significantly attenuated in OLETF rats (59 ± 7%) as compared to LETO rats (92 ± 2%) (Fig. 1, p < 0.05). That this effect reflected diminished endothelial synthesis of NO, rather than decreased sensitivity of the vascular smooth muscle to NO, was confirmed by the observations that the NOS inhibitor L-NAME completely abolished acetylcholine-induced aortic relaxation in both groups, and that there was no difference in the maximum relaxations evoked by exogenous NO liberated from the vasodilator sodium nitroprusside (data not shown).

In addition, renal klotho mRNA expression was significantly reduced in OLETF rats (Fig. 2). Acetylcholine (10^-5 M)-induced vasorelaxation showed a significant positive correlation with renal klotho mRNA expression in OLETF and LETO rats (r = 0.80, p < 0.01, n = 14).

Effects of Troglitazone on Endothelial Function and Renal klotho mRNA Expression in OLETF Rats

Following 10 weeks of treatment with troglitazone, systolic blood pressure in OLETF rats had declined to 141 ± 1 mmHg (p < 0.05), and plasma glucose and triglycerides had declined to 259 ± 12 mg/dl and 148 ± 19 mg/dl, respectively (Table 1, p < 0.05). Troglitazone also increased acetylcholine-induced aortic relaxation in OLETF rats to 85 ± 3%
Fig. 2. Renal expression of klotho mRNA is significantly diminished in OLETF rats. Troglitazone administration significantly increases renal expression of klotho mRNA in OLETF rats. *p<0.05 vs. LETO rats.

(Fig. 1) and significantly increased renal klotho mRNA expression (Fig. 2, p<0.05). By contrast, troglitazone had no effect on acetylcholine-induced vasorelaxation or klotho mRNA expression in LETO rats (data not shown).

Discussion

Troglitazone is a new orally effective antidiabetic agent able to partially restore proper glucose and lipid metabolism in obese patients with insulin-resistant, type 2 diabetes mellitus (10, 11). For example, treatment with troglitazone reduced plasma triglycerides in insulin-resistant coronary patients (12), and decreased insulin resistance, improved glucose tolerance and lowered blood pressures in obese subjects with either impaired or normal glucose tolerance (13). Ogishara et al. also reported that troglitazone treatment improved both glucose metabolism and blood pressure control in essential hypertensive patients with diabetes mellitus (14). Our findings indicate that oral administration of troglitazone has a similar effect in lowering plasma glucose, triglycerides, total cholesterol and blood pressure in OLETF rats.

Insufficient expression of the klotho gene is characterized by various phenotypes seen in human aging, including arteriosclerosis, pulmonary emphysema, osteoporosis, infertility and skin atrophy (1). Interestingly, adenovirus-mediated transfer of the klotho gene to homozygous klotho-deficient mice ameliorated these phenotypes (15), suggesting their suppression by the klotho gene product. We recently demonstrated that endothelium-dependent vasodilation of aorta and arterioles was significantly attenuated in klotho-deficient mice (5) and that, although klotho mRNA or protein are abundantly expressed in the kidney, they are downregulated by sustained hemodynamic or metabolic stress (2). We have also found that there was a positive correlation between renal klotho mRNA expression and acetylcholine-induced aortic relaxation. Furthermore, we have recently demonstrated that in vivo klotho gene delivery partially improved endothelial dysfunction in OLETF rats (16). In the context of the findings that vasodilatation induced by endothelium-derived NO is impaired in hypertension (17, 18), diabetes mellitus (19) and hyperlipidemia (20, 21), these observations lead us to hypothesize that decreased renal klotho gene expression is associated with endothelial dysfunction and may thus represent an atherogenic risk factor common to all of the aforementioned ailments.

Troglitazone is known to activate the peroxisome proliferator-activated receptor-γ (PPARγ), a member of the nuclear receptor superfamily of ligand-activated transcription factors. PPARγ binds to a specific sequence in the promoter of the target gene called the PPAR response elements sequence (22). There are no PPAR response elements in the promoter lesion of the klotho gene. Troglitazone is useful in the treatment of diabetes mellitus and protects rats with metabolic disorders from progressive renal injury, as PPARγ induces glucose transporter isoform 4 expression and then increases the rate of glucose transport in response to insulin. Therefore, the increase in renal expression of klotho mRNA may be due to a renoprotective effect of troglitazone, but not a direct effect of troglitazone on activating transcription of the klotho gene. We have recently reported that homozygous klotho mice showed an augmented expression of glucose transporter isoform 4 in skeletal muscle and an increase in insulin sensitivity as compared to wild-type mice (23). However, we were unable to determine the specific effect of klotho gene transfer on blood levels of glucose, triglyceride, and cholesterol, which showed significant reductions in klotho-treated as well as lacZ-treated OLETF rats. The klotho gene may preferentially affect the vascular endothelium as compared to the metabolic system. However, further study will be needed to clarify the specific effects of the klotho gene on metabolic regulation.

The present study demonstrates that troglitazone partially
restores vascular endothelial function and augments renal klotho mRNA expression in OLETF rats, which exhibit multiple atherogenic risk factors. We suggest that the capacity of troglitazone to increase renal klotho gene expression likely accounts for its capacity to improve endothelial function and makes troglitazone a potentially useful therapeutic tool for patients presenting with multiple risk factors. Moreover, our finding that troglitazone has potentially beneficial effects on serum lipid profiles suggests it may also be useful in patients with cardiovascular dysmetabolic syndrome associated with macrovascular diseases such as myocardial infarction, stroke and peripheral vascular disease.

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References