Short-term insulin treatment and aortic expressions of IGF-1 receptor and VEGF mRNA in diabetic rats

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Kobayashi, Tsuneo, and Katsuo Kamata. Short-term insulin treatment and aortic expressions of IGF-1 receptor and VEGF mRNA in diabetic rats. Am J Physiol Heart Circ Physiol 283: H1761–H1768, 2002; 10.1152/ajpheart.00248.2002.—We investigated the relationship between the changes in vascular responsiveness and growth factor mRNA expressions induced by 1-wk treatment with high-dose insulin in control and established streptozotocin (STZ)-induced diabetes. Aortas from diabetic rats, but not those from insulin-treated diabetic rats, showed impaired endothelium-dependent relaxation in response to ACh (vs. untreated controls). The ACh-induced nitrite plus nitrate (NOx) level showed no significant difference between controls and diabetics. Insulin treatment increased NOx only in diabetics. In diabetics, insulin treatment significantly increased the aortic expressions of endothelial nitric oxide synthase (eNOS) mRNA and VEGF mRNA. The expression of IGF-1 mRNA was unaffected by diabetes or by insulin treatment. In contrast, the mRNA for the aortic IGF-1 receptor was increased in diabetics and further increased in insulin-treated diabetics. In aortic strips from age-matched control rats, IGF-1 caused a concentration-dependent relaxation. This relaxation was significantly stronger in strips from STZ-induced diabetic rats. These results suggest that in STZ-diabetic rats, short-term insulin treatment can ameliorate endothelial dysfunction by inducing overexpression of eNOS and/or VEGF mRNAs possibly via IGF-1 receptors. These receptors were increased in diabetics, perhaps as result of insulin deficiency.

Acetylcholine; endothelium; nitric oxide synthase; relaxation; streptozotocin; insulin-like growth factor I; vascular endothelial growth factor; messenger ribonucleic acid

DIABETES MELLITUS is an important risk factor for the development of atherosclerosis. An accumulating body of evidence (10, 18, 21, 27, 31, 32, 39) indicates that endothelium-dependent relaxation is weaker in streptozotocin (STZ)-induced diabetic rats and diabetic Zucker rats. In STZ-induced diabetic animal models, starting chronic insulin treatment from the onset of glycosuria has been shown to prevent the impairment of the ACh-induced endothelium-dependent relaxation that is otherwise seen in mesenteric resistance arteries or aortic rings isolated from diabetic rats (15, 30, 37). Although this effect of insulin treatment could be secondary to its restorative effect on the plasma glucose level, there is preliminary evidence indicating that insulin itself may contribute to the regulation of vascular tone (4). When administrated in vitro, insulin enhances endothelial vasorelaxation by potentiating nitric oxide (NO) synthase and increasing the expression of its mRNA, suggesting that insulin itself can have a vasodilator effect (23, 25, 36). Indeed, we (22) have also shown that short-term, high-dose insulin treatment by inducing an overexpression of endothelial NO synthase (eNOS) can normalize the impaired endothelium-dependent relaxation seen in the diabetic rat. However, it remains unclear by what mechanism an early improvement in endothelial function might occur during treatment with insulin.

IGF-1 is a homologue of insulin and shares many signaling components and cellular responses with insulin itself (5). Moreover, high concentrations of insulin are known to activate IGF-1 receptors as well as insulin receptors (44). IGF-1 is present in high concentrations in the circulation and is locally produced by the cells of the cardiovascular system, where its role as a growth promoter is involved in several cardiovascular diseases (3, 14). A functional IGF-1/IGF-1 receptor autocrine loop is required for the mitogenic effects of growth factors such as platelet-derived growth factor (9, 35), epidermal growth factor (11), and VEGF (34). Indeed, both insulin itself and IGF-1 have been shown to induce an expression of VEGF mRNA in some cell systems (13, 26, 41).

VEGF has recently been championed as a potential new therapeutic agent for occlusive vascular disease. VEGF has been shown to increase intracellular calcium and to affect vascular tone through constitutive NOS, resulting in endothelium-dependent relaxation in coronary arteries (24). Furthermore, several recent studies (16, 28, 32) indicate a strong association between VEGF and an upregulation of eNOS expression. On the other hand, high levels of IGF and VEGF have been reported in some patients with diabetic retinopathy (1, 29), Inoue et al. (17) have demonstrated an expression of VEGF and its receptors in atherosclerotic segments of human coronary artery, but not in normal...
segments, and it has recently been reported (7) that recombinant human VEGF is capable of mobilizing macrophages and monocytes while simultaneously enhancing atherosclerotic plaque formation and progression. Thus VEGF may be involved in the progression of endothelial dysfunction [perhaps via an increased expression of VEGF (7)] and/or in its prevention [perhaps via an upregulation of eNOS (16, 28, 33)]. However, whether IGF and VEGF are involved in the endothelial dysfunction in macrovessels that is seen in diabetes has not been investigated in detail. In particular, few studies have directly assessed the possible relationship between the expressions of vascular growth factors and endothelial-dependent relaxation in insulin-treated and insulin-untreated established diabetes.

This study involved short-term, high-dose insulin treatment of rats that are controls and established STZ-induced diabetics. We investigated the effects of such treatment on the relationships between endothelium-dependent relaxation and IGF-1/VEGF expressions in the aorta. Furthermore, we also studied whether control and established diabetic rats given the same insulin treatment might differ in their expressions of IGF-1 receptor and VEGF.

MATERIALS AND METHODS

Animals and Experimental Design

Male Wistar rats (8 wk old and 180–250 g wt), received a single injection via the tail vein of 65 mg/kg STZ 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 National Institutes of Health Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (accorded by the Ministry of Education, Science, Sports and Culture of Japan).

Insulin Treatment

The STZ-induced diabetic (9 wk after the STZ injection) and control rats were treated with a gradually increasing dose of insulin (human insulin 5–30 U-kg⁻¹·day⁻¹) for 1 wk. Ten weeks after the STZ injection, the rats were euthanized by decapitation under diethyl ether anesthesia. Control rats were euthanized in the same way 10 wk after receiving their buffer injection.

Measurement of Plasma Glucose and Insulin

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

Measurement of Isometric Force

As mentioned above, rats were anesthetized with diethyl ether and euthanized by decapitation 10 wk 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 KHS (Krebs-Henseleit solution). The solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO₃, 1.8 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgSO₄, and 11.0 dextrose. The aorta was cleaned of loosely adhering fat and connective tissue and then 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 (model TB-611T; Nihon Kohden). 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 l-norepinephrine (NE) (5 × 10⁻⁸–3 × 10⁻⁷ M). When the NE-induced contraction reached a plateau level, ACh (10⁻⁹–10⁻⁵ M), sodium nitroprusside (SNP) (10⁻⁹–10⁻⁵ M), or IGF-1 (10⁻⁹–3 × 10⁻⁸ M) was added in a cumulative manner. When the effects of N⁷-nitro-l-arginine (l-NNA) (10⁻⁴ M) and indomethacin (10⁻⁵ M) on the response to the relaxant agents were examined in the diabetic aorta, one of these agents was added to the bath 20 min before the administration of NE.

Measurement of Nitrite and Nitrate

Concentrations of nitrite (NO₂⁻) and nitrate (NO³⁻) in the effluent from each type of tissue were assayed by the method described by Yamada and Nabeshima (43). Briefly, the NOx in the perfusate were separated by means of a reverse-phase separation column packed with polystyrene polymer (4.6 × 50 mm; NO-PAK, Eicom), after which NO₃⁻ was reduced to NO₂⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). NO₂⁻ 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 containing 0.15 M NaCl/NH₄Cl and 0.5 g/l 4 Na-EDTA. The Griess reagent, which was 1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l N-naphthylethylenediamine, was delivered at a rate of 0.1 ml/min. For the determination of NOx, the samples were collected during a 40-min period of stimulation by 10⁻⁷ M ACh or without ACh stimulation. The concentration of NO₃⁻ or NO₂⁻ in KHS and the reliability of the reduction column were examined in each experiment.

Measurement of Expressions of mRNAs for eNOS, IGF-1/Receptor, and VEGF/Receptor

Oligonucleotides. The primers used in this study are summarized in Table 1.

RNA isolation and RT-PCR. RNA was isolated by the guanidinium method (8). Briefly, rat aortas were carefully isolated and cleaned of adhering parenchyma and connective tissue. The aortas were homogenized in RNA buffer, and RNA was isolated 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 twenty-eight PCR cycles (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) were performed using one-half of the reverse-transcription mixture. The PCR products so obtained were analyzed on ethidium bromide-stained agarose (1.5%) gels. The PCR products were quantified by scanning densitometry with the amount of each product being normalized with respect to the amount of GAPDH product.

Drugs

STZ, NE hydrochloride, SNP, and recombinant human IGF-1 were all purchased from Sigma (St. Louis, MO). ACh
controls, STZ-diabetic rats, and insulin-treated rats.

**Statistical Analysis**

The contractile force developed by aortic strips from control and diabetic rats is expressed in milligrams of tension per milligrams 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 analysis of variance (ANOVA), and a probability level of $P < 0.05$ was considered 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 ($P < 0.05$ again being considered significant).

**RESULTS**

**Plasma Glucose and Insulin Levels**

As shown in Table 2, plasma glucose levels were significantly elevated in STZ-induced diabetes, whereas short-term treatment with high-dose (5–30 U·kg$^{-1}$·day$^{-1}$ for 1 wk) insulin in our established diabetic rats produced a plasma glucose concentration that was not different from that of the controls. In control rats, plasma glucose levels were lower (but not significantly) in insulin-treated animals than in untreated ones. Plasma insulin levels were significantly lower in STZ-induced diabetics than in controls and significantly higher in each insulin-treated group than in the corresponding insulin-untreated group.

**Relaxation Responses to ACh, SNP, Insulin, and IGF-1**

When the NE (5 × 10$^{-8}$–3 × 10$^{-7}$ M)-induced contraction had reached a plateau, ACh (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. After the administration of insulin for 1 wk, aortic strips from STZ-induced diabetic rats relaxed in a normal way to ACh. In contrast, treating control rats with insulin had no significant effect on the relaxation caused by ACh. The aortic relaxation caused by SNP (10$^{-9}$–10$^{-5}$ M) was not significantly different among the various groups (data not shown). In aortic strips from age-matched control rats, insulin and IGF-1 (10$^{-9}$–3 × 10$^{-8}$ M) each caused a concentration-dependent relaxation. These relaxation responses were significantly stronger in strips from STZ-induced diabetic rats (Fig. 2). After the administration of insulin for 1 wk, the relaxation responses to both IGF-1 and insulin were further increased (Fig. 2). Both the IGF-1- and insulin-induced relaxation responses in diabetic aortas were greatly diminished by preincubation with the NOS inhibitor.

![Fig. 1. Concentration-response curves for ACh-induced relaxation of aortic strips obtained from age-matched controls, untreated diabetic rats, and rats given short-term, high-dose insulin treatment. The y-axis shows the relaxation of aortic strips as a percentage of the contraction induced by an equieffective concentration of norepinephrine (5 × 10$^{-8}$–3 × 10$^{-7}$ M). Each data point represents the mean ± SE of 6–8 experiments; the SE is included only when it exceeds the dimension of the symbol used. ++++$P < 0.001$, diabetic vs. control; ††††$P < 0.001$, diabetic vs. insulin-treated diabetic.](image-url)
L-NNA (10⁻⁴ M) (Fig. 2), but not by preincubation with indomethacin (10⁻⁵ M) (data not shown).

Measurement of NOx

ACh increased the NOx level in the perfusate from aortic strips. The ACh-induced NOx level was not different between controls and diabetics. However, NOx was significantly increased in aortas from insulin-treated diabetics, although it was not different between controls, insulin-treated controls, and insulin-untreated diabetics (Table 3).

Expression of mRNA for eNOS

To investigate the possible mechanisms underlying the impaired ACh-induced relaxation seen in STZ-induced diabetic rats and its normalization by insulin treatment for 1 wk, we examined whether the expression of the mRNA for eNOS might have been changed by the insulin treatment. With the use of RT-PCR on the total RNA isolated from the aortas of age-matched controls, untreated diabetic, and chronic insulin-treated control and diabetic rats, we made the following discovery. First, the expression of GAPDH mRNA showed no differences among the four groups. Second, the expression of the mRNA for eNOS was significantly increased in aortas from insulin-treated diabetics (vs. both insulin-untreated diabetic and insulin-treated controls), but it was not different among controls, insulin-treated controls, and insulin-untreated diabetics (Fig. 3).

Expressions of mRNAs for IGF-1/IGF-1 Receptor and VEGF/VEGF-1 Receptor

We then tried to determine whether administration of insulin for 1 wk might increase the expressions of the
mRNAs for IGF-1/IGF-1 receptor and VEGF/VEGF-1 receptor. The expression of IGF-1 mRNA showed no differences among the various groups (Fig. 4, A and B). The expression of the mRNA for VEGF was significantly increased in aortas from insulin-treated diabetics (vs. both insulin-untreated diabetes and insulin-treated controls), but it was not different among control, insulin-treated controls, and untreated diabetics (Fig. 4, A and C). When the expression of the mRNA for the IGF-1 receptor was studied, it was significantly increased in the aortas from diabetic rats (vs. controls), and (perhaps significantly) further increased in those from high-dose insulin-treated diabetics (Fig. 5, A and B). The expression of VEGF-1 receptor mRNA showed no differences among the various groups (Fig. 5, B and C).

**DISCUSSION**

The main conclusion to be drawn from the present study is that in rats with established STZ-induced diabetes, short-term, high-dose administration of insulin not only normalizes the impaired endothelium-dependent relaxation in the aorta, but also upregulates the expressions of the mRNAs for eNOS and VEGF. Furthermore, these expressions were higher in insulin-treated diabetics than in the controls. These effects in diabetes may be related to the observed increase in the expression of the IGF-1 receptor in the diabetic aorta (insulin untreated and insulin treated).

Our studies are consistent with previous reports (20, 22) on aortas from rats with established STZ-induced diabetes, in which short-term, high-dose administration of insulin normalized the impaired endothelium-dependent relaxation together with an associated increase in expression of the mRNA for NOS and an enhancement of the production of NO. The normalization of endothelial function by insulin treatment in our study was accompanied by an increased level of NOx, which is composed of NO metabolites. Although arterial smooth muscle cells and endothelial cells express receptors for both insulin and IGF-1 (19), recent re-
ports (6, 12, 40, 42) suggest that the vasodilation induced by insulin may be mediated primarily via its stimulatory effects on the IGF-1 receptor. Furthermore, several studies (20, 40) have suggested that the vasorelaxation induced by insulin or IGF-1 is most likely mediated by the production of vascular NO. We found in the present study that the expression of IGF-1 receptor mRNA was increased in aortas from both diabetic and insulin-treated diabetic rats. Indeed, although the ACh-induced relaxation was impaired, the concentration-dependent relaxations induced by IGF-1 and insulin, which are also endothelium dependent, were greater in diabetic rats. This is supporting evidence for an increased expression of the IGF-1 receptor in diabetes. In our STZ-diabetic rats, the plasma insulin level was significantly lower than in the controls (Table 2), and in this condition administration of high-dose insulin may lead to a significant stimulation of IGF-1 receptors. Indeed, we found in the present study that the expression of the mRNA for VEGF, which is regulated by IGF-1 (see below; 13, 26, 41), was significantly increased only in insulin-treated diabetic rats.

IGF-1 is able to induce the expression of several genes, including those that encode VEGF. Indeed, IGF-1 strongly increases the expression of VEGF mRNA and the production of VEGF protein by culture cells (13, 26, 41). Hence, any increase in the expression of the IGF-1 receptor in established diabetes (for example, by high-dose insulin treatment; see Fig. 5) could promote VEGF expression. Evidence from several studies using different cell types has shown that the IGF-1 receptor is at a convergence point in the control of cell growth and function. Interestingly, a recent study (34) has demonstrated that administration of an IGF-1 receptor antagonist suppresses retinal neovascularization and that an interaction between IGF-1 and the IGF-1 receptor is necessary for the induction of neovascularization by VEGF. In fact, in our study, the expression of IGF-1 receptor mRNA in the aorta was higher in the insulin-treated diabetic group than in the untreated diabetic one. Thus it is indeed likely that the observed enhancement of IGF-1 receptor expression in insulin-treated diabetes was responsible for the promotion of VEGF expression we observed in that group (see Fig. 4).

The biological activities of VEGF are mediated through two high-affinity receptor tyrosine kinases: fms-like tyrosine kinase-1 (Flt-1)/VEGF receptor 1 and fetal liver kinase-1/VEGF receptor 2 (12, 38). In the present study, an increased expression of the mRNA for VEGF was found in aortas from high-dose insulin-treated diabetic rats. However, the expression of the mRNA for VEGF receptor 1/Flt-1 was unaffected by diabetes or insulin treatment. Acutely in vivo, VEGF has been shown to induce an endothelium-dependent vasodilation accompanied by a decrease in mean arterial blood pressure (24), whereas in cultured endothelial cells VEGF upregulates eNOS mRNA and protein and stimulates eNOS production (16, 28, 33). Thus the amount of VEGF would be expected to be closely related to the magnitude of the associated NO production and hence to the size of any effect on endothelial function. We found that the expressions of eNOS and VEGF were both increased in aortas from insulin-treated diabetics rats. These results strongly suggest that the high insulin level in the plasma in this group leads to an upregulation of VEGF expression and to a turn in an increase in NOS in the vascular wall and that this tends to normalize the impaired endothelium-dependent vasodilation otherwise seen in the diabetic rat aorta. In agreement with our findings, it has been reported (2) that the administration of VEGF results in improved endothelium-dependent responses in relatively large collateral vessels.

VEGF has emerged as a potentially exciting therapeutic agent for coronary and peripheral occlusive vascular disease. In the present study, insulin treatment proved able to reverse the impairment of endothelium-dependent relaxation seen in established diabetes in the rat through overexpressions of eNOS and VEGF mRNAs. On the other hand, several studies (7, 17, 14, 44) have suggested that VEGF itself and the IGF-1 receptor may promote the process of atherosclerosis, although high-dose insulin treatment in established diabetes seems able to restore endothelial function by enhancing eNOS, VEGF, and IGF-1 receptor expressions. More recently, Celletti et al. (7) reported that recombinant human VEGF enhances the progression and potential destabilization of atherosclerotic plaques. For the improvement effect of insulin, a possible explanation is the following: 1) the IGF-1 receptor is upregulated in STZ-induced diabetic rats; 2) insulin, which binds to the IGF-1 receptor, may significantly stimulate this receptor; 3) the stimulation of the IGF-1 receptor may lead to an increased expression of VEGF; and 4) the increased VEGF may upregulate eNOS, thereby resulting in an improvement in endothelial function in STZ diabetes. Of particular interest in this connection, it has been reported (26) that VEGF induction by insulin and IGF-1 occurs via different signaling pathways, the former involving phosphatidylinositol 3-kinase/protein kinase B and the latter mitogen-activated protein kinase. Although we do not have direct evidence for interactions among insulin, IGF-1, VEGF, and eNOS, these interactions may be altered in the diabetic state. To establish causal relationships will require research focusing, for example, on time-course changes in the expressions of the mRNAs for IGF-1, VEGF, and eNOS.

One week of insulin treatment increased the plasma insulin level in both the control and diabetic groups (Table 2), and it also ameliorated endothelial dysfunction and increased eNOS mRNA, suggesting that the hyperinsulinemia present in insulin-treated established STZ-induced diabetic rats may be responsible for these changes.

In conclusion, we found that 1 wk of insulin treatment in diabetic rats led to an enhanced expression of the IGF-1 receptor. This presumably increased the expression of VEGF mRNA, and the increased VEGF presumably upregulated eNOS, thereby resulting in an amelioration in the endothelial dysfunction otherwise
seen in diabetic rats. Furthermore, these expressions were higher in insulin-treated diabetic than in control (nondiabetic) rats. On the downside, an increase in the expression of the IGF-1 receptor may be a key event in the progress of atherosclerosis in diabetes. The above effects may follow from the increased IGF-1-receptor expression that occurs in both the insulin-untreated diabetic and insulin-treated diabetic aorta.

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