Gender Differences in Vascular Reactivity of Aortas from Streptozotocin-Induced Diabetic Mice

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Diabetes mellitus is a syndrome of disordered metabolism, usually due to a combination of hereditary and environmental causes, resulting in abnormally high blood glucose levels. The two most common forms of diabetes are due to either a diminished production of insulin (type 1) or a diminished response to insulin (type 2). Diabetes is a risk factor in the development of both macro- and microvascular diseases. Indeed, it increases the incidence of ischemic heart disease, cerebral ischemia, and atherosclerosis, conditions in which endothelial dysfunction plays a role in pathogenesis. One of the most important functions of the endothelium is the production of nitric oxide (NO) in response to various hormonal, mechanical, and chemical stimuli. NO has a variety of effects, including the induction of vascular relaxation. In patients with either type 1 or type 2 diabetes, the forearm blood-flow (dilator) responses to acetylcholine (ACH) are reduced, suggesting endothelial dysfunction. In streptozotocin (STZ)-induced diabetic rats or mice, which are models of type 1 diabetes, the endothelium-dependent relaxation induced by ACH is impaired, and reactive oxygen species and polyol products have been suggested to be involved in this impairment. Moreover, there is evidence that endothelial function is impaired in patients with diabetes mellitus in a manner similar to that seen in STZ-treated animals. Thus, STZ-induced diabetic rats and mice may provide models for studies of the underlying causes of endothelial-cell damage in diabetes mellitus, and a means of assessing the contribution of diabetes mellitus to cardiovascular disease states.

Gender differences in blood pressure and vascular reactivity have been reported in rats and mice. Although in the general (non-diabetic) population, cardiovascular diseases are more frequent in men than in premenopausal women, diabetes may produce a greater impairment in the female cardiovascular system, with the result that the above difference between men and premenopausal women disappears in diabetic patients. Although some studies have been carried out on the effects of exogenous sex hormones in diabetes, few studies have compared the influence of diabetes on vascular reactivity between males and females.

Against this background, we decided to assess whether there are gender differences in the effects of type 1 diabetes on the endothelium-dependent vascular relaxations to various stimuli. For this, we employed aortas isolated from STZ-induced diabetic mice. Although there have been many reports that male diabetic-model mice or rats exhibit endothelial dysfunction, little is known about gender differences in endothelial function in diabetes, particularly in murine models. That was our rationale for investigating gender differences in vascular reactivity in the aorta using STZ-induced type 1 diabetic-model mice.

Key words diabetes; gender difference; endothelium-dependent relaxation; adiponectin; mouse

MATERIALS AND METHODS

Reagents STZ, clonidine hydrochloride, insulin from porcine pancreas, norepinephrine (NE), and Nω-nitro-arginine (l-NNA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium nitroprusside dehydrate (SNP) was from Wako (Osaka, Japan), while acetylcholine chloride (ACH) was from Daichi Pharmaceuticals (Tokyo, Japan). All drugs were dissolved in saline, unless where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath.

Animals and Experimental Design Male and female ICR mice aged 4 weeks were housed under constant climatic conditions (room temperature 21—22 °C, room humidity 50±5%), and food and water were allowed ad libitum to all
animals. Mice were randomly allocated to diabetic and control groups. At five weeks of age, to induced diabetes mice received a single injection of STZ (200 mg/kg) via the tail vein, as in previous studies. The age-matched controls were injected with a similar volume of citrate buffer. Finally, for isolation of their aortas, mice were anesthetized with diethyl ether and euthanized by decapitation 10 weeks after treatment with STZ or buffer. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan).

**Measurement of Plasma Glucose, Cholesterol, and Triglyceride** Plasma parameters were measured as described previously.

**Measurement of Isometric Force** Each aorta ring was placed in a bath containing 10 ml modified Krebs–Henseleit solution (KHS; bubbled with 95% O₂ plus 5% CO₂, and kept at 37 °C), and one end of each ring was connected to a tissue holder and the other to a force-displacement transducer, as previously described. The above solution contained (mM): NaCl 118.0, KCl 4.7, NaHCO₃ 25.0, CaCl₂ 1.8, NaH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11.0. For the relaxation studies, rings were precontracted with an equieffective concentration of prostaglandin F₂α (PGF₂α) (10⁻⁶—3×10⁻⁶ mol/l). When the PGF₂α-induced contraction had reached a plateau level, ACh (10⁻⁹—10⁻⁵ mol/l), clonidine (10⁻⁹—10⁻⁵ mol/l), insulin (10⁻⁸—3×10⁻⁵ mol/l), or sodium nitroprusside (SNP) (10⁻¹⁰—10⁻⁵ mol/l) was added in a cumulative manner.

**Measurement of NO₂⁻ and NO₃⁻** The concentrations of nitrite and nitrate in the effluent from each tissue were sampled and assayed by the method described previously (ENO-20; Eicom). Each aorta was cut into transverse rings 5 mm in length. These were placed in 0.5 ml Krebs–Henseleit solution at 37 °C. Samples were collected on two occasions as follows: for one 20-min period before and one after application of 10⁻⁷ mol/l ACh. The amount of NOx was calculated as follows: agonist-stimulated NOx (10⁻⁷ mol/min·g⁻¹) = sample/20 (min) · g (weight of the frozen aorta). The concentrations of NO₂⁻ and NO₃⁻ in the Krebs–Henseleit solution and the reliability of the reduction column were examined in each experiment.

**Statistical Analysis** Data are expressed as the mean± S.E. mean. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after one-way analysis of variance. Statistical comparisons between concentration–response curves were made using one-way ANOVAs, with Bonferroni’s correction for multiple comparisons being performed post hoc. In each test, p<0.05 was regarded as significant.

**RESULTS**

**General Parameters** Ten weeks after treatment with STZ, male and female mice showed hyperglycemia, hypercholesterolemia, hypertriglyceridemia, and lower body weights than the corresponding age-matched control mice (Table 1). The plasma cholesterol level was significantly lower in diabetic females than in diabetic males. The plasma insulin level was below the limit of detection in STZ-induced diabetic male and female mice. The plasma adiponectin level was significantly higher in nondiabetic females than in nondiabetic males (females; 43.7±3.0 μg/ml vs. males; 14.5±1.1 μg/ml). In each gender, the plasma adiponectin level was lower in diabetic mice than in the controls, but it was significantly higher in diabetic females than in diabetic males (females, 13.0±1.2 μg/ml vs. males; 6.15±0.5 μg/ml) (Fig. 1).

**Relaxation Responses to Acetylcholine and Sodium Nitroprusside** When the prostaglandin F₂α (10⁻⁶—3×10⁻⁶ mol/l)–induced contraction had reached a plateau, ACh (10⁻⁹—10⁻⁵ mol/l) or SNP (10⁻¹⁰—10⁻⁵ mol/l) was added cumulatively in aortic rings from controls and diabetic mice of either gender. ACh induced dose-dependent relaxation. By comparison with male control mice, male diabetic mice showed significantly blunting of the ACh-mediated endothelium-dependent relaxation (Fig. 2A). In contrast, there was no significant difference in the ACh-mediated relaxation between the female control and female diabetic groups (Fig. 2B). The SNP-induced endothelium-independent relaxation was not different between control and diabetic aortas whether they were from male or female mice (Figs. 3A, B).

**NO₃⁻ Production Induced by Acetylcholine** In view of the published evidence that the endothelium derived nitric oxide synthase (eNOS)/NO signaling pathway plays a major role in endothelium-dependent relaxation in the aorta, we performed an analysis of this pathway using ACh-stimulated aortas from control and diabetic mice of either gender. ACh increased the NO₃⁻ (NO₂⁻ plus NO₃⁻) level in the perfusate from each group of aortic rings (Fig. 2C). This effect of ACh (10⁻⁷ mol/l) on NO₃⁻ production was weaker in STZ-induced diabetic male mice than in either the control male or diabetic female groups.

**Contractile Responses to Norepinephrine** Figure 4 shows response curves for the contractile responses of aortic
rings to norepinephrine (NE). In aortic rings from controls and diabetic mice of either gender, the concentration–response curve for NE (10^{-10}—10^{-7} \text{ M}) was bell-shaped. The aortic contraction induced by NE was enhanced in rings obtained from diabetic female mice, compared with those from age-matched female control mice (Fig. 4B). When aortic rings were incubated with l-NNA (10^{-5} \text{ M}), a nitric oxide synthase (NOS) inhibitor, the NE-induced bell-shaped curves were changed to sigmoid-shaped curves, and the responses were greatly increased especially at the higher concentrations in all groups (Figs. 4C, D; note the different ordinate scales between Figs. 4A—D). In the presence of l-NNA, NE-induced contractions were not significantly different between aortas from controls and diabetic mice (whether male or female) (Figs. 4C, D).

Relaxation Responses to Clonidine and Insulin We previously reported that in mouse aortas treated with clonidine or insulin, both the endothelium-dependent relaxation and the enhanced NO production were regulated by the phosphatidylinositol 3 (PI3)-kinase/Akt/NOS signal pathway, rather than by the classical Ca^{2+}/calmodulin/NOS pathway. In the present study, when the contraction induced by prostaglandin F_{2\alpha} (10^{-6}—3\times10^{-8} \text{ M}) had reached a plateau, clonidine (10^{-9}—10^{-5} \text{ M}) or insulin (10^{-8}—3\times10^{-8} \text{ M}) was added cumulatively to yield a concentration–response curve. In aortas from male mice, the clonidine-induced endothelium-dependent relaxation was not different between the controls and diabetics (Fig. 5A). On the other hand, in aortas from female mice this relaxation was...
Likewise, we found that ACh-stimulated NO production endothelium-dependent aortic relaxation is impaired.27) It is unclear at present, however, which missing acute exposure to high glucose impairs ACh-induced relaxation.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic aorta, and indeed the effect might be initiated by oxygen species.23—25) The present data suggest that ACh-independent aortic relaxation exhibited by streptozotocin-induced diabetic weaker in the diabetics (Fig. 5B). Similarly, the endothelium-dependent aortic relaxation induced by insulin was significantly weaker in the female diabetic group than in the female controls, but not different between the male diabetic and male control groups (Figs. 6A, B).

DISCUSSION

In the present study, the impairments of endothelium-dependent aortic relaxation exhibited by streptozotocin-induced diabetic mice were found to differ between the genders, as follows. (a) The ACh-induced endothelium-dependent relaxation was impaired by diabetes only in the aortas from male mice. (b) The NE-induced contractile response was enhanced by diabetes only in aortas from female mice. (c) Both the clonidine-induced and insulin-induced endothelium-dependent relaxations were impaired by diabetes only in aortas from female mice. (d) The plasma adiponectin level was higher in nondiabetic females than in nondiabetic males, and although it was lower in diabetic mice than in the controls in each gender, it was higher in diabetic females than in diabetic males.

An attenuation of NO releases from the endothelium in certain disease conditions has been put forward as one of the important factors contributing to impaired endothelium-dependent relaxation. The impairment of the acetylcholine-induced vascular relaxation observed in disease conditions has been postulated to be due to a decrease in the bioavailability of NO, in turn caused by an increased generation of reactive oxygen species.23—25) The present data suggest that ACh-induced endothelium-dependent aortic relaxation is impaired by STZ-induced diabetes only in male mice, not in female ones. Likewise, we found that ACh-stimulated NO production was decreased by diabetics in aortas from male mice, but not in those from female ones (Fig. 2C). This is consistent with previous observations of a dysfunction of the aortic endothelium in diabetic male rats and mice.9,26) On the other hand, Goel et al. reported that only in the female rat aorta, acute exposure to high glucose impairs ACh-induced relaxation.27) It is unclear at present, however, which missing action of plasma adiponectin in endothelial cells might be responsible for impaired ACh-induced relaxation in the male diabetic aorta, and indeed the effect might be initiated by changes in the plasma glucose level or in the level of any of several hormones, including adiponectin. Indeed, the plasma levels of glucose, triglyceride, and total cholesterol were all raised in diabetic mice of either gender, but the plasma glucose or triglyceride not differed between diabetic males and diabetic females. In contrast, there was a gender difference in the plasma adiponectin level in diabetic mice (Fig. 1). Low adiponectin levels are associated with endothelial dysfunction and a predisposition to vascular injury.29,30) In cultured endothelial cells, adiponectin has been shown to exhibit various anti-inflammatory effects, in particular those that counter the adverse cellular influences of increased oxidative stress or stimulation with cytokines such as tumor necrosis factor-α (TNF-α).31) Moreover, adiponectin enhances NO production by endothelial cells.32,33) To judge from these reports, although it is only one of the possible factors the difference in plasma adiponectin level between males and females might contribute to endothelium protection in female diabetic mice.

In addition, it is possible that the signaling cascade downstream of NO release from endothelial cells is malfunctioning in disease conditions, and that this could account for the reduced endothelium-dependent relaxation observed in diabetic aortas. To explore this possibility, the aortic relaxation induced by SNP, a general NO donor was evaluated. However, there was no evident difference in the magnitude of the SNP-induced vascular relaxation between control and diabetic aortas from either gender (Fig. 3). This result suggests that there was no change in the aortic sensitivity to NO in our diabetic mice regardless of whether they were male or female.

To explore other possible gender differences in vascular reactivity, we examined the aortic contractile response to NE. This response was markedly increased in all groups by treatment with an inhibitor of nitric oxide synthase, suggesting that the NE-induced contractile response is negatively regulated by nitric oxide released from the endothelium. Since the ACh-induced endothelium-dependent aortic relaxation was impaired by STZ-induced diabetes in male mice, the NE-induced contractile response might be expected to be enhanced in the male diabetic group. However, in our male mice it was not different between the controls and diabetics. Interestingly, though, it was enhanced in the female diabetic mice (vs. their age-matched controls).

In mouse aortic rings, the observed NE-induced contractile response may be the result of a summation of a contraction mediated by α2-adrenoceptors on the smooth muscle and a relaxation mediated by α2-adrenoceptors on the endothelium. We therefore examined the relaxation response to clonidine, an α2-adrenoceptor agonist, in diabetic aortas from each gender. The clonidine-induced endothelium-dependent relaxation response was found to be blunted by STZ-induced diabetes in female aortas, but not in male ones. These results suggest that the enhancement of the NE-induced contractile response in aortas from female diabetic mice was caused by an impairment of the action of endothelial NO on the α2-adrenoceptor. In addition to the α2-adrenoceptor-activator clonidine, we also explored whether the effect of an other physiological relaxing agent, insulin, might be affected by diabetes in either gender. When insulin was applied cumulatively, the endothelium-dependent relaxation response was impaired by STZ-induced diabetes in female aortas, but not in male ones (like the con-
centration-dependent response to clonidine). Many stimuli (including α₂-agonists, insulin, vascular endothelial growth factor, β-agonists, estrogen, and shear-stress signals) regulate NO production by activating eNOS via Ser-1177 phosphorylation through the PI3-kinase/Akt pathway. We previously found that in the mouse aorta, neither addition of LY294002 [an inhibitor of phosphatidylinositol 3 (PI3)-kinase] nor addition of Akt inhibitor had any significant effect on the ACh-induced relaxation or on ACh-induced NO/cGMP production, whereas the clonidine- and insulin-induced relaxation responses were completely abolished by each of these inhibitors. These observations suggest that in mouse aortas exposed to clonidine or insulin, both endothelium-dependent relaxation and NO production may be mediated or regulated by the PI3-kinase/Akt signal pathway. The present results suggest that in aortas from female diabetic mice, but not from male diabetic mice, there may be impairments of endothelium-dependent relaxation responses that depend on the PI3-kinase/Akt/eNOS pathway.

Interestingly, although our data indicate that endothelium-dependent relaxation was impaired by diabetes in both males and females, the affected pathways leading to NO production may have differed between the genders. In endothelial cells, the main signal-transduction pathway for agonist-stimulated eNOS activation depends on Ca²⁺/calmodulin/caveolin-1. At the cellular level, there is growing evidence that for some agonists, such as acetylcholine, histamine, and bradykinin, a rise in intracellular Ca²⁺ is necessary for NO production. In contrast, as already mentioned, for other stimuli (such as α₂-agonists, insulin, and estrogen) a rise in Ca²⁺ is not required, and NO production is activated by eNOS through the PI3-kinase/Akt pathway. The attenuation of the vasorelaxation induced by ACh suggests that the former pathway (i.e., that through Ca²⁺/calmodulin/caveolin-1/eNOS) is impaired in male diabetic mice. In contrast, in female diabetic mice the latter pathway (i.e., the PI3-kinase/Akt/eNOS pathway) is evidently impaired. Since epidemiological studies have established the possession of female gender as a protective factor in the development of various cardiovascular diseases (including atherosclerosis and hypertension), the endothelium-dependent relaxations induced by ACh, clonidine, and insulin might be expected not to be different between female control and female diabetic mice. However, we found that both the clonidine- and insulin-induced vasorelaxations were attenuated (vs. the age-matched controls) in female diabetic mice, but not in male diabetic mice. This development in female diabetic mice might be taken as evidence that an impairment of cardiovascular responses occurs not only in males, but also in females in diabetic conditions, even though females may be at lower risk. Concerning the difference between males and females in diabetes, the sex hormone estrogen has been proposed as one of the factors contributing to the impairment of endothelium-dependent relaxation in female diabetic mice. It should be noted that estrogen activates eNOS through the PI3-kinase/Akt pathway, which is impaired in female diabetic mice. Although estrogen may exert an endothelium-protecting influence in normal females, it might have a reduced influence in diabetic conditions. One difference between males and females might therefore be such an impairment of endothelium-dependent relaxation through the PI3-kinase/Akt pathway in female mice. However, this issue will require further investigation.

Collectively, our data demonstrate that an impairment of endothelial function in the aorta is present in STZ-induced diabetic mice, both male and female. However, this impairment may occur through separate pathways that differ between the genders. This gender-related difference may result, at least in part, from the presence of higher plasma levels of adiponectin and estrogen in females.

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REFERENCES


