Effect of chronic insulin treatment on NO production and endothelium-dependent relaxation in aortae from established STZ-induced diabetic rats

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Received 12 January 2000; received in revised form 12 May 2000; accepted 17 June 2000

Abstract

The hypothesis that the impaired endothelial function seen in streptozotocin (STZ)-induced diabetic rats may result from an increased nitric oxide (NO) metabolism was tested. Acetylcholine (ACh) increased the nitrite NO$_2^-$ and nitrate (NO$_3^-$) levels in the perfusates from both control and diabetic aortic strips, although the level of NO$_2^-$ was significantly lower in diabetic rats while the NO$_3^-$ level was significantly higher. Both effects (decrease in NO$_2^-$ and increase in NO$_3^-$) were ameliorated by chronic administration of insulin to diabetic rats but NO$_x$ (NO$_2^-$ plus NO$_3^-$) was increased. The expression of endothelial nitric oxide synthase (eNOS) was significantly increased by chronic administration of insulin to diabetic rats. A decrease in NO$_2^-$ and an increase in NO$_3^-$ occurred following treatment of control aortae with hypoxanthine/xanthine oxidase. Incubating diabetic aortic strips with superoxide dismutase (SOD) normalized the production of both NO$_2^-$ and NO$_3^-$. Both the basal and the ACh-stimulated production of O$_2^-$ were significantly higher in diabetic rats than in controls. These results demonstrate that the ACh-induced relaxation of aortic strips was significantly impaired in diabetic rats and that this impairment may be due to an abnormal oxidative metabolism of NO, rather than to a decrease in NOS mRNA and NO production. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Insulin; Diabetes; Endothelium; Nitric oxide; Relaxation; Streptozotocin

1. Introduction

Vascular disease is a complicating feature of diabetes mellitus in humans. An accumulating body of evidence indicates that the relaxation responses induced in aortic strips by endothelium-dependent agents are weaker in streptozotocin (STZ)-induced diabetic rats [1–5]. When a superoxide anion (O$_2^-$) reacts with nitric oxide (NO), it produces the less potent vasodilators, peroxynitrite, nitrite (NO$_2^-$) and nitrate (NO$_3^-$) [6]. Indeed, it has been reported that an enhanced formation of this radical species may lead to an accelerated inactivation of NO [7,8]. The idea of an important role for an increased O$_2^-$ in the abnormal endothelial function seen in diabetes is strengthened by the observation that a variety of pharmacological free-radical scavengers, including superoxide dismutase (SOD), improve endothelial function in arteries obtained from diabetic animals [9–11]. The chemical lability of NO in cells and tissues has been attributed to its rapid oxidation to both NO$_2^-$ and NO$_3^-$. It has been reported that the primary inactivation product of NO in aerobic aqueous solution is NO$_2^-$ and that further oxidation to NO$_3^-$ requires the presence of oxidizing species such as oxyhaemoglobin and O$_2^-$ [12]. Increases in the NO$_2^-$ + NO$_3^-$ concentration in both urine and plasma have been reported in experimental diabetes [13,14] but, because the source of this increased NO$_2^-$ + NO$_3^-$ is not easily ascertained, drawing conclusions about endothelial cell NO production on the basis of such measures alone is hazardous.

In animal models of STZ-induced diabetes, chronic insulin treatment starting from the onset of glycosuria has been shown to prevent the impairment of the acetylcholine (ACh)-induced endothelium-dependent...
relaxation that is seen in otherwise mesenteric resistance arteries or aortic rings from diabetic rats [15–17]. Although this effect of insulin treatment may be secondary its restorative effect on the plasma glucose level, there is preliminary evidence indicating that insulin itself may contribute to the regulation of vascular tone. In fact, in diabetic patients with hypertension, an administration of insulin is believed rapidly to lower blood pressure by a direct action on blood vessels [18]. It has been also reported that there is a balance between insulin-induced pressor (sympathetic activation) and depressor (stimulation of NO release) actions [19]. Recent data obtained has indicated that insulin enhances endothelial vasorelaxation by potentiating endothelial NO production in humans [20–24], and it potentiates the vasodilation induced by ACh in healthy humans and essential hypertensive patients [25].

In the present study, one set out first to determine whether the impairment in endothelial function seen in the diabetic rat aorta results from a decrease in NO production and/or a defective NO metabolism. The second objective was to investigate whether the expected improvement in endothelial function in aortae from diabetic rats given high-dose, short-term insulin treatment is mediated by increased NO production.

2. Methods

2.1. Animals and experimental design

Male Wistar rats, 8 weeks old and 180–250 g in weight, received a single injection via the tail vein of STZ 75 mg/kg dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given 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).

2.2. Insulin treatment

Nine weeks after the STZ injection, the STZ-induced diabetic rats were treated with a gradually increasing dose of insulin (human insulin 5–30 U/kg per day) for 1 week. Ten weeks after the STZ injection, the rats were killed by decapitation under ether anaesthesia. Control rats were killed in the same way 10 weeks after receiving their buffer injection. Thus, animals were divided into three groups; controls, untreated diabetic rats, insulin-treated diabetic rats.

2.3. Measurement of plasma glucose and insulin

Ten weeks after the injection of STZ or buffer, plasma glucose and insulin were determined using a commercially available enzyme kit (Wako, Osaka, Japan).

2.4. Measurement of isometric force

As mentioned above, rats (controls, untreated diabetic rats, insulin-treated diabetic rats) were anaesthetized with diethyl ether and killed by decapitation 10 weeks after treatment with STZ or buffer. A section of the thoracic aorta from between the aortic arch and the diaphragm was then removed and placed in oxygenated, modified Krebs–Henseleit solution (KHS). The solution consisted of (mM): NaCl 118.0, KCl 4.7, NaHCO₃ 25.0, CaCl₂ 1.8, NaH₂PO₄ 1.2, MgSO₄ 1.2, dextrose 11.0. The aorta was cleaned of loosely adhering fat and connective tissue and cut into helical strips 3 mm in width and 20 mm in length. The tissue was placed in a well-oxygenated (95% O₂, 5% CO₂) bath of 10 ml KHS at 37°C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g (determined to be optimum in preliminary experiments). For the relaxation studies, the aortic strips, which were weighed at the end of each experiment, were precontracted with an equieffective concentration of noradrenaline (NA) (5 × 10⁻⁸–3 × 10⁻⁷ M). When the NA-induced contraction reached a plateau level, ACh (10⁻⁹–10⁻⁷ M), sodium nitroprusside (SNP) (10⁻⁹–10⁻⁵ M), NO⁻ (10⁻⁶–10⁻³ M) or NO₃⁻ (10⁻⁶–10⁻³ M) was added in a cumulative manner.

2.5. Measurement of NO⁻ and NO₃⁻

The concentrations of nitrite and nitrate in the effluent from each type of tissue was assayed by the method described by Yamada and Nabeshima [26]. Briefly, the NO⁻ and NO₃⁻ 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⁻ was reduced to NO₃⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). The NO₃⁻ was mixed with a Griese 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 Griese reagent,
which was 1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l N-naphthyl-\textit{N}-ethylenediamine, was delivered at a rate of 0.1 ml/min. For the determination of NO\textsubscript{2} and NO\textsubscript{3}, the samples were collected over a 0 or 40 min period during stimulation by 10^{-7} M ACh. When the effects of hypoxanthine (HX; 10^{-5} M)/xanthine oxidase (XO; 0.02 U/ml) or superoxide dismutase (SOD; 180 U/ml) on the response to acetylcholine were to be examined in control or diabetic aortae, respectively, the HX/OX or SOD was added to the bath 30 min before the administration of 10^{-7} M ACh. The concentration of NO\textsubscript{2} or NO\textsubscript{3} in KHS and the reliability of the reduction column were examined in each experiment.

2.6. Measurement of the expression of the mRNA for endothelial nitric oxide synthase (NOS)

2.6.1. Oligonucleotides

Rat endothelial nitric oxide synthase (eNOS) oligonucleotides (ON) were used with primers, as described previously [27]. The Primers (with the respective Gene Bank data library accession numbers and the coding sequence of the PCR-amplified product given in brackets) were: rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (X02231, position 492-799, amplification of a 308 bp sequence) ON 1: 5'-TCCCTCAGAATGTTCAAGC-3'. ON 2: 5'-AGATCCACAAAG-GATACATT-3'; rat eNOS (RNU02534, amplification of a 693 bp sequence) ON 3: 5'-TCCAGTAACACAGAGTGA-3'. ON 4: 5'-CAGGAAGTAAGTGAC-3'.

2.6.2. RNA isolation and RT-PCR

RNA was isolated by the guanidinium method [28]. Rat aortae were carefully isolated and cleaned of adhering parenchyma and connective tissue. The aortae were homogenized in RNA buffer and the RNA was quantified by ultraviolet absorbance spectrophotometry. For the RT-PCR analysis, first-strand cDNA was synthesized from total RNA using Oligo (dT)$_{12-18}$ and a cDNA SYNTHESIS KIT (Life Sciences). Twenty (GAPDH) or 28 PCR cycles (94°C for 1 min, 62°C for 1 min, 72°C for 1 min) were performed using a half of the reverse transcription (RT) mixture. The PCR products so obtained were analyzed on ethidium-bromide-stained agarose (1.5%) gels. The eNOS and GAPDH products were quantified by scanning densitometry. The amount of eNOS was normalized with respect to the amount of GAPDH product.

2.7. Quantification of superoxide anion by measurement of amount of NBT reduced

Aortic rings were incubated with nitro blue tetrazolium (NBT) to allow the O$_2^-$ generated by the tissue to reduce the NBT to blue formazan. The details of this assay have been published previously [29]. Briefly, aortic rings were cut into transverse rings 10 mm in length. The rings were placed in 5 ml buffer containing NBT (100 pmol/ml) in the presence or absence of ACh (10^{-7} M) for 1.5 h. After this incubation, they were minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg/ml of diethylenetriaminepentaacetic acid. The mixture was centrifuged at 20,000 x g for 20 min. The resultant pellet was resuspended in 1.5 ml of pyridine during heating at 80°C for 1.5 h to extract formazan. The mixture was then subjected to a second centrifugation at 10,000 x g for 10 min. The absorbance of the formazan was determined spectrophotometrically at 540 nm. The amount of NBT reduced ( = quantity of formazan), was calculated as follows: amount of NBT reduced = A x V / (T x Wt x g x l), where A is the absorbance, V is the volume of the solution, T is the time period during which rings were incubated with NBT, Wt is the blotted wet weight of the aortic ring, g is the extinction coefficient (0.71 mmol per mm) and l is the length of the light path. The results are reported in pmol/min per Wt mg.

2.8. Drugs

STZ, N\textsuperscript{G}-nitro-L-arginine, (-)noradrenaline hydrochloride, sodium nitroprusside, NO\textsubscript{2} and NO\textsubscript{3} were all purchased from Sigma (St. Louis, MO). Acetylcholine chloride was purchased from Daiichi (Tokyo, Japan). All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath.

2.9. Statistical analysis

The contractile force developed by aortic strips from control and diabetic rats is expressed in mg tension/mg tissue. Data are expressed as the mean ± S.E. mean and median values. In some experiments, statistical differences were determined by Dunnett’s test for multiple comparisons after a one-way analysis of variance, a probability level of P < 0.05 being regarded as significant. Statistical comparisons between concentration-response curves were made by a two-way ANOVA with Bonferroni’s correction for multiple comparisons being performed post hoc. P < 0.05 was considered significant.

3. Results

3.1. Plasma glucose and insulin levels

As indicated in Table 1, plasma glucose levels were significantly elevated in STZ-induced diabetes, short-term treatment with high-dose (5–30 U/kg per day for
Table 1
Plasma glucose and insulin levels in age-matched controls, STZ-diabetic rats and insulin-treated STZ-diabetic rats

<table>
<thead>
<tr>
<th>Plasma parameter</th>
<th>Control (8)</th>
<th>Diabetic (8)</th>
<th>Insulin-treated diabetic (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>138.2 (129-152)</td>
<td>518.4 (482-571)***</td>
<td>89.5 (49.6-271)**</td>
</tr>
<tr>
<td>Insulin (µU/dl)</td>
<td>35.1 ± 6.1</td>
<td>6.1 ± 0.7***</td>
<td>201.3 ± 30.3***</td>
</tr>
</tbody>
</table>

* Number of determinations is shown in parenthesis, the glucose data are expressed as the median values.
*** P<0.001 vs. control; **+ P<0.001 vs. diabetic.

1 week) insulin in the established diabetic rats produced a plasma glucose concentration that was not different from that of the controls. Plasma insulin levels were significantly lower in STZ-induced diabetes than in controls. The plasma concentrations of insulin was higher in the insulin-treated group than in the untreated diabetic rats.

3.2. Relaxation response to ACh or SNP

When the NA (5 x 10^-8-3 x 10^-7 M)-induced contraction had reached a plateau, ACh (10^-9-10^-5 M) or SNP (10^-9-10^-5 M) was added cumulatively. The results are summarized in 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 weaker in strips from STZ-induced diabetic rats (Fig. 1A). After chronic administration of insulin, aortic strips from STZ-induced diabetic rats relaxed in a normal way to ACh (Fig. 1A). The relaxation caused by SNP (10^-9-10^-5 M) was not significantly different in the aortic strips from the different groups (Fig. 1B). SNP primarily releases NO intracellularly within smooth muscle cells, a location in which it is inaccessible to extracellular free radicals [30].

3.3. Relaxation response to NO_2^- or NO_3^- (Fig. 2)

In aortic strips from control rats, nitrite (NO_2^-; 10^-6-10^-3 M) but not nitrate (NO_3^-; 10^-6-10^-3 M) caused a concentration-dependent relaxation, with the maximum response at 10^-3 M (Fig. 2). Aortae from diabetic rats showed a weaker relaxation than the controls in response to nitrite (NO_2^-; 10^-6-10^-3 M) (Fig. 2).

3.4. Expression of the mRNA for eNOS

To investigate the possible mechanisms underlying the impaired ACh-relaxation seen in STZ-induced diabetic rats and its normalization in chronic insulin-treated diabetic rats, one examined whether the expression of the mRNA for eNOS might have been changed by the chronic insulin treatment. Using RT-PCR on the total RNA isolated from the aortae of age-matched controls, untreated diabetic and chronic insulin-treated diabetic rats, the following were found. The expression of GAPDH mRNA showed no change among the three groups. The expression of the mRNA for eNOS was significantly increased in aortae from insulin-treated diabetics but there was no difference between control and untreated diabetic rats (Fig. 3). In control rats, the chronic administration of insulin did not affect the mRNA for eNOS (data not shown).
Fig. 2. Concentration-response curves for $\text{NO}_2^-$ or $\text{NO}_3^-$-induced relaxation of aortic strips obtained from age-matched controls and diabetic rats. The ordinate shows the relaxation of aortic strips as a percentage of the contraction induced by an isometric concentration of noradrenaline ($5 \times 10^{-5} - 3 \times 10^{-7}$ M). Each data point represents the mean $\pm$ S.E. of four to six experiments; the S.E. is included only when it exceeds the dimension of the symbol used. ** $P < 0.01$, *** $P < 0.001$; $\text{NO}_2^-$ vs. $\text{NO}_3^-$, control vs. diabetic.

3.5. Measurement of $\text{NO}_2^-$ and $\text{NO}_3^-$

ACh increased both $\text{NO}_2^-$ and $\text{NO}_3^-$ levels in the perfusate from aortic strips (Fig. 4). The ACh-induced release of $\text{NO}_2^-$ was lower in STZ-induced diabetic rats than in the age-matched controls. After chronic administration of insulin, the release of $\text{NO}_2^-$ was more than restored to normal. In contrast, the $\text{NO}_3^-$ level was significantly higher in diabetic rats than in the controls. This effect of diabetes was ameliorated by the chronic administration of insulin. The ACh-induced NOx ($\text{NO}_2^-$ plus $\text{NO}_3^-$) level showed no change between controls and diabetics. However, NOx was increased by the chronic administration of insulin (Fig. 4). Acute incubation of diabetic aorta in vitro with SOD normalized the production of both $\text{NO}_2^-$ and $\text{NO}_3^-$ (i.e. increased $\text{NO}_2^-$, decreased $\text{NO}_3^-$ versus diabetic group without SOD) (Fig. 5B). Incubating aortic strips from control rats with hypoxanthine/xanthine oxidase decreased the $\text{NO}_3^-$ level and increased the $\text{NO}_2^-$ level (Fig. 5A). Acute incubation of aorta from controls and insulin-treated diabetics in vitro with SOD had no effects on $\text{NO}_2^-$ and $\text{NO}_3^-$ (data not shown).

3.6. Quantification of superoxide anion by measurement of amount of NBT reduced

To judge from our measurements of the amount of NBT reduced by $\text{O}_2^-$ (Table 2), the basal $\text{O}_2^-$ level in aortic rings was greater in diabetic rats than in the controls. The $\text{ACh} (10^{-7})$-stimulated level was also greater in the diabetic group than in the controls.

Fig. 3. RT-PCR assay of expression of the mRNA for endothelial nitric oxide (NO) synthase in aortae from control, untreated diabetic and short-term, high-dose insulin treated diabetic rats. (A) Expression of the mRNA for endothelial nitric oxide synthase (eNOS) assayed by RT-PCR. (B) Quantitative analysis of expression of the mRNA for eNOS (by scanning densitometry). Control rats ($n = 6$, open column); streptozotocin (STZ)-induced diabetic rats ($n = 6$, closed column); insulin-treated diabetic rats ($n = 6$, stippled column). Values are mean $\pm$ S.E. of six determinations (eNOS/GAPDH). * $P < 0.05$, insulin-treated diabetic vs. control. The RT-PCR assay was performed as described in Section 2. Each total RNA preparation (1.0 $\mu$g) was reverse transcribed and half of the cDNA product was PCR-amplified using the various primers, 28 cycles being employed. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethidium bromide. Left lane, $\Phi$X174/Hinc II digest molecular size marker.

Fig. 4. Acetylcholine (ACh) ($10^{-7}$)-stimulated release of $\text{NO}_2^-$, $\text{NO}_3^-$ and NOx as measured in the perfusate from aortic strips. Each column represents the mean $\pm$ S.E. of six to eight experiments. * $P < 0.05$ diabetic vs. control, ** $P < 0.001$, *** $P < 0.001$ Insulin-treated diabetic vs. diabetic.
although the ACh-stimulated level in the diabetic group was actually smaller than the basal level in the same group of animals. The chronic administration of insulin normalized the increased basal or ACh-stimulated \( \text{O}_2^- \) level in diabetics (Table 2). Although \( N^\text{G}-\text{nitro-L-arginine (L-NOARG)} (10^{-4} \text{ M}) \) had no effects on \( \text{O}_2^- \) production in the ACh-stimulated aorta from controls, ACh-stimulated \( \text{O}_2^- \) level in diabetics was greater by treatment with this agent (Table 2).

**Table 2**

Quantification of superoxide anion production by measurement of amount of nitro blue tetrazolium (NBT) reduced in age-matched controls, streptozotocin (STZ)-diabetic and insulin-treated STZ-diabetic rats

<table>
<thead>
<tr>
<th>Reduced NBT (pmol/min per mg)</th>
<th>Control (10)</th>
<th>Diabetic (10)</th>
<th>Insulin-treated diabetic (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>21.5 ± 1.1</td>
<td>33.1 ± 1.2***</td>
<td>19.5 ± 0.7***</td>
</tr>
<tr>
<td>ACh-stimulated</td>
<td>18.6 ± 0.6</td>
<td>26.9 ± 1.2***</td>
<td>19.2 ± 1.1***</td>
</tr>
<tr>
<td>ACh-stimulated + L-NOARG</td>
<td>17.7 ± 1.5</td>
<td>36.4 ± 2.8***</td>
<td>20.4 ± 1.1***</td>
</tr>
</tbody>
</table>

*a Number of determinations is shown in parenthesis.

*** \( P<0.001 \) vs. control; \( ^* P<0.05 \) vs. basal; \( ^{***} P<0.001 \) vs. ACh-stimulated + \( N^\text{G}-\text{nitro-L-arginine (L-NOARG; 10}^{-4} \text{ M)} \); \( ^{**} P<0.001 \) vs. diabetic.

**4. Discussion**

The main conclusion to be drawn from the present study is that the impairment of the ACh-induced relaxation seen in aortic strips obtained from diabetic rats may be due to the presence of an abnormal oxidative metabolism of NO, rather than to a decrease in eNOS mRNA and NO production. Furthermore, in rats with established STZ-induced diabetes, short-term, high-dose administration of insulin (1) normalizes the impaired endothelium-dependent relaxation in the aorta, (2) upregulates the expression of eNOS mRNA, and (3) normalizes NO metabolism.

The reduction in endothelium-dependent relaxation seen in the diabetic rats in the present study is in agreement with the results of several other studies on aortae from STZ-induced diabetic rats [1,2,9,11,21]. To investigate the possible mechanisms underlying the impaired ACh-relaxation, one determined whether the expression of the mRNA for endothelial NO might be altered in STZ-induced diabetic rats. In fact, it was found that the expression was not different between control and diabetic rats. Furthermore, NOx production in the aorta was not different between control and diabetic rats. It has been reported that acute administration of L-arginine, a substrate for NO synthase, to diabetic rats normalizes both the defective cGMP production and the relaxation induced by ACh in the aorta [5]. This evidence indirectly suggested that the impaired endothelial function seen in diabetes might not be due to a reduced eNOS expression, although no direct measurement of eNOS expression has previously been reported. In the present study, direct evidence was obtained suggesting that the impaired aortic relaxation seen in diabetic rats is not due either to a decreased expression of eNOS or to a decreased total NO production in endothelial cells.

The idea that an elevated \( \text{O}_2^- \) level might play an important role in the abnormal endothelial function seen in diabetes is strengthened by the observation that SOD improves endothelial dysfunction in arteries from diabetic animals [9–11]. Indeed, it is confirmed in the present study that the level of \( \text{O}_2^- \) was significantly greater in aortae from diabetic rats than in those from
the controls (Table 2). The stimulation of \( \cdot \text{O}_2^- \) formation induced by hypoxanthine/xanthine oxidase administration to control decreased the \( \cdot \text{NO}_2^- \) level and increased the \( \cdot \text{NO}_3^- \) level, indicating that the presence of \( \cdot \text{O}_2^- \) leads to the formation of \( \cdot \text{NO}_3^- \) from NO (with a consequent deficiency of \( \cdot \text{NO}_2^- \)). Thus, our results are consistent with the idea [12] that the primary inactivation product of NO is \( \cdot \text{NO}_2^- \) and that further oxidation to \( \cdot \text{NO}_3^- \) requires the presence of \( \cdot \text{O}_2^- \). Indeed, it has been reported that when \( \cdot \text{O}_2^- \) reacts with NO, it produces the less potent vasodilators, peroxynitrite, \( \cdot \text{NO}_2^- \) and \( \cdot \text{NO}_3^- \) [6]. Having noted these previously, one decided to measure \( \cdot \text{NO}_2^- \) and \( \cdot \text{NO}_3^- \) in aortic strips from diabetic rats. As would have been predicted (on the basis of the above evidence), the \( \cdot \text{NO}_2^- \) level was significantly lower in diabetic rats than in the controls and, in marked contrast, the \( \cdot \text{NO}_3^- \) level was significantly higher in diabetic rats than in the controls. SOD, an \( \cdot \text{O}_2^- \) scavenger, restored the deficiency of \( \cdot \text{NO}_2^- \) in the diabetic aorta. These results strongly suggest that NO is metabolized by \( \cdot \text{O}_2^- \) to \( \cdot \text{NO}_3^- \), not just to \( \cdot \text{NO}_2^- \), and that the resulting rapid inactivation of NO may be responsible for the impairment of the endothelium-dependent relaxation of aortic strips seen in diabetic rats. It is unclear at present, however, whether production of \( \cdot \text{O}_2^- \) is actually increased in aortic strips from diabetic rats. Some recent observations support our ideas: it has been reported that SOD activity is reduced in aortae from diabetic rats and that a greater production of \( \cdot \text{O}_2^- \) may cause an impairment of endothelium-dependent relaxation [9,31]. Moreover, it was found that although \( \cdot \text{NO}_2^- \) may contribute to smooth muscle relaxation (presumably through NO formation), \( \cdot \text{NO}_3^- \) was not able to relax aortic strips.

It was found that in the diabetic aorta, the ACh-stimulated production of \( \cdot \text{O}_2^- \) was significantly lower than basal level in the same vessels (Table 2). Furthermore, although L-NOARG had no effects on \( \cdot \text{O}_2^- \) production in the ACh-stimulated aorta from controls, ACh-stimulated \( \cdot \text{O}_2^- \) level in diabetics was greater by treatment with this agent. This may indicate that in the diabetic aorta, \( \cdot \text{O}_2^- \) production in vascular smooth muscle cells is effectively scavenged by NO from endothelial cells. These results suggest that both a decreased SOD activity [11,31] and enhanced \( \cdot \text{O}_2^- \) production in smooth muscle cells may be factors leading to a greater production of \( \cdot \text{O}_2^- \) in the diabetic aorta. Indeed, it has been demonstrated that SOD restores the ACh-induced relaxation in STZ aortic strips [11].

Chronic insulin treatment starting from the onset of glycosuria has been shown to prevent the impaired relaxation seen in mesenteric resistance arteries and aortic rings from diabetic rats [15–17]. In contrast, in vitro experiments, it has been shown that acute insulin administration does not prevent the impaired endothelium-dependent vasorelaxation seen in diabetes [17]. In the present study, short-term, high-dose administration of insulin normalizes the impaired endothelium-dependent relaxation in aortae from rats with established STZ-induced diabetes. Furthermore, chronic administration of insulin upregulated the expression of NOS mRNA and increased total NO production by comparison with both controls and untreated diabetic animals. Plasma insulin levels were elevated following high-dose administration of insulin, by comparison with both the controls and the diabetic rats (Table 1). Insulin-treated diabetic rats showed an increased NO production and a restored endothelial relaxation, suggesting the possibility that high insulin levels in the plasma may lead to an upregulation of the mRNA for the expression of NOS. Actually, eNOS expression is regulated by several stimuli, including fluid shear stress and vascular endothelial growth factor [32,33]. It is unclear at present, however, which insulin-like factors might be responsible for increasing the level of this mRNA in the aorta; the effect might result from changes in the insulin level or in the level of any of several other hormones. In fact, high concentrations of insulin are known to activate IGF-1 receptors as well as insulin receptors even through the binding affinity of insulin for the IGF-1 receptor is ~100 times less than its affinity for the insulin receptor [34]. Although arterial smooth muscle cells and endothelial cells express both insulin and IGF-1 receptors [35], recent reports suggest that the vasodilation induced by insulin may be mediated primarily via its stimulatory effects on IGF-1 production [36,37]. Furthermore, a number of studies have suggested that the vasorelaxation induced by IGF-1 or VEGF is most likely mediated by the production of vascular NO [37–39].

In conclusion, no found evidence has been found suggesting that an enhanced \( \cdot \text{O}_2^- \) level in the diabetic aorta may lead to an abnormal NO metabolism and a subsequent impairment of endothelial-dependent relaxation. Furthermore, it has been shown that in rats with established STZ-induced diabetes, short-term, high-dose insulin treatment can normalize the impaired endothelium-dependent relaxation in the rat aorta, probably by inducing an overexpression of eNOS.

Acknowledgements

This study was supported in part by the Ministry of Education, Science, Sports and Culture, Japan.

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