Differential expression of $\alpha_2D$-adrenoceptor and eNOS in aortas from early and later stages of diabetes in Goto-Kakizaki rats

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Kobayashi, Tsuneo, Takayuki Matsumoto, Kazuyuki Ooishi, and Katsuo Kamata. Differential expression of $\alpha_2D$-adrenoceptor and eNOS in aortas from early and later stages of diabetes in Goto-Kakizaki rats. Am J Physiol Heart Circ Physiol 287: H135–H143, 2004; 10.1152/ajpheart.01074.2003.—The aim of the present study was to compare vascular dysfunction between the early (12 wk old) and later (36 wk old) stages of spontaneous diabetes in Goto-Kakizaki (GK) rats. We also evaluated the aortic expression of the $\alpha_2D$-adrenoceptor and endothelial nitric oxide synthase (eNOS). Vascular reactivity was assessed in thoracic aortas from age-matched control rats and 12- and 36-wk GK rats. Using RT-PCR and immunoblots, we also examined the changes in expression of the $\alpha_2D$, adrenoceptor and eNOS. In aortas from GK rats (vs. those from age-matched control rats): 1) the relaxation response to Ach was enhanced at 12 wk but decreased at 36 wk; 2) the relaxation response to sodium nitroprusside was decreased at both 12 and 36 wk; 3) norepinephrine (NE)-induced contractility was decreased at 12 wk but not at 36 wk; 4) the expressions of $\alpha_1B$- and $\alpha_1D$-adrenoceptors were unaffected, whereas those of $\alpha_2D$-adrenoceptor and eNOS mRNAs were increased at both 12 and 36 wk; and 5) NE- and Ach-stimulated NO$_x$ (nitrite and nitrate) levels were increased at 12 wk, although at 36 wk Ach-stimulated NO$_x$ was lower, whereas NE-stimulated NO$_x$ showed no change. These results clearly demonstrate that enhanced Ach-induced relaxation and impaired NE-induced contraction, due to NO overproduction via eNOS and increased $\alpha_2D$-adrenoceptor expression, occur in early-stage GK rats and that the impaired Ach-induced relaxation in later-stage GK rats is due to reductions in both NO production and NO responsiveness (but not in eNOS expression).

Type 2 diabetes; $\alpha_2D$-adrenoceptor; endothelium; nitric oxide synthase; relaxation

Diabetes mellitus is an important risk factor for the development of atherosclerosis. Numerous epidemiological studies have indicated that the insulin resistance and hyperinsulinemia associated with Type 2 diabetes make important contributions to the development of hypertension and atherosclerotic lesions. Although an accumulating body of evidence indicates that endothelium-dependent relaxation is weaker both in a Type 1 diabetic model, namely, streptozotocin (STZ)-induced diabetic rats (11, 26, 32, 39–41), and in Type 2 diabetic rats (28, 44, 50, 51), we (29) and others (6, 42) have noted an augmented or unaltered endothelium-dependent relaxation at an early stage in STZ-induced diabetes. In fact, there is clinical and experimental evidence of augmented blood flow at early stages of diabetes (9, 24). In the Type 1 model STZ-induced diabetic rat, Pieper (42) showed that there is an early (for 1 day) increase in endothelium-dependent relaxation, followed by a reversion phase (for 1–2 wk), in which relaxation is normal, and then a final phase (for 8 wk) of impaired relaxation. In a Type 2 model, the obese Zucker rat, the aorta has been reported to exhibit either increased endothelium-dependent relaxation (45, 48) or decreased reactivity (50). Because endothelium-dependent relaxation seems to be attenuated in the aorta in at least some diabetic rats when the disease is of long duration, it is possible that time-dependent changes in endothelial function may occur (e.g., a two-phase effect of diabetes on endothelium-dependent relaxation). However, there have as yet been no reports concerning such time-dependent changes in Type 2 diabetic rats.

It has been reported that activation of the $\alpha_2$-adrenoceptors located on endothelial cells stimulates the release of NO, an action that would tend to attenuate the vasoconstrictive production of NO by the endothelium (2, 10, 44, 49). $\alpha_2$-Adrenoceptors can be divided into three subtypes ($\alpha_2A$, $\alpha_2B$, and $\alpha_2C$-adrenoceptors; Ref. 21). Of these three subtypes, the $\alpha_2D$-adrenoceptor is coupled to endothelium-dependent NO-mediated relaxation (3), although there have been no reports concerning changes in the expression of the mRNA for this subtype in diabetic vascular tissue.

Insulin is able to inhibit vascular smooth muscle contraction by increasing the production of NO in normal vessels (20). A lack of this action of insulin could be related to the vascular insulin resistance associated with arterial hypertension and endothelial dysfunction. In fact, it has been reported that insulin resistance plays essential roles in the hypertension and impairment of endothelium-dependent relaxation seen in mice lacking insulin receptor substrate-1 or -2 (1, 33). When insulin is administered in vitro, it enhances endothelial vasorelaxation by potentiating NO synthase (NOS) and increasing the expression of its mRNA, suggesting that insulin itself can have a vasodilator effect (34, 36, 37, 47). Moreover, it has been reported that insulin enhances $\alpha_2$-adrenergic-induced vasorelaxation by potentiating endothelial NO production in the human forearm and rat aorta (36, 37). We and others have suggested that the decreased contractile response to norepinephrine seen in fructose-fed diabetic mice with hyperinsulinemia may be related to an increase in NO formation mediated by the $\alpha_2$-adrenoceptor (as evidenced by the response to its agonist clonidine; Ref. 25). Although it has been suggested that glucose may impair NO production, there is evidence that endothelium-dependent relaxation is actually increased in the early stages of STZ-induced diabetes (see above). Furthermore, exposure to a high concentration of glucose for 5 days was

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shown to increase both the expression of NOS and the production of NO (NO\textsubscript{3}− + NO\textsubscript{2}−) in human aortic endothelial cells (12). Thus the plasma insulin and glucose levels in diabetes may regulate the production of NO and the expression of the mRNAs for NOS and α\textsubscript{2}β-adrenoceptors. However, no studies of endothelial function in Type 2 diabetes have directly assessed the expression of endothelial NOS (eNOS) and the production of NO while focusing on time-dependent changes in endothelial function.

In the present study, we investigated aortic relaxation and contraction as well as the aortic expression of the mRNAs for eNOS and α\textsubscript{2}β-adrenoceptors in early- and later-stage diabetes in a model of Type 2 diabetes, Goto-Kakizaki (GK) rats. GK rats are a highly inbred strain of Wistar rats that spontaneously develop Type 2 diabetes (17). This genetic rat model is particularly relevant to human Type 2 diabetes because a defect in glucose-stimulated insulin secretion, peripheral insulin resistance, hyperinsulinemia, and hyperglycemia are seen as early as 4 wk after birth (17). Therefore, this model provides a valuable tool for dissecting the pathogenesis of insulin resistance.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

Male GK rats and Wistar control rats were obtained at the age of 4 wk (Clea, Tokyo, Japan). All animals were allowed a standard laboratory diet (MF: Oriental Yeast Industry, Tokyo, Japan) and water ad libitum. 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, Science, Sports and Culture, Japan). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its systolic blood pressure was measured by the tail-cuff method with a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan; Ref. 35). At the ages of 12 and 36 wk, groups of rats were killed by decapitation under diethyl ether anesthesia.

**Measurement of Plasma Glucose and Insulin**

Plasma parameters were determined with a commercially available enzyme kit (Wako Chemical, Osaka, Japan).

**Measurement of Isometric Force**

Rats were anesthetized with diethyl ether. A section of the thoracic aorta from between the aortic arch and the diaphragm was then aorta from between the aortic arch and the diaphragm was then cut into helical strips 3 mm in width and 20 mm in length. The tissue was placed in an oxygenated (95% O\textsubscript{2}-5% CO\textsubscript{2}) bath of 10 ml of KHS at 37°C, with one end connected to a tissue holder and the other to a force-relative transducer (TB-611T; Nihon Kohden). The tissue was equilibrated for 60 min under a resting tension of 1.0 g (determined to be optimal in preliminary experiments). During this period, the KHS in the tissue bath was replaced every 20 min. For the relaxation studies, the aortic strips, which were weighed at the end of each experiment, were precontracted with an equieffective concentration of norepinephrine (5 × 10\textsuperscript{−8}–3 × 10\textsuperscript{−7} M). This concentration produced 75–85% of the maximal response, with each strip developing a tension of ∼95 mg/mg tissue whether it was from an age-matched control rat or a diabetic rat. When the norepinephrine-induced contraction had reached a plateau level, ACh (10−9–10−5 M), sodium nitroprusside (SNP; 10−9–10−5 M), or forskolin (10−7–10−5 M) was added in a cumulative manner. For the contraction studies, norepinephrine (10−9–10−5 M) or isotonic high-K\textsuperscript{+} solution (10–80 mM) was added cumulatively to the bath until a maximal response was achieved. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist. To investigate the influence of 10−8 M L\textsuperscript{-}nitro-l-arginine (L-NNA) or 10−5 M indomethacin on the norepinephrine-induced contractile responses, the strip was incubated for 30 min in the appropriate medium before the cumulative addition of the agonist. In the present study, we first obtained dose-response curves to assess ACh-induced relaxation; second, we assessed norepinephrine-induced contractile responses; and third, we assessed norepinephrine-induced contractile responses in the presence of l-NNA.

**Measurement of NO\textsubscript{2} and NO\textsubscript{3}**

The concentrations of NO\textsubscript{2} and NO\textsubscript{3} in the effluent from each tissue were assayed by the method described by Yamada and Nabeshima (52). Briefly, the NO\textsubscript{2} and NO\textsubscript{3} in the perfusate were separated by means of a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 × 50 mm; Eicom), after which NO\textsubscript{2} was reduced to NO\textsubscript{3} in a reduction column packed with copper-plated cadmium filings (NO-RED; Eicom). The NO\textsubscript{3} was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven set at 35°C. The absorbance of the colored product dye at 540 nm was measured by means of a flow-through spectrophotometer (NOD-10; Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol.

**Table 1. PCR primers and pCR Protocols**

<table>
<thead>
<tr>
<th></th>
<th>Product Size</th>
<th>PCR Primer Sequences</th>
<th>PCR Protocols</th>
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</table>
| GAPDH           | 308 bp       | Up: 5\textsuperscript{-}TTCTTGGCAAGATTGTCCAGCA-3'  
Down: 5\textsuperscript{-}AGATGACACAAAGGATAGACATT-3' | 94°C/60 s, 56°C/60 s, 72°C/60 s, 20 cycles |
| eNOS            | 691 bp       | Up: 5\textsuperscript{-}TCCAGTTAGACAGAAAGAGATGACATT-3'  
Down: 5\textsuperscript{-}AGATGACACAAAGGATAGACATT-3' | 94°C/60 s, 62°C/60 s, 72°C/60 s, 28 cycles |
| α\textsubscript{1B} Adrenoceptors | 520 bp     | Up: 5\textsuperscript{-}TGGGAGGAAAGGATGTCTCT-3'  
Down: 5\textsuperscript{-}AGATGACACAAAGGATAGACATT-3' | 94°C/60 s, 58°C/60 s, 72°C/60 s, 28 cycles |
| α\textsubscript{1D} Adrenoceptors | 435 bp     | Up: 5\textsuperscript{-}ATCTGTCATGTACGAGGC-3'  
Down: 5\textsuperscript{-}AGATGACACAAAGGATAGACATT-3' | 94°C/60 s, 58°C/60 s, 72°C/60 s, 28 cycles |
| α\textsubscript{2D} Adrenoceptors | 875 bp     | Up: 5\textsuperscript{-}ATCTGTCATGTACGAGGC-3'  
Down: 5\textsuperscript{-}AGATGACACAAAGGATAGACATT-3' | 94°C/60 s, 64°C/60 s, 72°C/60 s, 34 cycles |

eNOS, endothelial nitric oxide synthase.
containing 0.15 M NaCl-NH₄Cl and 0.5 g/l 4Na-EDTA. The Griess reagent, which was 1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l N-(1-naphthyl)ethylenediamine, was delivered at a rate of 0.1 ml/min. For the determination of NO₂⁻ and NO₃⁻, aortas were cut into transverse rings 10 mm in length. These were placed in 1 ml of KHS buffer at 37°C. Samples were collected on three occasions as follows: sample 1 for a 40-min period before application of 10⁻⁵ M ACh or 10⁻⁶ M norepinephrine, sample 2 for a 40-min period after their application, and sample 3 for a 40-min period without either 10⁻⁵ M ACh or 10⁻⁶ M norepinephrine stimulation. The amount of NOx was calculated as follows: agonist-stimulated NOx (nmol·min⁻¹·g⁻¹) = (sample 2 − sample 1 − sample 3)/40 (min·g) (wt. wet of the aorta). The concentrations of NO₂⁻ and NO₃⁻ in KHS and the reliability of the reduction column were examined in each experiment.

When we measured ACh-induced relaxation, the aortic strip was precontracted with norepinephrine. Therefore, we also measured ACh-stimulated NOx in the presence of norepinephrine (5 × 10⁻⁶, 3 × 10⁻⁷ M). Unfortunately, the data for ACh-stimulated NOx showed considerable variability under norepinephrine.

**Measurement of Expression of mRNAs for eNOS by Competitive PCR**

Snap-frozen aortic tissues were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail. The protein concentration was determined by means of a bicinchoninic acid protein assay reagent kit (Pierce). Aliquot samples (500 μg) of tissue homogenate

**Table 2. Various parameter levels in age-matched controls and 12- and 36-wk GK diabetic rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>12-wk Control (8)</th>
<th>12-wk GK (8)</th>
<th>36-wk Control (8)</th>
<th>36-wk GK (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>430.6 ± 6.5</td>
<td>412.8 ± 11.1</td>
<td>471.8 ± 27.4</td>
<td>436.0 ± 9.2</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>97.2 ± 5.6</td>
<td>328.4 ± 65.1*</td>
<td>121.9 ± 4.2</td>
<td>381.9 ± 38.5†</td>
</tr>
<tr>
<td>Plasma insulin, pg/ml</td>
<td>2.046 ± 33</td>
<td>2.836 ± 179*</td>
<td>2.362 ± 208</td>
<td>3.424 ± 434†</td>
</tr>
<tr>
<td>Plasma NOx (× 10⁻⁶ M)</td>
<td>8.6 ± 0.5</td>
<td>9.6 ± 0.3</td>
<td>9.0 ± 0.5</td>
<td>7.9 ± 0.6</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>437 ± 11</td>
<td>464 ± 8</td>
<td>425 ± 10</td>
<td>391 ± 7</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>134.3 ± 1.9</td>
<td>95.5 ± 19*</td>
<td>128.3 ± 2.3</td>
<td>124.8 ± 2.2</td>
</tr>
<tr>
<td>Aorta weight, mg/cm²</td>
<td>20.6 ± 1.1</td>
<td>16.8 ± 1.1*</td>
<td>22.2 ± 0.7</td>
<td>15.5 ± 1.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of determinations shown in parentheses. GK, Goto-Kakizaki. *P < 0.05 vs. 12-wk control; †P < 0.05 vs. 36-wk control.
obtained from diabetic and age-matched control rats were then incubated with anti-eNOS antibody (Calbiochem, La Jolla, CA; 1:100, 4C, 4 h), followed by addition of protein G Sepharose (Amersham Biosciences) for 2 h at 4C. Immunoprecipitates were collected by centrifugation (13,000 g; 1 min), washed three times with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and protease inhibitor cocktail, and then resuspended in Laemmli buffer containing mercaptoethanol. Samples were resolved by electrophoresis on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Briefly, after the residual protein sites on the membrane were blocked with Block ace (Dainippon-pharm, Osaka, Japan), the membrane was incubated with anti-eNOS antibody (1: 1,000) in blocking solution. Horseradish peroxidase-conjugated anti-mouse antibody (Vector) was used at a 1:10,000 dilution in Tween-PBS, followed by detection with a SuperSignal (Pierce). 

**Statistical Analysis**

The contractile force developed by aortic strips from control and diabetic rats is expressed in milligrams of tension per milligram of tissue. Data are expressed as means ± SE. When appropriate, statistical differences were determined by Dunnett’s test for multiple comparisons after a one-way ANOVA, a probability level of P < 0.05 being regarded as significant. Statistical comparisons between concentration-response curves were made by means of a two-way ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc (P < 0.05 again being considered significant).

**RESULTS**

**Plasma Parameters, Systolic Blood Pressure, and Aorta Weight**

As indicated in Table 2, plasma glucose levels were significantly elevated in both 12- and 36-wk GK diabetic rats (vs. age-matched control rats), but the levels were not different between the 12- and 36-wk GK groups. In both 12- and 36-wk GK rats, plasma insulin levels were significantly higher than in the age-matched controls. The plasma level of NOx was unchanged (vs. control rats) in GK rats at 12 wk, but it was significantly decreased at 36 wk. Heart rate was significantly lower in the 36-wk GK rats. Systolic blood pressure was...
significantly lower in the 12-wk GK rats but not in the 36-wk GK rats (vs. respective control rats). The aorta weights were significantly decreased in both 12- and 36-wk GK diabetic rats (vs. age-matched control rats), but the levels were not different between the 12- and 36-wk GK groups.

**Relaxation Responses to ACh and SNP**

When the norepinephrine (5 × 10⁻⁸–3 × 10⁻⁷ M)-induced contraction had reached a plateau, ACh (10⁻⁹–10⁻⁵ M) was added cumulatively (Fig. 1). In aortic strips from age-matched control rats, ACh (10⁻⁹–10⁻⁵ M) caused a concentration-dependent relaxation, with the maximum response at 10⁻⁵ M. This relaxation was significantly stronger in strips from 12-wk GK rats than in the age-matched control rats (Fig. 1A). In contrast, the relaxation induced by ACh was weaker in aortic strips from 36-wk diabetic rats than in those from their age-matched controls (Fig. 1B). The ACh-induced relaxation responses in aortas were greatly diminished by preincubation with the NOS inhibitor L-NNA (10⁻⁴ M) but not by preincubation with indomethacin (10⁻⁵ M) (data not shown).

**Contractile Responses to Norepinephrine and Isotonic K⁺**

Exposure of aortic strips to norepinephrine (10⁻⁹–10⁻⁵ M) led to a concentration-dependent rise in tension in all experimental groups. Aortas from 12-wk GK diabetic rats showed decreases in norepinephrine sensitivity and maximum contractile force compared with those of age-matched control rats (Fig. 3A). In contrast, aortas from 36-wk GK rats showed no change (vs. their controls) in contraction induced by norepinephrine (Fig. 3B). In the presence of 10⁻⁴ M L-NNA, aortas from both 12- and 36-wk diabetic rats displayed a substantially greater sensitivity to norepinephrine than age-matched control rats (Fig. 4), and this was also true in the presence of L-NNA (10⁻⁴ M) plus indomethacin (10⁻⁵ M) (data not shown).
Exposure of aortic strips to isotonic high-K\(^+\) solution (10–80 mM) led to a concentration-dependent rise in tension in all experimental groups, and there was no significant difference in sensitivity among the age-matched control and diabetic groups (12 and 36 wk) (data not shown).

**Measurement of NO\(_2^-\) + NO\(_3^-\)**

ACh (10\(^{-5}\) M) increased the NO\(_x\) (NO\(_2^-\) + NO\(_3^-\)) level in the perfusate from aortic strips, as did norepinephrine (10\(^{-6}\) M). At an early stage (12-wk GK diabetic rats), the ACh- and norepinephrine-induced NO\(_x\) levels were significantly increased (vs. those in age-matched control rats; Fig. 5). In contrast, the ACh-induced NO\(_x\) level was significantly decreased in aortas from 36-wk GK rats (Fig. 5A), whereas the norepinephrine-induced NO\(_x\) level was unchanged in GK rats at that stage (Fig. 5B) (in each case vs. age-matched control rats).

**Expression of mRNA and Protein for eNOS**

To investigate the possible mechanisms underlying the impaired ACh-induced relaxation seen in 36-wk diabetic rats, we examined whether the expression of the mRNA for eNOS might have been changed by the diabetes. Our finding was that it was significantly increased in aortas from both 12- and 36-wk diabetic rats (vs. age-matched control rats), although it was not different between the 12- and 36-wk control rats (Fig. 6, A and C). In aortas obtained from 36-wk GK and age-matched control rats, immunoblots made with anti-eNOS antibody revealed immunoreactive proteins with molecular masses of 135 kDa, like that of eNOS protein. The levels were higher in the 36-wk GK rats than in the age-matched control rats (Fig. 6B). The mRNA expression for iNOS was at a very low level, and it was not significantly different among all groups (data not shown). The mRNA expression for nNOS was significantly decreased in aortas from both 12-wk and 36-wk diabetic rats (vs. age-matched controls), although it was not different between the 12-wk and 36-wk control rats (data not shown).

**Expression of mRNAs for Aortic α\(_{1B}\)− , α\(_{1D}\)− , and α\(_{2D}\)− Adrenoceptors**

To investigate the possible mechanisms underlying the impaired norepinephrine-induced contraction seen in 12-wk diabetic rats, we examined whether the expression of the mRNA for the α\(_{2D}\)-adrenoceptor might have been changed by the diabetes. Using RT-PCR on the total RNA isolated from the aortas of age-matched control rats, 12-wk diabetic rats, and 36-wk diabetic rats, we found the following. The expression of GAPDH mRNA was not different among the four groups. The expression of the mRNA for the α\(_{2D}\)-adrenoceptor was significantly increased in aortas from both 12-wk and 36-wk diabetic rats (vs. age-matched control rats; Fig. 7). When the expressions of the mRNAs for the α\(_{1B}\)- and α\(_{1D}\)-adrenoceptors were studied, neither of them showed any change in aortas from 12-wk diabetic rats (vs. age-matched control rats; Fig. 7A).

**DISCUSSION**

The main conclusion to be drawn from the present study, which compared aortas from GK (Type 2 diabetic) rats with those from age-matched control rats, is that in the early stages of diabetes (12 wk), endothelium-dependent relaxation is enhanced (via NO overproduction and eNOS overexpression).
n the aorta and mesenteric artery of STZ-induced or Zucker diabetic rats, in that these, too, showed an increase in endothelium-dependent relaxation in the early stages of the disease (42, 45, 48).

In contrast, at a later stage (36 wk), our diabetic aortas exhibited decreases in both the endothelium-dependent relaxation induced by ACh and the endothelium-independent relaxation induced by SNP. This is consistent with previous reports that aortas from rats with long-term GK diabetes show an impaired endothelium-independent relaxation to SNP (44, 51). Interestingly, our finding that both endothelium-dependent relaxation and ACh-stimulated NO production were impaired in aortic strips from 36-wk diabetic rats seems to be in conflict with our finding that the expressions of mRNA and protein for eNOS were increased in aortas. This observation suggests that NO synthesis may be regulated by factors present in the intact cell independently of changes in NOS enzyme activity. In 12-wk GK diabetic rats, the norepinephrine-induced contractile response was decreased in the aorta (vs. age-matched control rats). However, in GK diabetic rats at the same stage, the norepinephrine-induced contraction in the presence of L-NNA, a NOS inhibitor, was enhanced and the norepinephrine-stimulated NO$_x$ level was raised. These results strongly suggest that the impairment of the norepinephrine-induced contractile response is due to increased NO release from the endothelium. In fact, there are known to be functional $\alpha_2$-adrenergic receptors on the endothelium, and NO has been shown to inhibit the contractile effects of $\alpha$-adrenergic agonists in vascular smooth muscle (2, 10, 43, 49). We therefore wondered whether the expression of the mRNAs for the $\alpha_1$- and $\alpha_2$-adrenoceptors might have been changed by the diabetes. What we found was that the expression of the $\alpha_{2A/D}^+$/adrenoceptor was greater in the 12-wk diabetic rats than in the age-matched control rats. However, expression of the $\alpha_2B$-adrenoceptor could not be detected, a finding inconsistent with previous observations of expression of $\alpha_{2A/D}^+$ and $\alpha_2B$-adrenoceptors in the rat aorta (14). Furthermore, it has been shown that in the rat mesenteric artery the $\alpha_2$-adrenoceptor that is coupled to endothelium-dependent NO-mediated relaxation belongs to the $\alpha_{2A/D}^+$-subtype (3). Thus the impaired norepinephrine-induced contractile response seen at an early stage in the GK diabetic aorta may be the result of an NO-dependent relaxation mediated via an increased expression of the $\alpha_{2D}^+/\alpha_2$-adrenoceptor in the endothelium. We also suggest that, during the early stages of diabetes, enhanced endothelial $\alpha_2$-adrenergic-evoked NO production may be one of the mechanisms responsible for decreasing arterial pressure. Interestingly, Bockman et al. (4) reported that the norepinephrine-induced release of NO is enhanced in mineralocorticoid hypertension, suggesting that endothelial $\alpha_2$-adrenoceptors may play an important role in the regulation of blood pressure. Furthermore, the hypotensive response to administration of $\alpha_2$-adrenoceptor agonists is absent in mice lacking the $\alpha_2A$-adrenoceptor, demonstrating that the $\alpha_2A$-subtype plays the principal role in this response (38). These findings lead us to speculate that in hypertension and endothelial dysfunction the major defect in endothelial function may be an impairment in the production of endothelial $\alpha_2$-adrenergic-evoked NO. It is unclear at present, however, whether or not the hyperinsulinemia present at an early stage in diabetes might be responsible for increasing the mRNA for eNOS in the aorta; the effect might equally well result from changes in the level of any of a number of other hormones.

Fig. 7. RT-PCR assay of the expression of mRNAs for $\alpha$-adrenoceptors in aortas from age-matched control rats and 12- and 36-wk GK diabetic rats. A: expression of the mRNAs for $\alpha_{2D}$-, $\alpha_{1B}$-, and $\alpha_{1D}$-adrenoceptors assayed by RT-PCR. The RT-PCR assay was performed as described in MATERIALS AND METHODS. B: quantitative analysis of expression of the mRNA for the $\alpha_{2D}$-adrenoceptor by competitive PCR. Values are means ± SE of 6 determinations ($\alpha_{2D}$/adrenoceptor/GAPDH). *P < 0.05, diabetic vs. control.

whereas norepinephrine-induced contraction is decreased (via overexpression of $\alpha_{2D}$-adrenoceptor mRNA). At a later stage (36 wk), endothelium-dependent relaxation was impaired in the diabetic rats, an effect that may be due to reductions in both NO production and NO sensitivity but not to a decline in eNOS expression.

Aortas from 12-wk diabetic rats exhibited an enhanced endothelium-dependent relaxation to ACh in comparison with both the age-matched control rats and the 36-wk diabetic rats. Furthermore, both eNOS mRNA expression and ACh-stimulated NO synthesis were increased in aortas from the 12-wk diabetic rats. These results suggest that in the early stage of Type 2 diabetes in GK rats, both endothelium-dependent relaxation and NO production are enhanced via overexpression of the eNOS mRNA. As far as the early stage of diabetes is concerned, our study agrees with previous studies conducted on the aorta and mesenteric artery of STZ-induced or Zucker diabetic rats, in that these, too, showed an increase in endothelium-dependent relaxation in the early stages of the disease (42, 45, 48).
Although eNOS was originally termed a constitutive enzyme, recent studies have indicated that its expression can be regulated by a variety of stimuli, including several growth factors, insulin, and glucose (12, 22, 34). Therefore, an enhancement of endothelial function could be a direct response to an increased plasma level of insulin and/or glucose. In fact, in the 12-wk GK diabetic rats the plasma glucose and insulin levels were much higher than those of the control rats, and we recently demonstrated (30, 31) that in the STZ-induced diabetic rat, short-term high insulin administration enhances both NO production and expression of the eNOS mRNA. Thus it is possible that the elevation of plasma insulin is directly responsible for the enhancement of endothelial function that occurs early on in diabetes in association with hyperinsulinemia. In contrast, aortas from 36-wk diabetic rats showed an unchanged contraction in response to norepinephrine, although the expression of the mRNA for the α2D-adrenoceptor was increased at this stage. This result suggests that at this late stage, impairments in the NO sensitivity and NOS may compensate for the effect of an increased expression of the α2D-adrenoceptor. Indeed, the norepinephrine-induced NOX production in aortas from 36-wk diabetic rats was unchanged vs. that of the control rats.

There is some apparent conflict among our data in that both endothelium-dependent relaxation and ACh-stimulated NO production were impaired in aortic strips from 36-wk diabetic rats, whereas norepinephrine-stimulated NO production was not changed at this time. The reasons for this are not immediately apparent, but it may be attributable to differences in the agonists used, a muscarine-receptor agonist vs. an α2D-adrenoceptor agonist. Indeed, α2-adrenoceptors are closely linked to GTP-binding protein (5, 15), whereas ACh receptors may not be linked to such a protein (27, 37). Furthermore, vasoactive substances such as ACh that elevate intracellular Ca2+ regulate eNOS activity through a protein-protein interaction, such as eNOS–calmodulin or eNOS–caveolin-1 (18, 19). On the other hand, many other stimuli (including insulin, VEGF, and shear-stress signal) regulate NO production by activating eNOS via Ser1177 phosphorylation through the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway (13, 16, 53). More recently, it was reported that catecholamines in part increase eNOS via Ser1177 phosphorylation through the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway (22). Although we do not have direct evidence for differential effects on eNOS, our study raises the possibility that in aortic strips from GK diabetic rats, eNOS activation through the PI3-kinase/Akt pathway (as with norepinephrine stimulation) is already enhanced, while at the same time eNOS activation through Ca2+-calmodulin binding (as with ACh stimulation) is at a reduced level. Although the aortic relaxation induced by SNP was weaker in both 12-wk and 36-wk GK rats than in their age-matched controls, ACh-induced relaxation was greater only in 12-wk GK rats, strongly suggesting that the eNOS activity of the endothelium may be significantly enhanced in 12-wk GK rats.

In conclusion, the present study has revealed a two-stage change (enhancement followed by impairment) in endothelium-dependent relaxation within one and the same Type 2 diabetes model, the direction of the change being dependent on the stage of the disease. The present study has revealed that at an early stage of diabetes in GK rats, there is enhanced relaxation and impaired contraction due to enhanced NO production via overexpression of both eNOS and the α2D-adrenoceptor. On the downside, increases in these expressions could be a key event in the initiation of atherosclerosis in diabetes. At a later stage in diabetes, there is an impairment of ACh-induced relaxation that appears to be due to decreases in both total NO production and NO responsiveness in vascular smooth muscle cells but not to a decrease in eNOS expression.

REFERENCES


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H142 eNOS AND α2D-ADRENOCEPTOR IN DIABETES
(eNOS), hsps90, and caveolin-1 complex in vitro. Evidence that hsps90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1.


