Differential Effects of Selective Endothelin Type A Receptor Antagonist on the Gene Expression of Vascular Endothelial Growth Factor and Its Receptors in Streptozotocin-Induced Diabetic Heart

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Cardiovascular complications are an important feature of diabetes mellitus (DM). Abnormal and decreased coronary collateral development has been implicated in the pathogenesis of cardiac complications in DM. More recently, decreased expression of vascular endothelial growth factor (VEGF) and its receptors has been found in diabetic heart. To our knowledge, no study has focused on the therapeutic improvement associated with VEGF in diabetic heart. DM was induced by intraperitoneal injection of streptozotocin (65 mg/kg) in Sprague-Dawley rats, while control rats received only citrate buffer. After 1 week, the streptozotocin-treated rats were randomly divided into two groups: one group received the selective endothelin (ET) type A receptor antagonist TA-0201 at a dose of 1 mg/kg/day for 2 weeks by osmotic mini-pump, and the vehicle group received saline only. The plasma glucose level was 504 ± 75 mg/dl in the diabetic rats and was unchanged by treatment with ET antagonist. The body weight was decreased in the diabetic rats compared with the control rats, but the left ventricular (LV)–body weight ratio was increased in the diabetic group and was unaffected by treatment with ET antagonist. mRNA expression of VEGF and its receptors (Flt-1 and Flk-1) in the LV tissues was assessed using real-time polymerase chain reaction. VEGF expression was significantly decreased in diabetic heart and was greatly improved by treatment with ET antagonist. The expression of VEGF receptors was down-regulated in early diabetic heart but was not recovered by treatment with ET antagonist. ET and its receptor A might have differential regulation on the gene expressions of VEGF and its receptors in early diabetic heart. Exp Biol Med 231:902–906, 2006

Key words: diabetic heart; VEGF and its receptors; endothelin antagonist; endothelin type A receptor

Macrovascular and microvascular complications in multiple organ systems are associated with the metabolic abnormalities in diabetes mellitus (DM) (1). Epidemiologic and pathologic data demonstrate that DM is an independent risk factor for cardiovascular disease in men and women (2). Coronary artery disease is the leading cause of morbidity and mortality in patients with DM (3). When large vessels are affected by atherosclerosis, the development of collateral vessels can be viewed as an attempt to minimize the degree of ischemic damage. Vascular endothelial growth factor (VEGF), which is a major mediator of neovascularization in physiologic and pathophysiologic conditions, has crucial roles in developmental blood vessel formation and regulation of hypoxia-induced tissue angiogenesis (4). VEGF is an endothelial cell–specific mitogen *in vitro* and *in vivo* (5, 6). Two high-affinity VEGF tyrosine kinase receptors have been identified: fms-like tyrosine kinase 1 (Flt-1) (also known as VEGF-R1) and fetal liver kinase 1 (Flk-1) (or VEGF-R2). Both receptors are expressed almost exclusively in endothelial cells (7).

VEGF has potential roles in the development of collateral vessels in different cardiac diseases. The expression of VEGF is greatly increased after myocardial infarction (MI) in the hearts of nondiabetic patients (8), and upregulation of VEGF contributes to the development of collateral vessels in the advanced stages of coronary atherosclerosis (9). Patients with DM, however, exhibit

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inadequate collateral vascular formation in response to ischemia, which increases cardiovascular morbidity and mortality rates (9). Structural and functional abnormalities of the coronary collateral circulation have been reported in clinical and experimental DM (9, 10).

A recent study showed that the expression of mRNA and protein for VEGF and its receptors (Flt-1 and Flk-1) in the myocardium was significantly decreased in nonischemic short-term (4 weeks) experimental diabetic rats (11). In the same report, preliminary findings demonstrated 2-fold reductions in VEGF and Flk-1 in autoptic ventricular specimens from patients with DM compared with nondiabetic patients who had died acutely of MI (11).

The production of endothelin (ET)-1, a potent vasoconstrictor with vasoproliferative activity (12), is reported to be increased in DM (13). Several observations suggest a potential role of the activated ET-1 system in the pathophysiology of complications of DM. Increased plasma immunoreactive ET-1 has been demonstrated in some animal models of DM (14–16) and clinically in patients with DM (17, 18). The activity of endogenous ET-1 on ET type A (ET_A) receptor is enhanced in the resistance vessels of patients with DM (13, 19). Therefore, alterations in the ET system may play a significant role in cardiovascular complications associated with DM.

The present study was designed to investigate the mRNA expression of VEGF and its two receptors in the early diabetic heart. We then assessed the effects of a selective ET_A receptor antagonist on these expressions.

Materials and Methods

Animals and Drug Treatment. Male, 10-week-old, Sprague-Dawley rats were obtained from Charles River Japan, Inc. (Yokohama, Japan) and cared for according to the *Guiding Principles for the Care and Use of Animals* based on the 1964 Helsinki Declaration. The prediabetic rats received single 65 mg/kg intraperitoneal injections of streptozotocin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 0.1 mol/l citrate buffer (pH 4.5). Control nondiabetic animals ($n = 11$) were administered citrate buffer only. Animals with blood glucose levels higher than 250 mg/dl 48 hrs after the streptozotocin injection were considered diabetic. One week after the streptozotocin injection, the diabetic animals were randomly divided into two groups: one group ($n = 11$) received the selective ET_A receptor antagonist TA-0201 at a dose of 1 mg/kg/day for 2 weeks by osmotic mini pump (model 2004; Durect Corporation, Cupertino, CA), while the vehicle group ($n = 11$) was administered physiologic saline only. Before the start of the drug treatment, blood glucose levels were determined almost daily; after the treatment started, the diabetic status was assessed weekly. The rats were fed standard laboratory chow and were allowed free access to water in an air-conditioned room with a 12-hr light:dark cycle until they were humanely killed. After 2 weeks treatment, blood pressure was recorded, rats were humanely killed under anesthesia, and heart tissue was removed. Left ventricle (LV) was dissected from the heart tissue and was used for different types of experiments. The present experimental design was approved by the University of Tsukuba School of Medicine Animal Care and Use Committee.

Hemodynamic Measurements. On the day of the experiment, the rats were anesthetized with pentobarbital sodium (40 mg/kg body weight ip), and a microtip pressure transducer catheter (SPC-320; Millar Instruments, Houston, TX) was inserted into the left carotid artery. Arterial blood pressure and heart rate were monitored using a pressure transducer (model SCK-590; Gould, Cleveland, OH) and were recorded using a polygraph system (AP-601G amplifier, AT-601G tachometer, and WT-687G thermal pen recorder; Nihon Kohden, Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR). Total tissue (LV) RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene, Toyama, Japan). Briefly, the tissue was homogenized in Isogen (100 mg tissue/1 ml Isogen) with a Polytron tissue homogenizer (model PT10SK/35; Kinematica, Lucerne, Switzerland). The precipitated RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% (vol/vol) ethanol. The resulting RNA was resolved in diethyl pyrocarbonate–treated water, treated with DNase I (Takara, Shiga, Japan), and extracted again with Isogen to eliminate the genomic DNA. The RNA concentration was determined spectrophotometrically at 260 nm. Total tissue RNA was primed with 0.05 μg of oligo d $(pT)_{12–18}$ and reverse transcribed by omniscript reverse transcriptase using a first-strand cDNA synthesis kit (Qiagen, Tokyo, Japan). The reaction was performed at 37°C for 60 mins.

The mRNA expression levels of VEGF, Flt-1, and Flk-1 in the LV tissues were analyzed by quantitative RT-PCR with TaqMan probe using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster, CA). The gene-specific primers and TaqMan probes were synthesized using Primer Express v. 1.5 software (Perkin-Elmer Applied Biosystems) according to the published cDNA sequences for each of the following: VEGF, Flk-1, Flt-1, and glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The sequences of the oligonucleotides were as follows:

VEGF forward: 5′-TGAGACCCTGGTGGACATCTT-3′

VEGF reverse: 5′-CACACAGGACGGCTTGAAGA-3′

VEGF probe: 5′-CCCCGATGAGATAGAGTAT-3′

Flk-1 forward: 5′-GAAACTGAATGGCACCGTGTT-3′

Flk-1 reverse: 5′-GCAGGGAGGCATTCTGGAAT-3′

Flk-1 probe: 5′-CTAACAGCACAAACGACATCT-3′

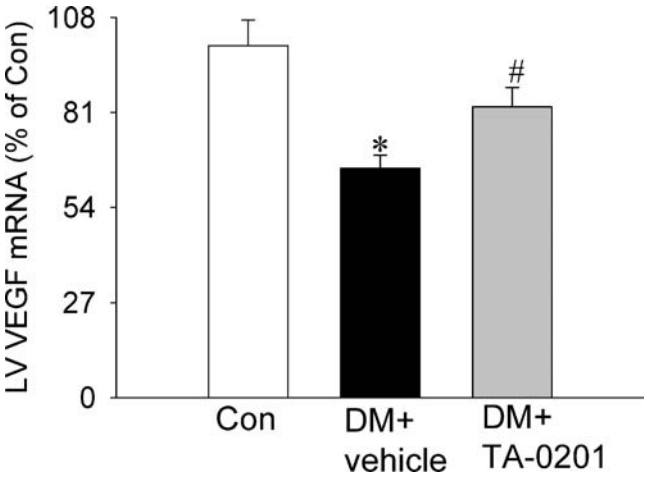

Figure 1. The mRNA expression of VEGF, determined using RT-PCR, in the LV tissues of control, diabetic vehicle, and diabetic TA-0201 rats. The data are means ± SD ($n = 10$) and are shown as the relative levels (the value of the control is defined as 100%). *$P < 0.01$ vs. control. #$P < 0.01$ vs. diabetic vehicle (one-way analysis of variance followed by Fisher protected least significant difference test for multicomparison).

Flt-1 forward: 5′-TCGGCTGTCCATGAAAGTGAAGT-3′

Flt-1 reverse: 5′-GCGGGTACGCCATCTTTTAAC-3′

Flt-1 probe: 5′-CCTCGCCAGAAGTCGTATG-3′

GAPDH forward: 5′-GTGCCAAAAGGGTCATCATCTC-3′

GAPDH reverse: 5′-GGTTCACACCCATCACAAACATG-3′

GAPDH probe: 5′-TTCCGCTGATGCCCC-3′

The expression of GAPDH mRNA was used as an internal control. The PCR mixture (25 μl total volume) consisted of 450 n*M* of forward and reverse primers for VEGF, Flk-1, Flt-1, and GAPDH; 200 n*M* of FAM-labeled primer probes; and TaqMan Universal PCR Master Mix (all from Perkin-Elmer Applied Biosystems). Each PCR amplification was performed in triplicate, using the following profile: 1 cycle of 95°C for 10 mins and 40 cycles of 94°C for 15 secs and 60°C for 1 min. For the standard curve in the quantitative RT-PCR, serial dilutions of rat heart cDNA were performed within the range of various concentrations (1×, 2×, 4×, 8×, and 16×). A no template (water) reaction mixture was prepared as a negative control.

Statistical Analysis. Values are means ± SD. Statistical assessment of the data was performed using one-way analysis of variance with multiple comparisons using the Fisher protected least significant difference test. Nonparametric data were analyzed using the Mann-Whitney *U* test or the Wilcoxon signed rank test. $P < 0.05$ was considered significant.

Results

There was no significant alteration in systolic blood pressure in the experimental animals. Values for the control, diabetic vehicle, and diabetic TA-0201 groups were 123 ± 15, 111 ± 15, and 115 ± 20 mm Hg, respectively.

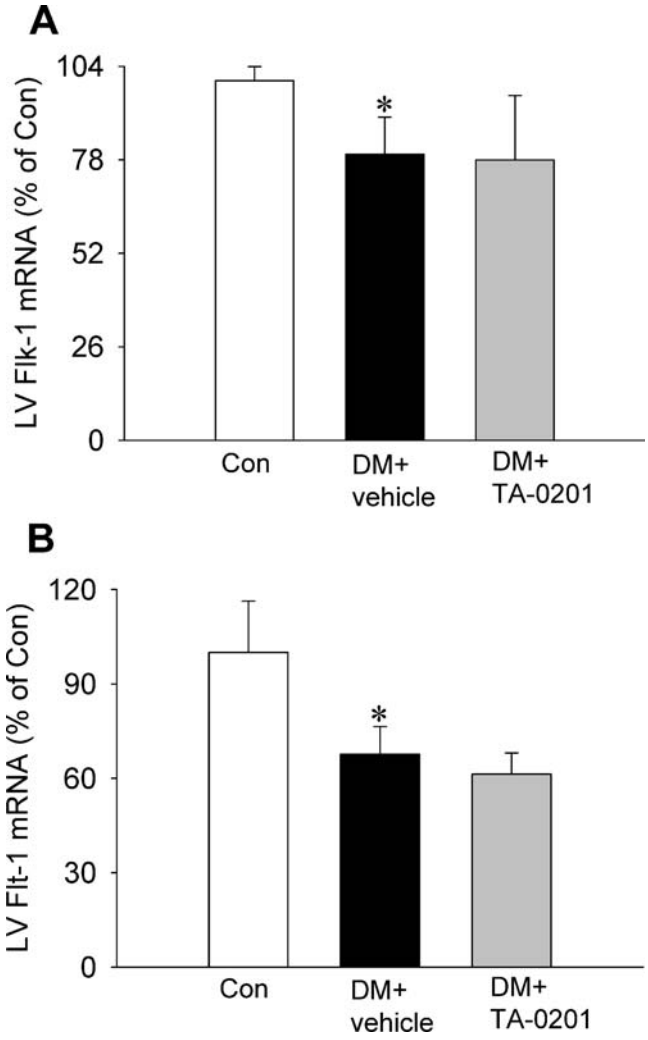

Figure 2. The mRNA expression of Flk-1 (A) and Flt-1 (B), determined using RT-PCR, in the LV tissues of control, diabetic vehicle, and diabetic TA-0201 rats. The data are means ± SD ($n = 10$) and are shown as the relative levels (the value of the control is defined as 100%). *$P < 0.01$ vs. control (one-way analysis of variance followed by Fisher protected least significant difference test for multicomparison).

A 35% decrease in VEGF mRNA expression in LV tissues in diabetic heart was observed, and this downregulation was significantly recovered by treatment with selective ET_A receptor antagonist (Fig. 1). The mRNA expression of the VEGF receptors Flk-1 and Flt-1 was significantly downregulated in diabetic heart, but this downregulation was not recovered by treatment with ET antagonist (Fig. 2A and B).

Discussion

In the present study, we investigated whether there is any treatment option that would reverse the downregulated VEGF and its receptor expression in early diabetic heart.

Our results demonstrated that the mRNA expressions of VEGF and its receptors were significantly downregulated during 2 weeks in streptozotocin-induced diabetic heart. The downregulated VEGF was greatly recovered by treatment with ET antagonist, while ET antagonist had no reversal effects on the decreased mRNA expression of VEGF receptors in diabetic heart.

The plasma glucose level was significantly higher in diabetic rats (504 ± 75 mg/dl) compared with nondiabetic control rats (115 ± 13 mg/dl) ($P < 0.001$). The plasma insulin level was remarkably decreased in diabetic rats (0.32 ± 0.29 ng/ml) compared with nondiabetic control rats (7.93 ± 2.44 ng/ml) ($P < 0.001$). The plasma glucose (505 ± 62 mg/dl) and insulin (0.23 ± 0.17 ng/ml) levels were not affected by treatment with ET antagonist. The systolic blood pressure was unchanged among the experimental animals. The animal models used in the present study were hyperglycemic with greatly reduced plasma insulin levels.

In recent years, there have been conflicting reports concerning the expression of VEGF and its receptors in diabetic heart. While Chou et al. (11) demonstrated a significant decrease in VEGF and its receptor expression in cardiac tissues in diabetic animals and in patients with DM (11), a recent study showed increased VEGF mRNA expression in the hearts of long-term (3 months) experimental diabetic rats, although, the mRNA transcripts of Flt-1 and Flk-1 receptors were unaffected by the diabetic state (20). The authors theorized that VEGF is decreased in short-term DM but is increased in long-term DM, owing to a compensatory reaction from desensitization or downregulation of the VEGF receptors in the diabetic state (20).

Normal mammalian heart expresses VEGF and its receptors. After cardiac ischemic events, coronary collateral blood vessel formation contributes to preserving myocardial function and viability by reducing myocardial ischemia and functional deficit (21), and VEGF is believed to play a significant role in this adaptive response. *VEGF* gene transfer is an important therapeutic option to induce vascular growth after critical ischemia (22, 23). It might be important to improve the decreased VEGF and its receptor levels in the early diabetic heart, and this has led us to search for a pharmacologic compound that would normalize the downregulated VEGF expression in diabetic heart, although a recent report has demonstrated the attenuation of DM-induced cardiomyopathic changes in streptozotocin-induced diabetic heart by *VEGF* gene therapy (24).

ET-1, which is a potent vasoconstrictor, has been implicated in the pathogenesis of a variety of cardiovascular diseases. Early blockade of ET-1 might have a potential role in arresting the VEGF-induced molecular and morphologic remodeling in diabetic retina (authors' submitted publication). Although the role of VEGF is detrimental in the diabetic eye, patients with DM have abnormal coronary collateral development, which might be due to the decreased myocardial VEGF level in subjects with DM. In the present study, ET antagonism partly normalized the *VEGF* gene expression in diabetic heart. Moreover, VEGF protein expression was 40% downregulated in diabetic heart compared with control heart in immunoblot analysis, and this downregulation was greatly normalized by treatment with ET antagonist ($P < 0$ .001) (authors' unpublished observation, 2005). It is difficult to speculate how VEGF expression is regulated by ET-1 in different tissues in diabetic models. For the diabetic eye, it might be simpler to consider that blockade of ET-1 could reverse the upregulated VEGF in diabetic retina as in ovarian carcinoma; ET-1 has been identified as the potent stimulator for increased VEGF expression via ET_A receptor and is associated with the induction of hypoxia-inducible factor (HIF)-1α (25). A recent observation is that ET-1 induces VEGF expression in neonatal ventricular cardiomyocytes *in vitro*. However, the mechanism behind the normalization of VEGF in diabetic heart by ET antagonist cannot be ruled out based on the results of the present study. Additional studies should aim at mechanistic insights concerning the reversal effect on downregulated VEGF by ET antagonist in diabetic heart.

ET-1 might have differential regulatory roles on the gene expression of VEGF receptors in diabetic heart. In future studies, the effects of ET antagonism on VEGF receptor expression at the protein levels should be examined. The differential effects of ET antagonism on the expression of VEGF and its receptors also raise the question whether the recovery of VEGF receptor requires longer treatment with ET antagonist. Other investigations should look at the capillary morphology of coronary circulation in the experimental animals used herein. In the present experimental setting, there was no echocardiographic heart abnormality.

In conclusion, we demonstrated that selective ET_A receptor antagonist could recover the downregulated *VEGF* gene expression in early diabetic heart. Decreased mRNA expression of VEGF receptors was not ameliorated by selective ET_A receptor antagonist.

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