Full Paper

Gender Differences in Endothelial Function in Aortas From Type 2 Diabetic Model Mice

Yasuhiro Takenouchi¹, Tsuneo Kobayashi¹, Kumiko Taguchi¹, Takayuki Matsumoto¹, and Katsuo Kamata¹,*

¹Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

Received April 27, 2009; Accepted July 24, 2009

Abstract. Type 2 diabetes mellitus is associated with high mortality and morbidity, mainly due to coronary artery disease and atherosclerosis, although female gender is a protective factor in the development of, for example, atherosclerosis and hypertension. Our main aim was to investigate gender differences in endothelial function in aortas from type 2 diabetic model mice. The nonfasting plasma glucose level was significantly elevated in diabetic mice (both males and females). The plasma insulin level was not different between controls and diabetics (either gender). The plasma adiponectin level was decreased by diabetes, and was lower in males. In control aortas (from males or females), the clonidine-induced relaxation was abolished by Akt-inhibitor treatment. In diabetic males (versus both control males and diabetic females): a) the clonidine- and insulin-induced endothelium-dependent aortic relaxations were impaired, but the acetylcholine (ACh)–induced and sodium nitroprusside (SNP)–induced aortic relaxations were not, b) the norepinephrine (NE)–induced aortic contractile response was enhanced, c) systemic blood pressure was elevated, and d) the clonidine-stimulated Ser-473 phosphorylation of Akt in the aorta was decreased. These results suggest that endothelial functions dependent on the Akt pathway are abrogated by type 2 diabetes only in male mice.

Keywords: diabetes, gender difference, endothelial cell, aorta

Introduction

Diabetes mellitus, a syndrome involving a disordered metabolism, usually has a combination of hereditary and environmental causes and results in abnormally high blood glucose levels. The two most commonly encountered forms of diabetes are due either to a diminished production of insulin (type 1) or to a diminished response to insulin (type 2). Diabetes is a risk factor in the development of both macro- and microvascular diseases (1 – 3). Indeed, it increases the incidence of ischemic heart disease, cerebral ischemia, and atherosclerosis, conditions in which endothelial dysfunction plays a pathogenetic role. One of the most important functions of the endothelium is the production of nitric oxide (NO) in response to a variety of hormonal, mechanical, and chemical stimuli. NO has a number of effects, including vascular relaxation. In patients with type 2 diabetes, the forearm blood flow responses to acetylcholine (ACh) are reduced, suggesting endothelial dysfunction (4, 5). In the aorta of db/db mice — an experimental model of type 2 diabetes associated with insulin resistance, hyperglycemia, and dyslipidemia — contractile responses are enhanced, while endothelium-dependent relaxations are impaired (6, 7). We recently observed that both the relaxation and the NO production induced by clonidine via the Akt pathway, but not those induced by ACh, are impaired in aortic rings from a type 2 diabetic model [nicotinamide + streptozotocin (STZ)–treated male mice] (8, 9).

NO plays a key role in the vascular homeostatic regulation of a wide range of functions, partly via local effects on blood vessel diameter and tissue blood flow. In endothelial cells, the main signal-transduction pathway for agonist-stimulated endothelial NO synthase (eNOS) activation depends on Ca²⁺/calmodulin/caveolin.
At the cellular level, evidence is accumulating that a rise in intracellular Ca\(^{2+}\) is necessary for the induction of NO production by agonists such as ACh, histamine, and bradykinin (10–12), but not by stimuli such as fluid shear stress (13), estrogen (14), and insulin/IGF (15). We and others have suggested a role for the Akt pathway in the induction of NO in the endothelium by insulin or \(\alpha\)-agonists, since inhibition of such agonist-induced activations of the Akt pathway leads to impaired NO availability (16, 17). There is some evidence that abnormal regulation of the Akt pathway may be one of several factors contributing to vascular dysfunction in diabetes. However, although there are known to be gender differences in the cardiovascular effects of diabetes (see below), it is not known whether gender differences exist in the endothelium-dependent relaxations induced by various stimuli (Akt-pathway agonists and non-Akt-pathway agonists) in diabetes.

Gender has been found to be associated with differences in blood pressure (16) and vascular reactivity (17–20) in both rats and mice. In the general (non-diabetic) population, cardiovascular diseases are more frequent in men than in premenopausal women (21, 22). However, 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 (23). Indeed, type 2 diabetes has been reported to negate the protective cardiovascular effects of female gender (24–26). Although some studies have been carried out on the effects of exogenous sex hormones in diabetes, the influence of type 2 diabetes on vascular reactivity has not been compared between males and females using a murine diabetic model.

Against this background, we decided to assess whether there are gender differences in the endothelium-dependent relaxations induced by various stimuli [ACh, clonidine, insulin, and sodium nitroprusside (SNP)] in thoracic aortas isolated from nicotinamide + STZ–induced type 2 diabetic mice (males and females).

Materials and Methods

Reagents

STZ, nicotinamide, clonidine hydrochloride, insulin from porcine pancreas, norepinephrine (NE), and \(N^\omega\)-nitro-L-arginine (L-NNA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SNP dehydrate was from Wako (Osaka), while ACh chloride was from Daichi Pharmaceuticals (Tokyo). The particular 1,6-hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate “Akt inhibitor” we used was manufactured by Calbiochem (Darmstadt, Germany). All drugs were dissolved in saline, unless 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 of 21°C–22°C, room humidity of 50 ± 5%), and food and water were allowed ad libitum to all animals. Mice were randomly allocated to diabetic and control groups. To induce diabetes, 5-week-old mice received an intraperitoneal injection of 1.5 g/kg body weight of nicotinamide dissolved in saline 15 min before an injection via the tail vein of STZ (200 mg/kg) dissolved in a citrate buffer, as in previous studies (8, 9). The age-matched controls were injected with a similar volume of citrate buffer. Finally, mice were anesthetized with diethyl ether and euthanized by decapitation 12 weeks after this treatment with nicotinamide-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, triglyceride, and blood pressure

Plasma parameters were measured as described previously (27, 28). Briefly, plasma glucose, cholesterol, and triglyceride levels were each determined by the use of a commercially available enzyme kit (Wako). The plasma insulin (Shibayagi, Shibukawa, Gunma) and plasma adiponectin (Otsuka Pharmaceutical, Tokyo) levels were each measured by enzyme immunoassay. Systolic blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo) while the mice were in a constant-temperature box at 37°C (8).

Measurement of isometric force

Each aorta was separated from the surrounding connective tissue and cut into rings, as previously described (8, 9). For the relaxation studies, rings were precontracted with an equieffective concentration of prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)) (1 × 10\(^{-6}\)–3 × 10\(^{-6}\) M). When the PGF\(_{2\alpha}\)-induced contraction had reached a plateau level, ACh (1 × 10\(^{-9}\)–1 × 10\(^{-7}\) M), clonidine (1 × 10\(^{-9}\)–1 × 10\(^{-2}\) M), insulin (1 × 10\(^{-8}\)–3 × 10\(^{-5}\) M), or SNP (1 × 10\(^{-10}\)–1 × 10\(^{-5}\) M) was added in a cumulative manner. When the effects of an Akt inhibitor (7 × 10\(^{-7}\) M) or a NOS inhibitor (L-NNA) (1 × 10\(^{-5}\) M) on the response to a given relaxant agent were to be examined, the appropriate inhibitor was added to the bath 30 min
Before the application of PGF₂α.

Measurement of the protein expressions of Akt and phospho-Akt (by Western blotting)

Aortas (3 pooled vessels per group total protein 200 μg) were homogenized in ice-cold lysis buffer, as previously described (9). Samples (20 μg/lane) were resolved by electrophoresis on 7.5% SDS-PAGE gels and then transferred onto PVDF membranes. The membrane was incubated with anti-Akt antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Akt (Ser473) antibody (1:1000, Cell Signaling Technology), or β-actin (1:5000, Sigma) in blocking solution. Horseradish-peroxidase–conjugated, anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) was used at a 1:4000 dilution in Tween PBS, followed by detection using SuperSignal (PIERCE, Rockford, IL, USA). To normalize the data, we used β-actin as a housekeeping protein. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β-actin band.

Statistical analyses

Data are expressed as the mean ± S.E.M. 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 ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc. In each test, P<0.05 was regarded as significant.

Results

Plasma glucose, insulin, adiponectin, cholesterol, and triglyceride levels, and systolic blood pressure

The experimental model employed here (adult mice or rats given STZ and partially protected with a suitable dose of nicotinamide) was devised a few years ago (8, 9, 29 – 32). In this model, the diabetic syndrome shares a number of features with human type 2 diabetes. It is characterized by stable moderate hyperglycemia, glucose intolerance, altered but significant glucose-stimulated insulin secretion, altered in vivo and in vitro responsiveness to tolbutamide, and a reduction in pancreatic β-cell mass (30, 32). As shown in the Table 1, the nonfasting plasma glucose level and the plasma total cholesterol level were each significantly elevated (vs. the age-matched controls) in nicotinamide + STZ–induced diabetic mice (both males and females). Body weight was not different between control and diabetic mice of either gender (Table 1). Plasma insulin was not different between diabetic and control males, but it was lower in diabetic than in control females (Table 1). The plasma adiponectin level was a) higher in females than in males, whether the animals were controls or diabetics, and b) was significantly lower in the diabetics than in the controls (of either gender) (Table 1). Systolic blood pressure was higher in diabetic male mice than in control male mice, but not between the female diabetic and control groups (Table 1).

Aortic relaxations

When the PGF₂α (1 × 10⁻⁶ – 3 × 10⁻⁶ M)–induced contraction had reached a plateau, ACh (1 × 10⁻⁹ – 1 × 10⁻⁵ M), clonidine (1 × 10⁻⁵ – 1 × 10⁻⁷ M), insulin (1 × 10⁻⁸ – 3 × 10⁻⁶ M), or SNP (1 × 10⁻⁵ – 1 × 10⁻³ M) was added cumulatively. In aortic rings from controls and diabetic mice of either gender, ACh caused a dose-dependent relaxation. The ACh-induced relaxation was not different between rings from diabetic mice and those from control mice, whether the mice were males or females (Fig. 1: A and B). The relaxations to clonidine and insulin were very weak in rings from diabetic male mice (Figs. 2A and 3A). In contrast, there was no significant difference

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (10)</td>
<td>Diabetic (8)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>49.8 ± 1.7</td>
<td>47.1 ± 1.8</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>172.0 ± 15.2</td>
<td>648.8 ± 19.1***</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>135.4 ± 6.0</td>
<td>187.3 ± 14.4***</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>142.5 ± 24.2</td>
<td>111.9 ± 17.2</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.3 ± 0.3</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Adiponectin (μg/ml)</td>
<td>12.1 ± 0.9</td>
<td>9.4 ± 1.4*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>107.2 ± 2.1</td>
<td>114.9 ± 2.9*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. The number of determinations is shown in parenthesis. *P<0.05, **P<0.001, vs. male-control mice; †††P<0.05, †††P<0.001, vs. male-diabetic mice; †††P<0.001 vs. female-control mice.
Fig. 1. Concentration–response curves for ACh-induced relaxation in isolated thoracic aortic rings from control and diabetic mice, together with effects of an Akt inhibitor (7 × 10⁻⁷ M) and L-NNA (1 × 10⁻⁴ M) on these relaxations. Line graphs show concentration–response curves for the ACh-induced relaxation of aortic rings obtained from diabetic and age-matched control mice, data for male and female mice being shown separately (A and B, respectively). Ordinates show relaxation as a percentage of the contraction induced by an equieffective concentration of prostaglandin F₂α (1 × 10⁻⁶ – 3 × 10⁻⁶ M). Each data point represents the mean ± S.E.M. from 5 or 6 experiments (S.E.M. is included only when it exceeds the dimension of the symbol used). ***P<0.001, as indicated by brackets.

Fig. 2. Concentration–response curves for clonidine-induced relaxation in isolated thoracic aortic rings from control and diabetic mice, together with effects of an Akt inhibitor (7 × 10⁻⁷ M) and L-NNA (1 × 10⁻⁴ M) on these relaxations. Line graphs show concentration–response curves for the clonidine-induced relaxation of aortic rings obtained from diabetic and age-matched control mice [male (A), female mice (B)]. Ordinates show relaxation as a percentage of the contraction induced by an equieffective concentration of prostaglandin F₂α (1 × 10⁻⁶ – 3 × 10⁻⁶ M). Each data point represents the mean ± S.E.M. from 4 to 7 experiments (S.E.M. is included only when it exceeds the dimension of the symbol used). ***P<0.001, as indicated by brackets.
in either the clonidine-induced or insulin-induced relaxation between the female control and female diabetic groups (Figs. 2B and 3B). The ACh-induced relaxation was not changed by treatment with an Akt inhibitor at $7 \times 10^{-7} \text{M}$ (Fig. 1: A and B) in rings from any of the four groups of mice. In control male mice, the aortic relaxations induced by clonidine and insulin were almost completely abolished by preincubation with the Akt inhibitor at that concentration (Figs. 2A and 3A). However, in the diabetic male group, these relaxations were already very weak and they were not weakened further by the Akt inhibitor (Figs. 2A and 3A). In female mice, the clonidine-induced relaxation was almost completely abolished by the Akt inhibitor in both the controls and the diabetic group (Fig. 2B). In contrast, the insulin-induced relaxation was not changed by such treatment in either the control or diabetic female mice (Fig. 3B). In all groups, all three of the above relaxation responses were abolished by preincubation with the NOS inhibitor L-NNA at $10^{-4} \text{M}$ (Figs. 1 – 3). The SNP-induced endothelium-independent relaxation was not different between the control and diabetic groups, whether the mice were male or female (data not shown).

**Contractile responses to NE**

Figure 4 shows concentration–response curves for the contractile responses of aortic rings to NE ($10^{-10} - 10^{-5} \text{M}$). In rings obtained from controls and diabetic mice of either gender, the curve was bell-shaped, as in a previous study (33). The contraction induced by NE was enhanced in the male diabetic group, compared with the male control group (Fig. 4A). However, it was not different between the female control and female diabetic groups (Fig. 4B). When aortic rings were incubated with L-NNA ($10^{-4} \text{M}$), a NOS inhibitor, the NE-induced bell-shaped curves were changed to sigmoid-shaped curves and the responses were greatly increased at the higher concentrations ($10^{-8} - 10^{-5} \text{M}$) in all groups (Fig. 4: C and D). Thus, in mouse aortic rings, the observed NE-induced contractile response may represent a summation between a contraction mediated by $\alpha_1$-adrenoceptors on the smooth muscle (at relatively low concentrations) and a relaxation mediated by $\alpha_2$-adrenoceptors on the endothelium (at relatively high concentrations). In the presence of L-NNA, the NE-induced aortic contraction was not significantly different between the control and diabetic groups, whether the mice were male or female (Fig. 4: C and D).

**Protein expressions of Akt and phosphorylated Akt, and effects of clonidine**

By the use of anti-Akt and anti-phospho-Akt (Ser473)
antibodies, which allowed detection of an immunoreactive protein with a molecular weight of 60 kDa, we next evaluated clonidine-induced Ser-473 Akt phosphorylation. This phosphorylation was significantly weaker in aortas from male diabetic mice than in those from the male controls (Fig. 5). In contrast, there was no such significant difference between the female control and female diabetic groups (Fig. 5).

**Discussion**

The present investigation reveals a gender difference
in endothelial function in the type 2 diabetic mouse aorta and discloses a potential mechanism that might underlie the relative preservation of this function in female mice. The principal findings made in this study were that in aortas from diabetic males (vs. those from control males and diabetic females), 1) the clonidine-induced and insulin-induced endothelium-dependent relaxations were impaired, but the ACh-induced endothelium-dependent relaxation and the SNP-induced endothelium-independent relaxation were not, 2) the NE-induced vascular contraction was enhanced, and 3) clonidine-stimulated Ser-473 phosphorylation of Akt was decreased.

To induce endothelium-dependent relaxation in the isolated aorta, we used ACh, clonidine, and insulin (Figs. 1 – 3). In the present preparations, we found that the ACh-induced relaxation was not significantly different between the control and diabetic groups (whether the mice were males or females), whereas the clonidine-induced and insulin-induced relaxations were each impaired by diabetes in male aortas, but not in female ones. This is consistent with previous observations indicating a dysfunction of the aortic endothelium in diabetic male mice (8, 9). Since these aortic responses to clonidine and insulin were not altered by diabetes in female mice, it seemed possible that the downstream signaling cascade activated after the release of NO from endothelial cells might be malfunctioning under diabetic conditions only in males. To explore this possibility, the aortic relaxation induced by SNP, a general NO donor, was evaluated, but there was no significant difference in the magnitude of this relaxation between the diabetic and control groups of either gender. This result suggested that type 2 diabetes led to no change in the sensitivity of the NO-mediated response in either males or females.

As mentioned in the Introduction, we recently observed that both the relaxation and NO production induced by clonidine via the Akt pathway are impaired in aortic rings obtained from nicotinamide + STZ–induced type 2 diabetic male mice (8). In the part of the present study devoted to the male mouse aorta, both the ACh-induced relaxation and the clonidine-induced relaxation were abolished in control aortas following treatment with L-NNA, an inhibitor of NO synthase. However, application of an Akt inhibitor to such aortas led to no significant change in the ACh-induced relaxation, whereas the clonidine-induced and insulin-induced responses were significantly reduced, or even abolished. The above results indicated that in male mice, the endothelium-dependent relaxations induced by clonidine and insulin, but not that induced by ACh, are regulated by the Akt signal pathway. Interestingly, it has been reported that in diabetic states accompanied by hypoadiponectinemia, there is decreased Akt expression and activity (34). Our data show that the factors that differed between control and diabetic male mice included the levels of plasma adiponectin (lower in diabetics, see Table 1), endothelial function, and aortic Akt activity (Fig. 5). Reportedly, low adiponectin levels are associated with both endothelial dysfunction (35) and a predisposition to vascular injury (36, 37). Even though adiponectin is only one of the possible causative factors, the low plasma adiponectin levels in the present male diabetic mice might have contributed to the lower Akt activity levels seen in their aortas.

In female mice, although the clonidine-induced relaxation was almost completely abolished by the Akt inhibitor both in the controls and in the diabetic group, the insulin-induced relaxation was unchanged by such treatment in either the controls or the diabetics. These observations suggest that in female mice, the insulin-induced relaxation may be, at least in part, due to NO production through an Akt non-dependent pathway. Since the major pathway for insulin signaling is the PI3-K/Akt pathway, NO synthase in endothelial cells can be, at least in part, regulated by serine/threonine kinases, including cAMP-dependent protein kinase (38, 39) and protein kinase C (40, 41). We believe that the preservation of insulin-induced relaxation in the diabetic female aorta may be mediated via a downstream mechanism that preserves not only the Akt pathway, but also the Akt non-dependent pathways. However, the mechanisms by which insulin acts on the female aorta to cause NO production will require further investigation.

In the present examination of endothelium-dependent relaxations (including those involving the Akt pathway), neither the clonidine-induced nor the insulin-induced relaxation was impaired in female diabetic aortas (vs. the female controls), in contrast to the results obtained for male aortas. Furthermore, the clonidine-stimulated Ser-473 phosphorylation of Akt did not differ between the female control and female diabetic groups. As a possible explanation for the relative preservation of endothelial function in aortas from type 2 diabetic females, a gender-related difference in the plasma adiponectin level was considered. In our data, plasma glucose, cholesterol, triglyceride, and insulin were not different between males and females. Indeed, the main gender difference we detected in the plasma of our diabetic mice, with there being a low plasma adiponectin level in males. In cultured endothelial cells, adiponectin has been shown to exhibit various anti-inflammatory effects, in particular those that counter the adverse cellular influences exerted either by increased oxidative stress or by stimulation with cytokines such as tumor necrosis factor-α (TNF-α) (42). Adiponectin also enhances NO
production by endothelial cells (34, 43). From these reports, we speculate that even though the vascular protection afforded by the female sex hormone estrogen may be attenuated under diabetic conditions, the relative abundance of plasma adiponectin in female mice might have a beneficial effect on endothelial function via the Akt pathway.

There are known to be functional α₂-adrenoceptors and insulin receptors on the endothelium, and the increased nitric oxide production mediated via each receptor has been shown to inhibit the contractile effects of α-adrenergic agonists and catecholamines on vascular smooth muscle (44, 45). In our study, the diabetic male group showed both enhanced NE-induced aortic contraction and elevated systolic blood pressure compared to the male controls. In contrast, these responses were not different between the diabetic and control “female” groups. When aortic rings were incubated with a NOS inhibitor, the NE-induced contraction was greatly enhanced in all four groups (especially at higher NE concentrations). In the presence of this NOS inhibitor, the NE-induced aortic contractions were not different between control and diabetic mice (whether these were male or female). Thus, in mouse aortic rings, the observed enhancement of the NE-induced aortic contractions were not different between control and diabetic mice (whether these were male or female). Therefore, the responses were not different between the diabetic and control “female” groups. When aortic rings were incubated with a NOS inhibitor, the NE-induced contraction was greatly enhanced in all four groups (especially at higher NE concentrations). In the presence of this NOS inhibitor, the NE-induced aortic contractions were not different between control and diabetic mice (whether these were male or female). Thus, in mouse aortic rings, the observed enhancement of the NE-induced contractile response may represent a summation between a contraction mediated by α₁-adrenoceptors on the smooth muscle (at relatively low concentrations) and a relaxation mediated by α₂-adrenoceptors on the endothelium (at higher concentrations). These results suggest that in aortas from male diabetic mice, the observed enhancement of the NE-induced contractile response may have been due to a diabetes-related impairment of the endothelial NO generation that occurs via the α₂-adrenoceptor. Such an enhancement might conceivably accelerate the progression of hypertension.

Collectively, our data demonstrate that an impairment of certain endothelial functions mediated via the Akt pathway is present in the aorta in male mice, but not in female mice. We consider it possible that the low plasma adiponectin levels present in males might contribute to this dysfunction. Furthermore, we believe that this preservation of the Akt pathway in females (which may, at least in part, be related to their higher levels of plasma adiponectin), may serve to limit endothelial dysfunction in females with type 2 diabetes.

Acknowledgments

This study was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology, Japan and by the Open Research Center Project.

References

19. Matumoto T, Kakami M, Kobaayashi T, Kamata K. Gender differences in vascular reactivity to endothelin-1 (1-31) in