Protective effect of taurine on diabetic rat endothelial dysfunction

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ABSTRACT
As increasing evidence suggest that oxidative stress plays an important role in the developing angiopathy in diabetes, we studied the effects of taurine, a free radical scavenger, on diabetes-induced angiopathy in the rat aorta. Six-week-old male Wistar rats were randomly divided into three groups; control group (Cont), diabetes group (DM) and diabetes group treated with taurine for four weeks, 500 mg/kg/day, intraperitoneally (i.p.) (DM+T). Diabetes was induced by streptozotocin (50 mg/kg i.p.). Four weeks after the induction of diabetes, serum glucose and malondialdehyde concentrations were measured. Additionally, organ bath studies and real-time PCR on muscarinic M₃ receptor and eNOS were performed. Although taurine treatment failed to decrease serum glucose levels, the increased serum malondialdehyde levels in diabetic rats were significantly decreased after taurine treatment. Norepinephrine-induced hyper-contractility as well as acetylcholine-induced, endothelium-dependent hypo-relaxation in diabetes were significantly prevented after taurine treatment. The differences in the expressions of muscarinic M₃ receptor mRNAs were statistically non-significant between groups. Moreover, diabetes-induced up-regulation of eNOS mRNAs was slightly prevented after taurine treatment. These data suggest that taurine acts beneficially against the diabetes-induced vascular dysfunction. Its potential action as a radical scavenger ameliorates the vascular disorders in diabetes.

Vascular disease is a prominent sequela of both type-1 and type-2 diabetes (7). Cardiovascular disease alters vascular responsiveness to several vasoconstrictors and vasodilators and is a major factor underlying the development of this disease (21). Most of the complications in diabetes are caused by the increased serum glucose and the increased generation of oxygen-derived free radicals, which lead to endothelium dysfunction and neuropathy. Although strict glycemic control delays the onset and slows down the progression of diabetic vascular complications (8, 25), this strategy is not successful in all patients.

It has been demonstrated that there are functional changes in various smooth muscle cells in diabetic animals (16). Oxidative stress plays a pivotal role in the development of diabetes complications, both microvascular and cardiovascular. The metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as well as in the myocardium. The increased superoxide production causes the activation of five major pathways involved in the pathogenesis of microvascular and cardiovascular damage: polyol pathway flux, increased formation of advanced glycation end products (AGEs), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C...
isoforms, and overactivity of the hexosamine pathway (16). It also directly inactivates two critical anti-atherosclerotic enzymes, endothelial nitric oxide synthase (eNOS) and prostacyclin synthase. Through these pathways, increased intracellular reactive oxygen species (ROS) cause defective angiogenesis in response to ischemia, activate a number of pro-inflammatory pathways, and cause long-lasting epigenetic changes that drive persistent expression of proinflammatory genes after glycemia is normalized (16). It has been shown that vessels from diabetic animals exhibit abnormal endothelium-dependent vascular relaxation to acetylcholine (15).

The diabetes-induced functional changes may be associated with endothelial dysfunction in diabetes. Impaired endothelium-dependent vasodilatation may arise from several mechanisms: decreased production of one of the endothelium-derived relaxing factors (EDRFs), including nitric oxide (NO) and prostacyclin (prostaglandin I₂), enhanced inactivation of EDRF, impaired diffusion of EDRF to the underlying smooth muscle, and enhanced generation of endothelium-derived constricting factors (EDCF) (5). In particular, NO plays an important role in vasodilation. NO is synthesized by three different NO-synthase (NOS) isoforms: inducible NOS (iNOS), brain-type NOS (bNOS), and eNOS (9). Furthermore, previous studies using pharmacological approaches with muscarinic receptor agonists and antagonists suggest that the muscarinic M₁ receptor mainly mediates vasodilation via the actions of eNOS in the rat aorta (4, 11).

Taurine is a semi-essential amino acid for humans, and exists naturally in seafood and meat (27). Plasma levels of this amino acid are highly dependent on food content; vegans have 50% lower taurine plasma levels compared to omnivores (17). Health drinks containing taurine are marketed for athletic and mental performance and for the treatment of various conditions, for improvement of athletic and mental performance and for general well being (2). Taurine plays a pivotal role in several essential biological processes such as membrane stabilization and immunity; it has antioxidant and anti-inflammatory effects (20). In the present study, we tried to investigate the possible role of taurine on diabetes-induced angiopathy in the rat.

MATERIALS AND METHODS

Animal models. All experiments were performed in accordance with the guidelines set by Tottori University Committee for Animal Experimentation. Six-week old male Wistar rats were divided randomly into three age-matched groups. One group included non-diabetic control animals (Cont group, n = 10), which were administered 0.1 M citrate-phosphate buffer (pH 4.2) with normal saline intraperitoneally (i.p.). In the other two groups diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (50 mg/kg) dissolved in 0.1 M citrate-phosphate buffer (pH 4.2) (19). One day after STZ injection, the induction of diabetes was confirmed by measuring urinary glucose with Pretest 3aIl (Wako Pure Chemical, Osaka, Japan) and diabetic animals having more than (3+) of urinary glucose levels were included in the study. Two days after the induction of diabetes, the rats of one diabetic group were treated for another four weeks with normal saline intraperitoneally (DM group, n = 14), whereas the rats of the other diabetic group were treated with taurine intraperitoneally at a daily dose of 500 mg/kg, for another four weeks (DM+T group, n = 8). All animal groups were kept under identical conditions and had access to food and drinking water ad libitum. Four weeks after the induction of diabetes, the rats were sacrificed with an overdose of pentobarbital (60 mg, i.p.). Blood samples were collected from the vena cava, and the aorta was removed from each animal and placed in Krebs-Henseleit solution comprised of (mM): NaCl 118.0, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 24.9, glucose 5.6 and sodium pyruvate 2.0, for use in functional and biochemical studies.

Serum glucose and malondialdehyde (MDA) measurements. Serum glucose concentrations in the experimental rats were measured by the hexokinase method (Glucose CII; Wako Pure Chemical, Osaka, Japan), which was carried out according to the kit manufacturer’s instructions. In order to investigate oxidative stress, the serum MDA concentrations were measured by colorimetric assay, according to the manufacturer’s instructions (NWLSSTM Malondialdehyde Assay; Northwest Life Science Specialties, LLC, Vancouver, WA).

In vitro organ bath experiments. Functional studies were performed according to the methods described in previous reports of this laboratory (12, 23). The thoracic aortas were cut into approximately 3-mm-long ring segments. Each ring was suspended on a wire hook in an organ bath (25 mL) containing Krebs-Henseleit solution, and bubbled with 5% CO₂ and 95% O₂ (37°C). One hook was suspended from a transducer (type 45196A; San-ei Instruments, To-
kyo, Japan), and the lower hook was fixed to a plastic support leg to a micrometer (Mitutoyo, Tokyo, Japan). Each ring was equilibrated unstretched for 30 min. A load of 0.5 g was applied to each ring by micrometer adjustment, and the load was readjusted to this level 30 min later. Changes in the tone were recorded by a force transducer on a personal computer (Macintosh G3; Apple Computer, Cupertino, CA) by use of Chart v 3.6.9 software and a PowerLab/16sp data acquisition system (AD Instruments, Castle Hill, Australia). Following a 30-min period of equilibration, the rings were exposed to 100 mM KCl. In the aorta rings, the contractile response to norepinephrine (1 × 10⁻⁹–3 × 10⁻⁴ M) was determined cumulatively. After a 30-min washout period, propranolol (1 × 10⁻⁶ M) was added to prevent the involvement of β-adrenoceptors. Endothelium-mediated relaxation was measured as a concentration-response curve to acetylcholine (3 × 10⁻⁸–1 × 10⁻⁴ M) in rings precontracted with the submaximal dose of norepinephrine (3 × 10⁻⁷ M). Endothelium-independent aortic relaxation in response to nitroglycerin (1 × 10⁻⁶ M) was also measured in the rings.

Real-time polymerase chain reaction (PCR) of muscarinic M₃ receptor and eNOS mRNAs in the aorta. Real-time PCR was performed according to our previous reports (23). Muscarinic M₃ receptor and eNOS mRNAs in the aorta were measured by real-time PCR method. The mRNAs were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The reverse transcriptase (RT) mixture (20 μL) containing 2 μg of total RNA was prepared and incubated at 37°C for 60 min. Fifteen micro liters of the mixture were used for real-time PCR, which was carried out using a Light Cycler thermal cycler system with a LightCycler-FastStart Hybridization Probe kit (Roche Diagnostics, Tokyo, Japan). The primers and probe sequences specific to the genes of muscarinic M₃ receptors (GeneBank Accession: NM_012527), eNOS (GeneBank Accession: AJ011116), and β-actin (GeneBank Accession: NM_031144) were used according to our previous reports (23). The primer and probe of the β-actin used were from the LightCycler-Primer/Probe Set (rat), and was used as the internal standard. A total of 5 μL of cDNA solution was used for the sample.

Data analysis and statistical analysis. The data for the contractions induced by norepinephrine were normalized by the contractions induced by 100 mM KCl. The relaxation responses with submaximal contraction caused by 3 × 10⁻⁷ M norepinephrine were expressed as percentages of nitroglycerin. The EC₅₀ and E₇⁰ values were obtained using a Macintosh computer (G3) loaded with Chart v3.6.9 software and a PowerLab/16sp data acquisition system. The EC₅₀ values were calculated as geometric means, whereas the E₇⁰ values were calculated as arithmetic means. A statistical comparison of differences between groups was performed using analysis of variance and Fisher’s multiple comparison tests. P < 0.05 was regarded as the level of significance.

Drugs and chemicals. Taurine, propranolol and norepinephrine were purchased from Sigma-Aldrich (St. Louis, MO). Nitroglycerin (millisrol®) was purchased from Nihonkayaku Co., Ltd. (Tokyo, Japan). STZ was purchased from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals were available commercially and of reagent grade.

RESULTS

Body weight, serum glucose levels and serum MDA levels of experimental rats

The general features of the experimental animals are presented in Table 1. The STZ-induced diabetic rats showed significantly smaller body weights in comparison with the control animals. Treatment with taurine significantly increased the body weight compared to those of the DM group. However, the body weight in the DM+T group was still significantly smaller than those in the Cont group. The serum glucose levels in the DM group were significantly
higher than those of the Cont group. Treatment with taurine failed to reduce the serum glucose levels. The serum MDA levels in the diabetes were significantly larger than those in the Cont group. Treatment with taurine significantly prevented diabetes-induced increases in serum MDA levels.

**Measurement of contraction and relaxation**

Functional data obtained from organ bath studies are shown in Fig. 1 and Table 2. The maximum contraction ($E_{\text{max}}$) values and $EC_{50}$ values for the contractile responses of the aorta rings to norepinephrine and KCl (100 mM) were determined (Table 2). The $E_{\text{max}}$ values in response to norepinephrine in the diabetic rats were significantly higher than those in the control rats. This increase in maximum contraction was significantly prevented by treatment with taurine. The maximum relaxation ($E_{\text{max}}$) and $EC_{50}$ values are also shown in Table 2. In the aortic rings precontracted by norepinephrine with intact endothelium obtained from all groups, the relaxation was produced in a dose-dependent manner. The relaxation produced by acetylcholine was markedly reduced in the diabetic aorta. The attenuated relaxation was sig-

![Fig. 1](image-url)  
**Fig. 1** Contractile response and relaxation in the rat aorta. Left panel: contractile response of rat aortic rings to norepinephrine. The data of contraction induced by norepinephrine were normalized by the KCl (100 mM). Right panel: endothelium-dependent relaxation of rat aortic rings to acetylcholine. In the right panel, the data of relaxation were normalized by the nitroglycerin. Cont: control rats. DM: diabetic rats. DM+T: diabetic rats treated with taurine. NE: norepinephrine. ACh: acetylcholine. Data are shown as mean ± S.E.M. of ten separated determinations in each group. *p < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>DM</th>
<th>DM+T</th>
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<tr>
<td>$E_{\text{max}}$/KCl</td>
<td>1.58 ± 0.83</td>
<td>2.20 ± 0.55*</td>
<td>1.96 ± 0.56*</td>
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<tr>
<td>$EC_{50}$ (1 × 10^{-8} M)</td>
<td>4.11 ± 1.59</td>
<td>4.03 ± 0.96</td>
<td>2.00 ± 0.31</td>
</tr>
<tr>
<td>Maximum relaxation (%)</td>
<td>67.6 ± 11.1</td>
<td>33.3 ± 5.0*</td>
<td>57.4 ± 5.3*</td>
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<tr>
<td>$EC_{50}$ (1 × 10^{-7} M)</td>
<td>3.47 ± 1.80</td>
<td>3.21 ± 3.48</td>
<td>1.83 ± 0.62</td>
</tr>
<tr>
<td>Maximum relaxation (%)</td>
<td>104.6 ± 17.2</td>
<td>101.3 ± 15.8</td>
<td>107.0 ± 11.0</td>
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Cont: control group, DM: diabetic group, DM+T: diabetic group treated with taurine daily (500 mg/kg/day)  
*) Significantly different from Cont group. #) Significantly different from DM group. (P < 0.05)
Effect of taurine on diabetes

Effect of taurine on diabetes. However, there were no significant differences in EC₅₀ values between any groups in contractile or relaxation responses. The nitroglycerin-induced relaxation was not altered by the induction of diabetes or the treatment with taurine (Table 2).

Measurement of muscarinic M₃ receptor and eNOS mRNAs in the aorta

The expression of muscarinic M₃ receptor and eNOS mRNAs in the aorta are presented in Fig. 2. The expressions of muscarinic M₃ receptor mRNA levels were slightly but not significantly higher in diabetic animals than those in the control animals, while the expressions of eNOS mRNAs were significantly higher in the diabetic animals than those in control animals. Treatment with taurine prevented slightly those up-regulations but did not reach any statistical significance.

DISCUSSION

In the present study, we demonstrated that diabetes induces vascular hyper-reactivity to norepinephrine and impairment of endothelium-dependent relaxation by acetylcholine. While the expressions of muscarinic M₃ receptor mRNA levels were not changed, the expressions of eNOS mRNA were significantly increased in the diabetic rat aorta. Although taurine did not improve the serum glucose levels in the diabetic rats, it significantly improved the increased contraction produced by the α₁-agonist norepinephrine and the decreased endothelium-dependent relaxation induced by acetylcholine. Taurine also ameliorated serum MDA levels in the diabetic animals.

Taurine is reported to have anti-oxidant and anti-inflammatory effects (20). Sethupathy et al. (22) demonstrated that the oxidant activity, measured by thiobarbituric acid reactive substances (TBARS), was significantly less in the plasma of male Wistar rats fed a high-fat diet supplemented with 50 mg/kg/day taurine for six months (1.6 nmol/mL) compared to rats fed a high-fat diet without taurine supplementation (2.4 nmol/mL). Serum TBARS were also significantly lowered in apolipoprotein E-deficient mice after 2% taurine supplementation for 12 weeks (8.6 nmol/mL), compared to mice without taurine supplementation (11.1 nmol/mL) (13). Taurine is also known to react with hypochlorous acid (HOCl), a powerful oxidant, to create a more stable taurine chloramine (TauCl) in vivo to block the production of proinflammatory cytokines. The adhesion of circulating leukocytes to endothelial cells and their transendothelial migration is an initiating step of atherosclerosis (18). The expression of intracellular adhesion molecule-1 (ICAM-1), which mediates cell-cell adhesion, was decreased by taurine in Sprague-Dawley rats with impaired ROS scavenging capability. Taurine given intravenously at 200 mg/kg for 5 days before the induction of inflammation prevented a significant increase in the expression of ICAM-1 in the post-capillary venules (high endothelial cell region) (6). The production of tumor necrosis factor-α (TNF-α), an important pro-inflammatory cytokine, has been shown to be down-regulated

![Fig. 2](image-url) Expressions of muscarinic M₃ receptor and eNOS mRNAs in the rat aorta. Left panel: expressions of muscarinic M₃ receptor mRNAs in the rat aorta normalized by β-actin. Right panel: expressions of eNOS mRNAs in the rat aorta normalized by β-actin. Cont: control rats. DM: diabetic rats. DM+T: diabetic rats treated with taurine. Data are shown as mean ± S.E.M. of five to seven separated determinations in each group. *) Significantly different from the Cont group. (P < 0.05)
by taurodilione, a derivative of taurine. Taurine blocked the production of TNF-α by 50–90% in human peripheral blood mononuclear cells from healthy donors stimulated by lipopolysaccharide and interferon-α (3). Thus, taurine possesses powerful anti-oxidant and anti-inflammatory effects on mammals.

Kamata and his associates reported that the acetylecholine-induced concentration-dependent relaxation was significantly attenuated in aortic rings that were taken from cholesterol-fed and STZ-induced diabetic mice (10). Additionally, the impaired endothelium-dependent vasodilatation encountered in both cholesterol-fed and STZ-diabetic mice could be normalized by the chronic administration of taurine. This effect might be, at least in part, due to lowering of serum low-density lipoprotein levels (10). Wang et al. reported that taurine improved vascular endothelial dysfunction induced by experimental type 1 diabetes (26). According to the authors, this effect might be associated with down-regulation of lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) and ICAM-1 expression on aortic vascular endothelium via its anti-oxidative property (26). Abebe in his study suggests that taurine ameliorates or prevents vascular reactivity alterations in diabetes (1). This effect of the nutrient on diabetic arteries appeared to be associated with greater inhibition of calcium mobilization and PKC-mediated responses. Impairment of endothelial-dependent vasorelaxation observed in diabetic rat aortas in response to acetylcholine was also reversed by taurine. Similarly, high glucose-induced enhancement of aortic smooth muscle contractility and depression of endothelium-mediated aortic relaxation in response to phenylephrine and acetylcholine, respectively, were prevented by co-incubation of the tissues with taurine (1). From the previous reports it can be deduced that taurine ameliorates the endothelial dysfunction caused under pathological conditions such as diabetes.

Oxidative stress as well as nitrosative stress are important to understand the pathogenesis of diabetes-induced aortic dysfunction. Increased extracellular glucose concentration induces a dysregulation of reactive oxygen and reactive nitrogen generating pathways. Endothelial cells placed in an increased extracellular glucose condition respond with a marked increase in mitochondrial superoxide formation. Superoxide, when combining with NO generated by eNOS, leads to the formation of peroxynitrite, a cytotoxic oxidant. Reactive oxygen and reactive nitrogen species trigger endothelial cell dysfunction through a multitude of mechanisms including substrate depletion and uncoupling of eNOS (24). It is important to understand another possibility about taurine’s favorite effect on NO-NOS pathway. From our real-time PCR studies, although there were no significant differences of expressions of muscarinic M1 receptor mRNAs in each group, diabetes-induced up-regulation of eNOS mRNAs was slightly but not significantly prevented by treatment with taurine. From these data, it is assumed that the translation from eNOS mRNAs to eNOS protein and the subsequent downstream of eNOS activation should be impaired after the induction of diabetes. NO production from constitutive activation of eNOS by phosphorylation contributes to vascular homeostasis. Recently, we reported that treatment with edaravone, a radical scavenger, significantly improved the increased diabetes-induced serum MDA levels, the decreased penile eGMP concentrations, the increased diabetes-induced norepinephrine-mediated contractions, and the decreased acetylecholine-mediated relaxation in the rat penile tissue (14). In the same study we clearly demonstrated that the expressions of phosphorylated nNOS as well as phosphorylated eNOS in the diabetes in the rat corpus cavernosum were significantly lower than those in the controls, and that these down-regulations of phosphorylated nNOS and phosphorylated eNOS were significantly recovered by the treatment with edaravone (14). Under the light of the above findings we may hypothesize that diabetes could induce down-regulation of phosphorylated eNOS, which could be prevented by treatment with taurine. Taurine could ameliorate the NO-NOS pathway and thus prevent, at least, partially the developing angiopathy in diabetes mellitus in the rat. In the present study, since we had only limited volume of aortic tissue, we were unable to perform western blot analysis of eNOS or phosphorylated eNOS.

In conclusion, we demonstrated that taurine prevented diabetes-induced angiopathy in the rat, and that taurine worked as a radical scavenger. However, further studies are required to reveal the mechanisms of taurine on diabetes-induced angiopathy.

REFERENCES

Effect of taurine on diabetes

peripheral blood mononuclear cells. Cytokine 3, 568–575.


