# Effects of a Selective Endothelin A Receptor Antagonist on the Expressions of iNOS and eNOS in the Heart of Early Streptozotocin-Induced Diabetic Rats

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**Vascular tone is regulated through the actions of locally produced agents. Among the vasoconstrictors, the most potent agent is endothelin (ET), which exerts its vasoconstrictor actions principally through ET type A ($ET_A$) receptors. Of the vasodilators, nitric oxide (NO) seems to be the most important contributor to the acute regulation of vascular tone. Vasculopathy is an important feature of diabetes mellitus (DM). Endogenous ET-mediated vasoconstrictor tone is augmented in diabetic states, and conflicting results persist concerning the NO system in diabetes. The present study investigated the expressions of inducible NO synthases (iNOS) and endothelial NOS (eNOS) in the heart of diabetic animals and the effects of a selective $ET_A$ receptor antagonist on these alterations. Type I diabetes was induced by intraperitoneal injection of streptozotocin (65 mg/kg) in Sprague-Dawley rats, while control (Con) rats received only citrate buffer. After 1 week, the streptozotocin-administered rats were randomly divided into two groups: the selective $ET_A$ receptor antagonist–administered group (DM+TA-0201, 1 mg/kg/day, by osmotic minipump for 2 weeks) and the DM+vehicle group (comprising the diabetic rats that received saline). The random blood glucose level was 405 ± 103 mg/dl in DM animals, and this level was unchanged by ET antagonism. Body weight was more greatly decreased in DM rats than in Con rats, but the left ventricle to body weight ratio was increased in the DM group and was unaffected by ET antagonism. Protein expressions of eNOS and iNOS were assessed in the left ventricular tissues. eNOS expression was significantly increased in DM heart and was greatly inhibited by the treatment with ET antagonist. The expression of iNOS was also increased in early DM heart but was reversed by the ET antagonist. Thus, endothelin antagonism might be beneficial for DM heart by reversing the upregulated eNOS and iNOS expressions.** Exp Biol Med 231:925–931, 2006

**Key words:** diabetic heart; eNOS; iNOS; endothelin type A receptor; endothelin antagonist

Cardiovascular complications of diabetes are the leading causes of morbidity and mortality among diabetic patients (1). A variety of mechanisms, including coronary atherosclerosis, autonomic neuropathy, macroangiopathy, and diabetic cardiomyopathy, have been implicated in the development of cardiovascular disease in patients with diabetes (2, 3).

Vascular complications play a vital and crucial role in the morbidity and mortality of patients with diabetes mellitus (DM) (4). The mechanisms underlying the development of microvascular and macrovascular angiopathy in patients with Type I (insulin-dependent) DM are complex and poorly understood. Vascular tone is determined by a balance between vasoconstricting and vasodilating factors. Nitric oxide (NO), generated from L-arginine by NO synthases (NOS) (5), is an important endogenous vasodilator and has been involved in the regulation of blood flow observed in diabetes (6). Thus, impaired NO activity could be implicated in the development of diabetic vasculopathy. Endothelium-derived NO has several potential antiatherogenic actions, including inhibition of platelet aggregation, adhesion molecule expression, and vascular smooth muscle cell proliferation (7), and defective production or activity of NO enhances atherosclerosis in experimental models (8). Various defects in NO pathways have been reported in Type I diabetes in both humans and animal models (9). However,

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the *in vivo* data are inconsistent (4) and conflicting, and it is not clear whether the defect is in basal or stimulated NO synthesis, NO bioavailability, responsiveness to NO, or perhaps all of these.

NO is a labile molecule and may carry out important biological roles both within the cell in which it is synthesized and in interactions with nearby cells and molecules (10, 11). Since NO may be either stabilized or degraded through its interactions with diverse intracellular or extracellular chemical moieties, the localization of NOS within the cell might be expected to influence the biological role and chemical fate of the NO produced by the enzyme. NO is synthesized by three different isoforms of NOS. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and synthesize small amounts of NO under basal conditions and on stimulation by various stimuli, including, in the case of eNOS, shear stress and insulin (12). In contrast, inducible NOS (iNOS) is expressed when stimulated by inflammatory cytokines and can produce up to 1000-fold more NO than eNOS (13), which, while important for the immune response, can have detrimental effects on different cell types, including vascular cells (14). Excess NO generation resulting from iNOS induction is important not only for nonspecific host defense, *via* its cytotoxic and bactericidal effect (5), but also for regulation of cell growth and programmed cell death (apoptosis) (15).

As Type I diabetes is associated with vascular endothelial abnormalities, vasoactive mediators such as endothelins and reactive oxygen species are modulated in diabetic patients. A growing body of evidence indicates that the potent vasoconstrictor endothelin-1 (ET-1) may play an important role in the pathogenesis or reinforcement of cardiovascular diseases (16). Diabetes caused significant increases in ET-1 mRNA and protein expression in the heart at all time points of 6 months in streptozotocin (STZ)-induced diabetic rats, indicating that ET-1 induces functional and structural changes in the myocardium in diabetes (17).

The present study was designed to investigate the expressions of iNOS and eNOS in the heart of early STZ-induced diabetic rats and the effects of a selective ET type A ($ET_A$) receptor antagonist on these alterations.

## 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 Helsinki Declaration (1964). The rats to be diabetic received single 65 mg/kg intraperitoneal injection of STZ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 0.1 mol/l citrate buffer (pH 4.5). Control nondiabetic animals were administered citrate buffer only (non-DM control). Animals with blood glucose levels greater than 250 mg/dl 48 hrs after the STZ injection were considered to be diabetic. One week after the STZ injection, the diabetic animals were randomly divided into two groups; one group received the selective $ET_A$ receptor antagonist (TA-0201) at a dose of 1 mg/kg/day for a total of 2 weeks (by osmotic minipumps, model 2004; Durect Corporation, Cupertino, CA) (DM+TA-0201), while the vehicle group was treated with physiologic saline only (DM+vehicle). Before the start of the drug treatment, blood glucose was determined almost every day, but after the treatment began, the diabetic status was assessed every week. The rats were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12:12-hr light:dark cycle until time of sacrifice. After 2 weeks of treatment, rats were sacrificed under anesthesia and the heart tissue was removed. Left ventricle (LV) was dissected from heart tissues and was used for different types of experiments. In another set of experiments, male 10-week-old Sprague-Dawley rats were divided into two groups; control vehicle animals were treated with physiologic saline only (control+vehicle) and another group of control rats received the selective $ET_A$ receptor antagonist (TA-0201) at a dose of 1 mg/kg/day for a total of 2 weeks by osmotic minipump (control+TA-0201). Then the heart tissues were processed, as mentioned above. The present experimental design was approved by the Tsukuba University School of Medicine Animal Care and Use Committee.

**Enzyme Immunoassay of iNOS and eNOS.** LV tissues from the study groups were homogenized and centrifuged, and the protein concentration was measured in the supernatant after centrifugation. These steps were performed according to the instructions of the immunoassay kits used for the eNOS and iNOS protein detection in the tissue extracts. The kits for the determination of iNOS and eNOS were purchased from R&D Systems (Minneapolis, MN). The enzyme immunoassays were performed according to the manufacturer's instructions.

**Western Blot Analysis.** After removing and rinsing the LV tissues in sterilized water on ice, the tissues were minced with scissors, homogenized, and centrifuged at 500 *g* for 15 mins to pellet any insoluble material. The protein concentration of supernatant was determined with the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Samples (20 μg) were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4%–12% polyacrylamide gel and were electrotransferred to polyvinylidine difluoride filter membrane. To reduce nonspecific binding, the membrane was blocked for 2 hrs at room temperature with 1% bovine serum albumin in phosphate-buffered saline (PBS) (137 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* $Na_2HPO_4$, and 1.5 m*M* $KH_2PO_4$) containing 0.1% Tween 20. Thereafter, the membrane was incubated overnight at 4°C with specific antibody for nNOS in PBS–Tween buffer. After washing three times with PBS–Tween buffer, the membrane was incubated with horseradish peroxidase–conjugated anti-mouse (Amersham, Little Chalfont, Buckinghamshire, UK) or anti-rabbit (Amersham) antibody diluted at 1 to 5,000–

10,000 in PBS–Tween buffer at room temperature for 60 mins. Then the membrane was washed five times in PBS–Tween buffer. The blots were developed using the enhanced chemiluminescence detection system (Amersham). The chemiluminescence was visualized using a Lumino Image Analyzer (LAS1000; Fuji Photo Film, Tokyo, Japan) or was exposed to x-ray film (Fuji Photo Film).

To check for protein loading/transfer variations, all blots were stained with Ponceau Red (washable, before incubation with antibodies) and with Coomassie brilliant blue (permanent, after the enhanced chemiluminescence detection system). Intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was observed between samples. Anti-human nNOS rabbit polyclonal antibody (ZYMED Laboratories, San Francisco, CA) was used in the present study.

**Enzyme Immunoassay for Plasma and LV ET-1 Levels.** Concentration of ET-1 in LV tissue extracts and plasma was determined using an ET-1 Enzyme Immuno Assay Kit (Immuno-Biological Laboratories, Fujioka, Japan). This kit is a solid-phase sandwich enzyme-linked immunoassay (ELISA) using two kinds of high specific antibodies. Tetraethyl benzidine is used as a coloring agent (chromogen), the strength of which is in proportion to the quantities of ET-1. In plasma and LV tissue extract, an equal volume of 0.1% trifluoroacetic acid (TFA) in water was added. Then the sample was centrifuged at 3000 *g* for 15 mins at 4°C to clarify, and the supernatant was saved. After a 200-mg C18 Sep-Pak column (Part 235D1, Waters Inc., Milford, MA) was washed with $4 \times 1$ ml of 60% acetonitrile in 0.1% TFA followed by $4 \times 5$ ml of 0.1% TFA in water, the supernatant was applied to the column and washed with $4 \times 5$ ml of 0.1% TFA in water. The sample was eluted slowly by applying $3 \times 1$ ml of 60% acetonitrile in 0.1% TFA in water, and the eluant was collected in a plastic tube. The eluant was then evaporated to dryness using a centrifugal concentrator under vacuum. The sample was reconstituted with assay buffer and measured immediately according to the manufacturer's instructions.

**Quantitative Real-Time Polymerase Chain Reaction (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% (v/v) 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 (RT) using a first-strand cDNA synthesis kit (Qiagen). The reaction was performed at 37°C for 60 mins.

The mRNA expression level of eNOS in the LV tissues was 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 from Primer Express v.1.5 software (Perkin-Elmer) according to the published cDNA sequences for each of the following: eNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The sequences of the oligonucleotides were as follows: eNOS forward: 5′-GATCC-TAACTTGCCTTGCATCCT-3′; eNOS reverse: 5′-TGTAATCGGTCTTGCCAGAATCC-3′; eNOS probe: 5′-CTGGTATTGCACCCTTCC-3′; GAPDH forward: 5′-GTGCCAAAAGGGTCATCATCTC-3′; GAPDH reverse: 5′-GGTTCACACCCATCACAAACATG-3′; and GAPDH probe: 5′-TTCCGCTGATGCCCC-3′.

The expression of GAPDH mRNA was used as an internal control. The PCR mixture (25 μl total volume) consists of 450 n*M* of both forward and reverse primers for eNOS and GAPDH (Perkin-Elmer), 200 n*M* of FAM-labeled primer probes (Perkin-Elmer), and TaqMan Universal PCR Master Mix (Perkin-Elmer). 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 real-time quantitative PCR, serial dilutions of a rat heart cDNA performed within the range of various concentrations (1×, 2×, 4×, 8×, and 16×). No template (water) reaction mixture was prepared as negative control.

**Statistical Analysis.** Values are means ± standard deviations (SD). Statistical assessment of the data was made by one-way analysis of variance (ANOVA) with multiple comparisons by Fisher's protected least-significant difference *t* test. Nonparametric data were analyzed by the Mann-Whitney *U* test or Wilcoxon signed rank test. A $P$ value of $<0.05$ was taken as significant.

## Results

**Expressions of NOS.** In early diabetic heart, the expression of eNOS, as demonstrated by immunoassay, was significantly increased, and this increase was completely inhibited by selective $ET_A$ receptor antagonist (Fig. 1A). The inhibitory effect of ET selective receptor antagonist was also seen at upregulated eNOS mRNA levels in DM heart (Fig. 1B). When some control nondiabetic rats were treated with selective $ET_A$ receptor antagonist, there was no effect of ET antagonism on cardiac eNOS protein expression in control rats (Fig. 3A).

iNOS protein expression was also significantly higher in early DM heart and was greatly normalized by selective $ET_A$ receptor antagonist (Fig. 2A). But when some control nondiabetic rats were treated with selective $ET_A$ receptor antagonist, there was no effect of ET antagonism on cardiac iNOS protein expression in control rats (Fig. 3B).

nNOS protein expression in diabetic heart was

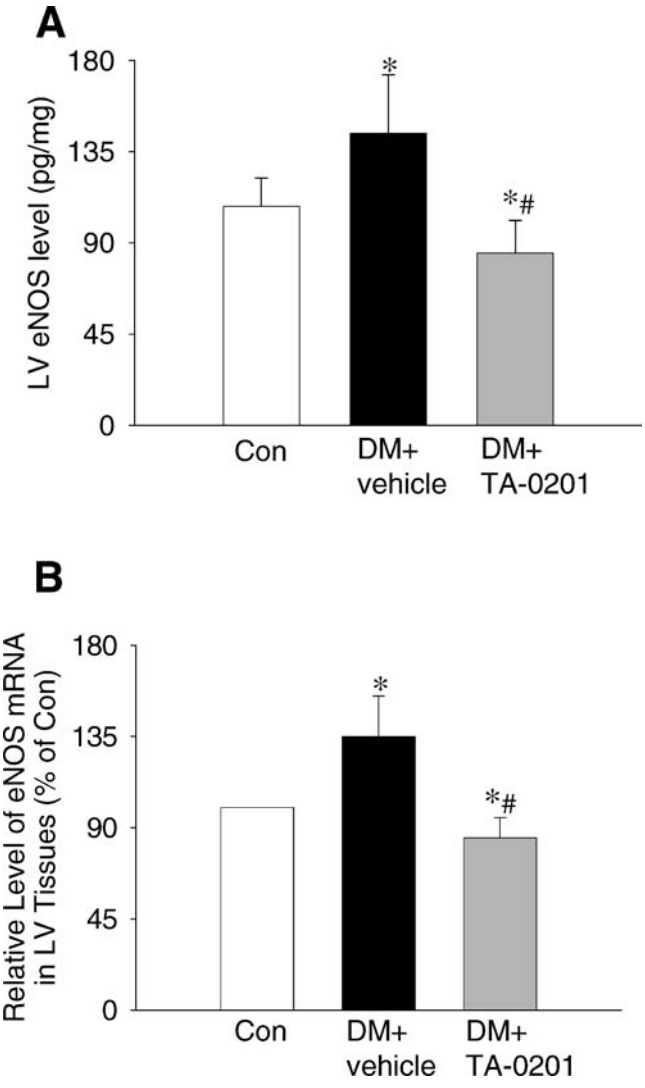


**Figure 1.** (A) eNOS protein levels by ELISA and (B) eNOS mRNA levels in LV tissues of control, DM+vehicle, and DM+TA-0201 rats. Data are means ± SD ($n = 11$). eNOS mRNA was assessed by real-time PCR. The results of eNOS mRNA are expressed as relative values (% of control). *, $P < 0.01$ versus control; #, $P < 0.01$ versus DM+vehicle (one-way ANOVA followed by Fisher's protected least-significant difference *t* test for multicomparison).

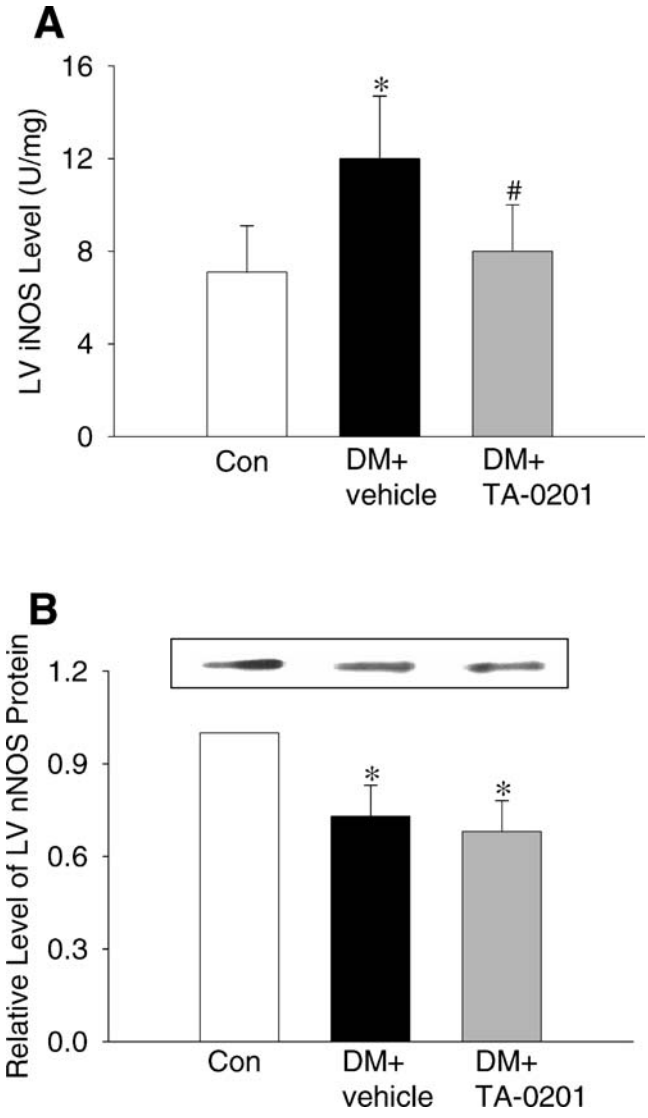


**Figure 2.** (A) iNOS protein level in LV tissues of control, DM+vehicle, and DM+TA-0201 rats. iNOS protein level was measured by ELISA. Data are means ± SD ($n = 12$). *, $P < 0.01$ versus control; #, $P < 0.01$ versus DM+vehicle (one-way ANOVA followed by Fisher's protected least-significant difference *t* test for multicomparison). (B) nNOS protein expression in LV tissues of control, DM+vehicle, and DM+TA-0201 rats. nNOS level was determined by immunoblotting. In immunoblot, the panel of bands, just above the histogram, shows representative blot of the type of animal, as described above. The net intensity of the bands was plotted as histogram, as shown below in each panel. In each of the experiments, the band obtained from control is normalized as 1.0. *, $P < 0.01$ versus control; #, $P < 0.01$ versus DM+vehicle (one-way ANOVA followed by Fisher's protected least-significant difference *t* test for multicomparison).

significantly decreased and was further decreased by the treatment with selective $ET_A$ receptor antagonist (Fig. 2B).

**Cardiac ET-I Level.** Cardiac ET-1 level was significantly ($P < 0.01$) upregulated in early diabetic rats (ratio of control to DM+vehicle: 1.5 ± 0.4 to 2.04 ± 0.5 [pg/mg], respectively).

**Plasma ET-1 Level.** In the present study, plasma ET-1 level was not significantly different in control and DM groups.

## Discussion

The main finding of the present study is that treatment of diabetes with a highly selective $ET_A$ receptor antagonist reduced the upregulated cardiac eNOS protein and gene expression despite the elevated blood glucose level in DM rats. The present study also demonstrated that the iNOS protein level was significantly increased in early diabetic heart and was greatly reversed by a selective $ET_A$ receptor antagonist. nNOS expression was downregulated in DM heart and was further downregulated by ET antagonism. The present study seems to be the first to demonstrate the

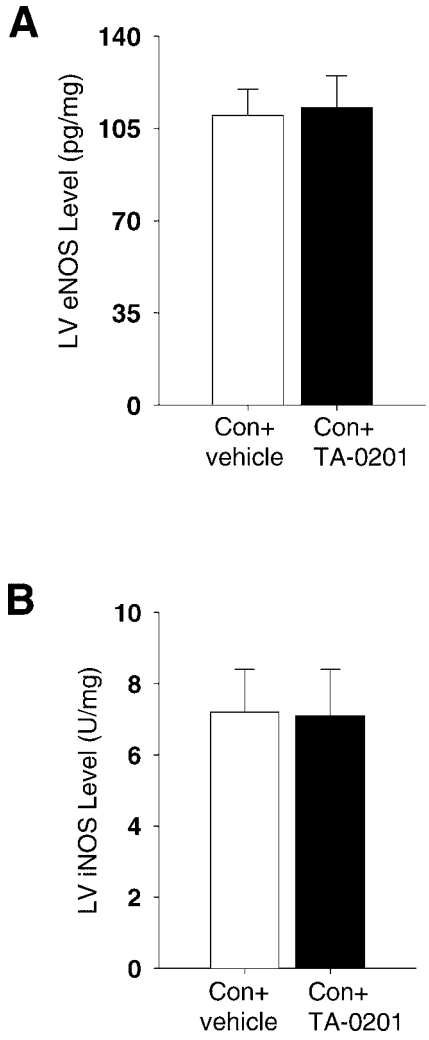


**Figure 3.** (A) eNOS and (B) iNOS protein levels by ELISA in LV tissues of control+vehicle and control+TA-0201 rats. Data are means ± SD ($n = 5$).

effects of a selective $ET_A$ receptor antagonist on the expressions of NOS in early DM heart.

The balance between endothelium-derived NO and ET-1 may contribute to altered vasomotor function in pathophysiologic states, including DM. Heterogeneity in distribution of NO and ET-1 throughout the vasculature is well recognized. The role of NO in the regulation of hemodynamics under hyperglycemic conditions has been controversial. Despite an impairment of endothelial function and reduced bioavailability of endothelium-derived NO, STZ diabetic rats are not hypertensive but instead are normotensive or hypotensive (18–21). The blood pressure changes in the present study were consistent with the above findings. In the present study, neither the systolic and diastolic blood pressures were different among the study groups. In addition, an increased dependence on a functional NO system at the onset of diabetes has been reported to prevent the development of hypertension in STZ diabetic rats (22). In the present study, the levels of nitrate and nitrite were also increased in early DM heart (unpublished observation).

It has been indicated that increased NO synthesis may be important to maintain adequate renal blood flow and to suppress the activated renin angiotensin system and sympathetic nervous system activities in diabetes. These findings imply the presence of increased NO to counteract the pressor effects of endogenous mediators in diabetes (23, 24). The same notion can also be applied to the heart in diabetic animals. Conflicting reports exist concerning the eNOS and iNOS expression in diabetic heart.

The findings in the present study were consistent with those of a previous report, which showed that diabetes causes a temporary increase in NOS activity and eNOS mRNA in the rat heart, which is presumably the consequence of an enhanced oxidative stress exerted by hyperglycemia (25). And in the same study, reductions of NOS activity and the amounts of mRNAs of eNOS and iNOS were only seen after a diabetes with duration longer than 20 weeks, a time at which a loss of endothelium has been described (25). In a recent investigation, the cause of the impairment of the endothelium-dependent vasodilatation of coronary arteries in diabetes has been pointed out to be the inactivation of NO by the formation of reactive oxygen intermediates, rather than defects of NOS (25). Nagareddy *et al.* (26) stated that the induction of iNOS in cardiovascular tissues is dependent on the duration of diabetes and contributes significantly to the depressed pressor responses to vasoactive agents and potentially to endothelial dysfunction. This study reports that at 3 weeks of diabetes, endothelial dysfunction already occurs and that the iNOS expression is increased with the duration of diabetes, but this study has shown decreased eNOS expression in cardiovascular tissues (26). In another study, diabetic hearts expressed iNOS protein, whereas eNOS expression was similar in diabetic and control groups (27). Diabetic hearts exhibit markedly abnormal posthypoxic relaxation, which is attributable to both reactive oxygen species and NO derived from iNOS (27). In another study in the rat heart model, diabetes does not influence the overall eNOS protein level or its mRNA level (28). However, there is a diminution in the deposition of eNOS in cardiac endothelial cells of diabetic rats versus nondiabetic controls, indicating a relation between eNOS and the loss of vasodilatory response that is observed in diabetes (28). Thus, it is difficult to make a comment with regard to which of the above studies our findings are similar. The present study measured the total iNOS and eNOS levels in heart tissues, so the specific structure of heart in which different types of NOS are specifically upregulated or downregulated cannot be understood from the present study. The upregulation of eNOS at both protein and mRNA levels in the present study can be explained as a compensatory mechanism in the diabetic heart. Regarding the iNOS expression, the result from the present study was almost consistent with the reports from other laboratories. Regarding the expression of

nNOS, we found in the present study that nNOS expression in diabetic heart is reduced. Previous studies did not find a change in the expression or activity of nNOS in STZ diabetic rats (29).

ET-1 acts *via* stimulation of NO release in several tissues. For example, ET-1 administration *in vivo* causes transient vasodilation followed by sustained vasoconstriction (30). The initial vasodilation is attenuated by blocking NO synthesis (31) but is dependent on activation of $ET_B$ receptors (32). Thus, endothelial $ET_B$ receptor activation stimulates NO release. ET-1–induced vasoconstriction, particularly at concentrations of less than $10^{-10}$ mol/l, was evoked *via* $ET_A$ receptors, since a specific antagonist for $ET_A$, BQ-123, substantially decreased it. BQ-123 also augmented the ET-1–evoked increase of NO release (32). Although the mechanism is unclear, this may have caused a further reduction in the vasoconstrictive activity of ET-1. The finding of the present study is that treatment of diabetes with a highly selective $ET_A$ receptor antagonist reduced cardiac eNOS protein and gene expression despite the elevated blood glucose level in DM rats. This indicates that hemodynamic factors associated with the DM are crucial for the induction of eNOS protein expression in DM rat heart, whereas the $ET_B$ receptor–mediated pathway seems to be less important. To further investigate the role of ET-1 in the regulation of eNOS gene expression, we used the highly selective $ET_A$ receptor antagonist. This enabled us to create a situation in which ET-1 plasma levels remain unchanged and only the $ET_A$ receptor, but not the $ET_B$ receptor, is blocked. On the assumption that TA-0201 exerts no effect on ET receptor expression, $ET_B$ receptor stimulation should be unaffected or should be even more pronounced when DM rats are treated with TA-0201. However, the observation that eNOS levels decreased during treatment provides further evidence that the $ET_B$ receptor–mediated pathway plays no crucial role in the stimulation of cardiac eNOS gene expression in DM rats *in vivo*. Nevertheless, in theory it might be that the increased $ET_B$ receptor stimulation indirectly downregulates eNOS gene expression *via* an NO-mediated negative feedback mechanism. In the present study, both the expression of eNOS and the levels of NO were increased in DM heart. Finally, we cannot rule out that TA-0201 influenced eNOS expression by altering the redox state of the cells. In addition to the increase in eNOS expression, increased cardiac iNOS protein expression has been found in DM animals in the present study. The underlying mechanisms are not yet clear, in particular whether this defect is caused by a direct effect of diabetes on the activity and the expression of NOS or indirectly by an enhanced inactivation of NO. Moreover, to have the more specific understanding between the relationship of eNOS expression and the $ET_B$ receptor level in DM heart, selective $ET_B$ receptor antagonist should be tried in the present study. Finally, one should keep in mind that the selective $ET_A$ receptor antagonist had no effect on eNOS and iNOS protein levels in heart tissue in nondiabetic control rats.

Now we are on the way to measuring the total level of NO after selective $ET_A$ receptor antagonism in diabetic heart. Before that, we need to assess the activity of each NOS in the present study. It would also be important to examine the level of VEGF and Akt in the current experimental setting, as NOS occupies an important position in the signal transduction for VEGF and Akt in heart. Since diabetic heart has the tendency to develop abnormal collaterals, the understanding about NOS in early DM heart is important. In conclusion, the present study demonstrates that selective $ET_A$ receptor antagonist is effective in reversing the upregulated eNOS and iNOS levels in the early STZ-induced diabetic heart.


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