Effects of Dual Endothelin Receptor Antagonist on Antiapoptotic Marker Bcl-2 Expression in Streptozotocin-Induced Diabetic Rats

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Erectile dysfunction (ED) affects approximately 50% of male patients with diabetes mellitus (DM) and is possibly due to the vascular and neuropathic complications of DM. Recently, apoptosis has been regarded as a downstream event in ED. More recently, the importance of alterations in apoptosis-related molecules in the mechanism of DM-induced ED has begun to be appreciated. Endothelin-1 (ET-1) plays a role via ET_A and ET_B receptors in the regulation of cavernosal smooth-muscle tone in penile tissues. We found that the ET-1 level in the penis of rats with DM was higher than that in the penis of control animals. The present study investigated a rat model in which DM was induced by a 3-week regimen of streptozotocin (STZ) to assess the expression of several apoptosis-related molecules in penile tissue and, concomitantly, the effects of ET antagonism on these changes. Male Sprague-Dawley rats (weight [±SD], 450 ± 26 g) received a citrate saline vehicle or STZ (65 mg/kg ip). DM was confirmed by the presence of hyperglycemia. Diabetic animals were further separated into two treatment groups 1 week after onset of disease: one group received $ET_{A/B}$ dual receptor antagonist (SB209670) by means of osmotic minipump at a dosage of 1 mg/day, and the other group received saline. Rats in both groups were treated for 2 weeks and then sacrificed. Plasma glucose levels (±SD) in rats with DM were significantly higher than those in rats without DM (506 ± 70 vs. 111 ± 11 mg/dl). In the penile tissue of rats with DM, a 35% decrease in the expression of Bcl-2 protein (an important antiapoptotic marker detectable by immunoblotting) was seen, and $ET_{A/B}$ dual antagonist was observed to significantly counteract this decrease. Real-time polymerase chain reaction revealed that the expression of Bcl-2 mRNA was consistent with Bcl-2 protein expression. Levels of Bax and caspase-3, two important proapoptotic markers, were not significantly altered in the present study. Thus, we conclude that, in the penis of rats with early stage DM, the protection against apoptosis has decreased but can be improved by ET antagonism. Exp Biol Med 231:1034–1039, 2006

Key words: diabetic penis; Bcl-2; endothelin antagonist

Erectile dysfunction (ED) is more frequent among men with diabetes mellitus (DM) than among men without DM (1, 2). Diabetic men are three times more likely than nondiabetic men to develop ED. The cause is multifactorial but most commonly reflects endothelial dysfunction and autonomic neuropathy. DM and vascular disease often coexist, and ED may be a marker for silent occlusive arterial disease, for which the patient should be screened. The mechanism by which DM causes ED is largely unknown and remains under investigation.

Penile erection is a hemodynamic process involving increased arterial inflow and restricted venous outflow. Although ED is not life threatening, this common problem can significantly affect quality of life and psychological and social well-being. Recently, alterations in different angiogenic and apoptotic factors have been implicated in the pathogenesis of ED. Vascular endothelial growth factor (VEGF), the potent angiogenic growth factor, has been shown in different studies to be effective in the improvement of erectile function (3–5). In rat and rabbit models of vasculogenic ED, intracavernosal delivery of VEGF restores erectile function within weeks and is associated with increased cavernosal endothelial cell content and restoration of neural and smooth-muscle integrity (6, 7). Apoptosis is

This study was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (14104009, 15390077, 15650130, and 16500391) and by a grant from the Miyauchi Project of Center for Tsukuba Advanced Research Alliance at the University of Tsukuba.

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Received October 4, 2005.
Accepted November 17, 2005.

1535-3702/06/2316-1034$15.00

an active process and was first described as shrinkage necrosis, which is a morphologically distinct form of cell death (8). Apoptosis also plays an important role in the development of several diseases. Diabetic rats have been shown to have an apoptotic cell count that is three times higher than that in control rats (9). Apoptosis may cause some reduction of the erectile tissue within the corpora. Klein *et al*. (10) have shown that cavernosal neurotomy of the rat induces the loss of rat erectile cells by apoptosis and may account for postoperative impotence or the perceived reduction in penile mass after radical prostate surgery. Because polyneuropathy occurs in DM, an increase in apoptosis is to be expected. Therefore, a high rate of apoptosis in diabetic erectile tissues may be caused by ketoacidosis, polyneuropathy, hypoxia, and/or excessive oxidative stress created by DM.

Endothelin-1 (ET-1) belongs to a family of potent vasoconstrictor peptides constituted by 21 amino acids. ET-1 plays a role via ET_A and ET_B receptors in the regulation of cavernosal smooth-muscle tone in penile tissues (11). Recently, investigators have analyzed the various risk factors involved in the pathogenesis of ED and their involvement in the action of ET-1 (11). At the time of writing, the roles of DM, hypertension, smoking, and dyslipidemia are each under investigation. The pharmaceutical industry has shown an interest in the development of ET antagonists for use in the treatment of various diseases, including ED (11). Currently, experimental ET-1 antagonists that may be of therapeutic benefit in ED are intensively under investigation in both basic and clinical researches.

The present study investigated whether the level of the antiapoptotic marker Bcl-2 is altered in the penile tissues of mice with early stage, streptozotocin (STZ)–induced DM and whether short-term treatment with a dual ET antagonist would have a favorable effect on any such alteration. We also investigate two important proapoptotic molecules, Bax and caspase-3, in penile tissues.

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 specified in the Helsinki Declaration of 1964. DM was induced by a single 65-mg/kg ip injection of STZ (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in 0.1 *M* citrate buffer (pH 4.5). Control nondiabetic animals received citrate buffer only (non-DM control group). Animals with blood glucose levels >250 mg/dl 48 hrs after receipt of STZ were considered to be diabetic. One week after the STZ injection, the diabetic animals were randomly divided into two treatment groups. One group received dual ET receptor antagonist (SB209670) at a dosage of 1 mg/day for 2 weeks by osmotic minipump (model 2004; Durect Corporation, Cupertino, CA) (DM-SB209670 group), and the other group received physiological saline only for 2 weeks (DM-vehicle group). Before the start of the drug treatment, the blood glucose level was determined almost every day, but after the start of treatment, the diabetic status was assessed every week. The rats were fed standard laboratory chow and had unrestricted access to water in an air-conditioned room with a 12:12-hr light:dark cycle. After receiving treatment for 2 weeks, rats were sacrificed under anesthesia, and the penile tissue was removed. The present experimental design was approved by the Tsukuba University School of Medicine Animal Care and Use Committee.

Enzyme Immunoassay for Plasma and Penile ET-1 Levels. The concentration of ET-1 in penile 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 immunosorbent assay that uses two kinds of highly specific antibodies. Tetraethyl benzidine is a coloring agent (chromogen), the strength of which is proportionate to the quantity of ET-1. In plasma and penile tissue extract, an equal volume of 0.1% trifluoroacetic acid (TFA) in water was added. The sample was then centrifuged at 3000 *g* for 15 mins at 4°C to clarify the solution, and the supernatant was saved. After a 200-mg C 18 Sep-Pak column (235D1; Waters Inc., Milford, MA) was washed four times with 1 ml of 60% acetonitrile in 0.1% TFA and four times with 5 ml of 0.1% TFA in water, the supernatant was applied to the column and washed four times with 5 ml of 0.1% TFA in water. The sample was eluted slowly by applying 1 ml of 60% acetonitrile in 0.1% TFA in water three times, and the eluant was collected in a plastic tube. The eluant was then evaporated to dryness with a centrifugal concentrator under vacuum. The sample was reconstituted with assay buffer and measured immediately according to the manufacturer's instructions.

Western Blot Analysis. The method of immunoblot used in the present study appears in an earlier study we published (12). Briefly, ice-cold whole penile 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 were boiled in reducing sodium dodecyl sulfate (SDS) sample buffer for 5 mins, loaded for SDS-polyacrylamide gel electrophoresis (4%–15% polyacrylamide) under reducing conditions, subjected to electrophoresis, and electrophoretically transferred to polyvinylidine difluoride filter membrane. To reduce nonspecific binding, the membrane was blocked for 2 hrs at room temperature with 5% nonfat milk in phosphate buffer solution (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 primary antibody in PBS-Tween buffer. After the membrane was washed three times with PBS-Tween buffer, it was incubated with a suitable secondary antibody coupled

to horseradish peroxidase for 60 mins at room temperature. The blots were washed five times in PBS-Tween buffer and subsequently visualized with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK) exposed to radiographic film (Fuji Photo Film, Tokyo, Japan). The intensities of the total protein bands in each lane were evaluated by densitometry. Negligible variation due to loading and transfer was observed between samples. Moreover, β-actin was used as a loading control (anti-*Xenopus laevis* β-actin mouse monoclonal antibody; Abcam, Cambridge, UK). Anti-human Bcl-2 polyclonal antibody was used in this study (Santa Cruz Biotechnology, Santa Cruz, CA).

Quantitative Real-Time Polymerase Chain Reaction (PCR). Total RNA in penile tissue was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene, Toyama, Japan). Total RNA penile tissue was primed with 0.05 μg of oligo $d(pT)_{12–18}$ and reverse transcribed by omniscript reverse transcriptase by means of a first-strand cDNA synthesis kit (Qiagen, Tokyo, Japan) as described elsewhere (13). The reaction was performed at 37°C for 60 mins.

The levels of expression of Bcl-2 mRNA, Bax mRNA, and caspase-3 mRNA in penile tissue were analyzed by quantitative reverse transcriptase (RT)–PCR with a TaqMan probe by use of an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster, CA), as previously described (13). The gene-specific primers and TaqMan probes were synthesized using Primer Express software, version 1.5 (Perkin-Elmer), according to the published cDNA sequences for Bcl-2, Bax, caspase-3, and GAPDH mRNA. The sequences of the oligonucleotides were as follows: Bcl-2 forward: 5′-TGCGCTCAGCCCT GTG-3′, Bcl-2 reverse: 5′-GGTAGCGACGAGAGAAGTC ATC-3′, and Bcl-2 probe: 5′-CCACCTGTGGTCCACC TG-3′; Bax forward: 5′-CAAGAAGCTGAGCGAGTGT CT-3′, Bax reverse: 5′-CAATCATCCTCTGCAGCTCCAT ATT-3′, and Bax probe: 5′-CCAGTTCATCTCCAATTCG-3′; Caspase3 forward: 5′-GCAGCTAACCTCAGAGAGAC ATTC-3′, Caspase3 reverse: 5′-ACGAGTAAGGTCATTT TTATTCCTGACTT-3′, and Caspase3 probe: 5′-ATGGCCCTGAAATAC-3′; and 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) consisted of 450 n*M* of both forward and reverse primers for Bcl-2, Bax, caspase-3, and GAPDH (Perkin-Elmer); 200 n*M* of FAM-labeled primer probes (Perkin-Elmer); and TaqMan Universal PCR Master Mix (12.5 μl; 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. To determine the standard curve in the real-time quantitative PCR analysis, serial dilutions of rat penis cDNA were performed using a range of concentrations (1×, 2×, 4×, 8×, and 16×). A water-based reaction mixture was prepared as a negative control.

Statistical Analysis. Data are expressed as means ± SD. Statistical assessment of the data was made by one-factor analysis of variance, with the problem of multiple comparisons addressed by the Fisher protected least-significant difference *t* test. Nonparametric data were analyzed by the Mann-Whitney *U* test or the Wilcoxon signed rank test. A *P* value of <0.05 was considered to be statistically significant.

Results

Biological Parameters of the Study Animals. In this study, the plasma glucose level in DM was significantly higher than that in non-DM control rats (506 ± 70 vs. 111 ± 11 mg/dl; $P < 0.001$). The plasma insulin level in DM was remarkably lower than that in non-DM control rats (0.34 ± 0.29 vs. 7.73 ± 2.20 ng/ml; $P < 0.001$). Plasma glucose and insulin levels were not affected by ET antagonism (507 ± 65 mg/dl and 0.25 ± 0.16 ng/ml, respectively). There was no significant alteration in systolic blood pressure in experimental animals (121 ± 13.8 mm Hg in the non-DM control group, 113 ± 14 mm Hg in the DM-vehicle group, and 116 ± 19 mm Hg in the DM-SB209670 group). On the basis of these findings, the animal models used in the present study were hyperglycemic with greatly reduced plasma insulin levels.

Plasma ET-1 Level. The plasma ET-1 level was similar or tended to be lower in rats with DM, compared with non-DM control rats, but after ET antagonism, the plasma ET-1 level was significantly increased ($P < 0.001$). ET-1 levels in plasma were as follows: 2.4 ± 0.7 pg/ml in the non-DM control group, 2.3 ± 0.5 pg/ml in the DM-vehicle group, and 7.4 ± 1.7 pg/ml in the DM-SB209670 group.

Penile ET-1 Expression. The penile ET-1 level in the DM-vehicle group was higher than that in the non-DM control group (20.74 ± 3.32 vs. 13.39 ± 4.3 pg/ml), and ET antagonism in the DM-SB209670 group was found to ameliorate this upregulation (ET-1 level, 13.53 ± 3.04 pg/mg) ($P < 0.01$).

Expression of Bcl-2 in Penile Tissues. A significant downregulation of Bcl-2 expression in penile tissues was observed at the protein and mRNA levels in rats with DM, and dual ET antagonism ameliorated this downregulation (Fig. 1).

Expression of Bax and Caspase-3 in Penile Tissues. There was no significant alteration in the expression of the genes encoding Bax and caspase-3 in this experimental setting (Fig. 2).

Discussion

The present study demonstrated that rats with STZ-induced DM had decreases in protein expression of Bcl-2 in penile tissues 2–3 weeks after onset of disease. There was a significant parallel correlation between decreases in Bcl-2

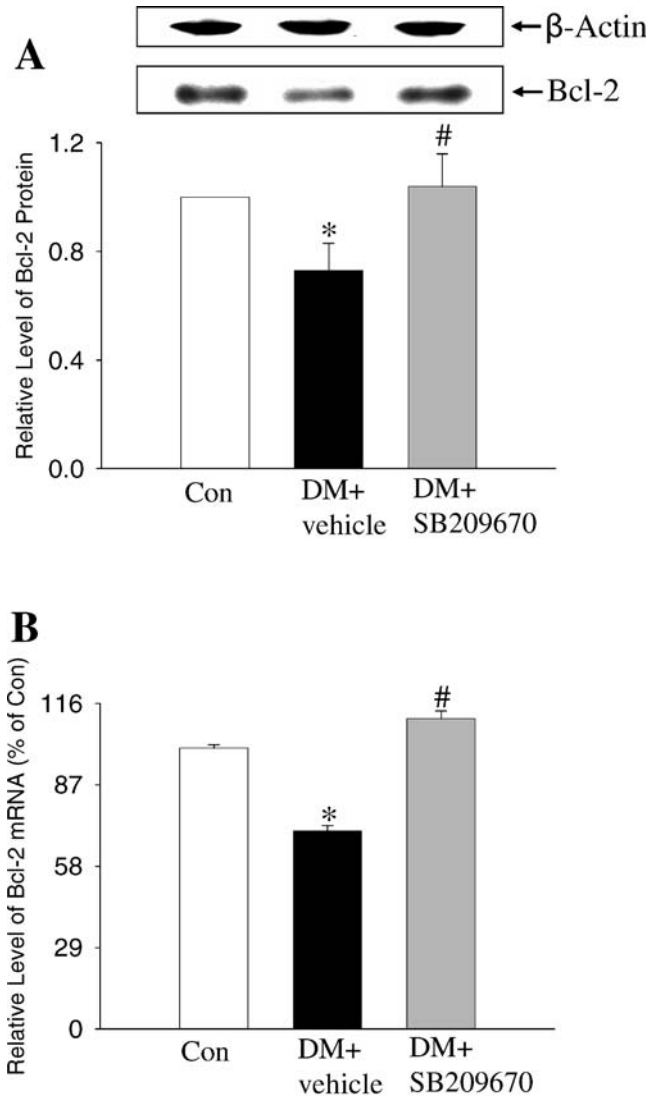

Figure 1. (A) Immunoblot analysis for Bcl-2 in the penile tissues of control, DM-vehicle, and DM-SB209670 rats. β-Actin served as a loading control. The panel of bands just above the histogram shows representative blots for each of the three treatment groups. The net intensity of the bands was plotted as a histogram, as shown below in the panel. In each of the experiments, the band associated with the control group is normalized as 1.0. Data are means ± SD for seven separate experiments. Statistical analysis was done for the basal data before normalization by means of one-way analysis of variance, followed by the Fisher protected least-significant difference *t* test. $^{*}P < 0.01$ vs. the control group; $^{\#}P < 0.01$ vs. DM-vehicle group. (B) Expression of Bcl-2 mRNA in the penile tissues of control, DM-vehicle, and DM-SB209670 rats. The mRNA expression was determined by real-time PCR. Data are means ± SD ($n = 8$). The data are shown as the relative levels (the value associated with the control group is defined as 100%). $^{*}P < 0.01$ vs. the control group; $^{\#}P < 0.01$ vs. the DM-vehicle group.

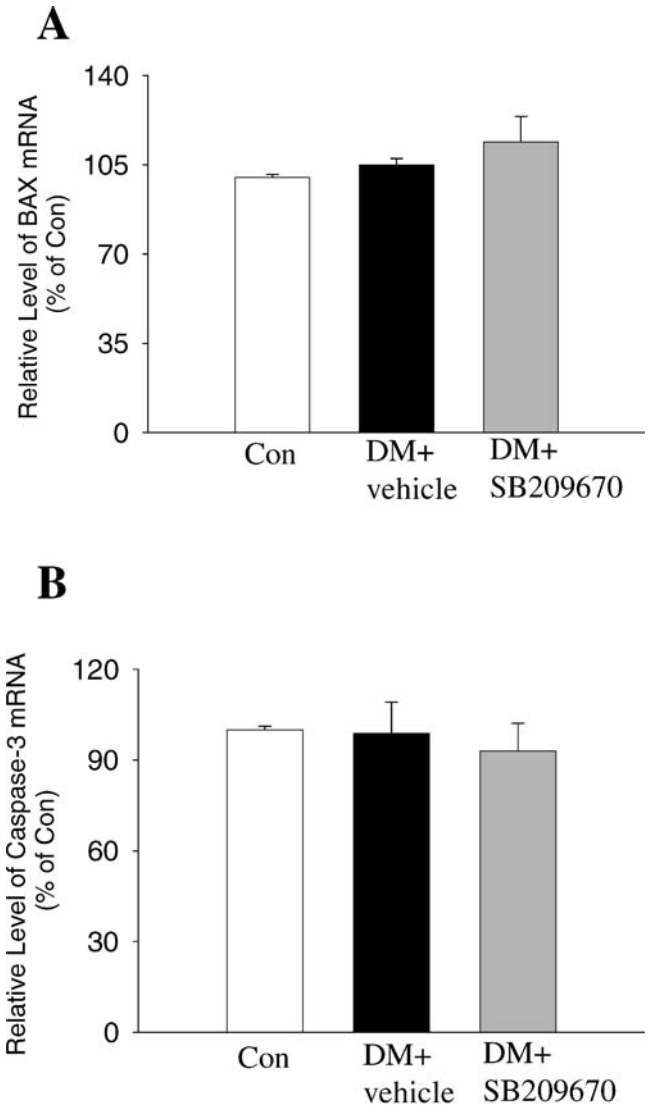

Figure 2. The expression of Bax mRNA (A) and caspase-3 mRNA (B) in the penile tissues of control, DM-vehicle, and DM-SB209670 rats. The mRNA expression was determined by real-time PCR. Data are means ± SD ($n = 6$). The data are shown as the relative levels (the value associated with the control group is defined as 100%).

protein levels and Bcl-2 mRNA levels in penile tissue of rats with DM, as determined by real-time quantitative PCR analysis. This parallel behavior of protein and mRNA expression implies that the decreases in the synthesis of Bcl-2 in penile tissues occur at the level of transcriptional regulation. The downregulation of Bcl-2 expression in the DM penis was greatly normalized by ET antagonist. The rats with DM had remarkably low plasma insulin levels, as well as a high plasma glucose level. Moreover, the plasma ET-1 level in rats with DM was similar to or lower than that in non-DM control rats, but after ET antagonism, the plasma ET-1 level in rats with DM was significantly higher than that in non-DM control rats. On the other hand, the penile ET-1 level was significantly increased in rats with DM, compared with non-DM control animals, and receipt of the ET antagonist was shown to significantly decrease this upregulation.

The etiopathogenesis of ED in DM is multifactorial, with vascular and neural factors being equally implicated. Recently, the role of apoptotic changes in penile tissues in ED has begun to be appreciated. Apoptosis has been regarded as a downstream event in ED (14). Moreover, proapoptotic factors (such as Bak and Bax) and antiapop-

totic factors (such as Bcl-2 and Bcl-x) are involved in the etiology of DM-induced ED (14). Furthermore, treatment of DM-associated ED with insulin has been observed on the basis of alterations in the protein expression of apoptotic factors in rat crura (14). In the present study, Bcl-2 is downregulated in penile tissue even at the early stages of DM (i.e., 2 weeks after onset), which suggests that the protection against apoptosis has been lost before the alterations in proapoptotic molecules. However, no significant alteration in the expression of the genes encoding Bax and caspase-3 were detected in penile tissue at this time.

Recently, we have reported that the expression of VEGF is downregulated in type II diabetic penile tissues (12). There is strong evidence that VEGF is a survival factor for endothelial cells (15). This prosurvival activity of VEGF requires the PI3K/Akt signal transduction pathway, and activation of Akt stimulates expression of Bcl-2 (an antiapoptotic protein) and phosphorylation of Bad (a proapoptotic protein), thereby inhibiting initiation of apoptosis. As expected from the finding of the reduced expressions of Bcl-2, rats with DM in the present study exhibited decreased expressions of VEGF, its receptors, and Akt in penile tissues (unpublished data). The antiapoptotic Bcl-2 protein plays important roles in inhibiting mitochondria-dependent extrinsic and intrinsic pathways of cell death (16). The present results are in good agreement with a previous report that showed a loss of Bcl-2 expression in cavernosal tissue from diabetic men with ED (17), although no change in Bcl-2 mRNA was detected in diabetic animals in another study (14). Compared with those in the control group, apoptotic cells were greater in number ($P < 0.01$), and the expression of Bcl-2 was absent in the penis cavernosal tissue from diabetic rats (18). Also, our findings support previously published data showing that there is a greater number of apoptotic cells in the erectile tissue of rats with STZ-induced DM (9). Although further research is needed to identify the role of apoptosis in the pathophysiologic mechanism of diabetic ED, we assume that apoptosis may cause some loss of erectile tissue within the corpora, leading to degeneration of penile cavernosum and replacement of the cavernosal smooth muscle with collagen.

The imbalance between vasoconstrictors and vasodilators may play an important role in the pathogenesis of ED. Conflicting results exist regarding the plasma ET-1 level in DM subjects. In rats with STZ-induced DM, plasma ET-1 levels have been undetectable (19), unchanged (20), enhanced (21, 22), or suppressed (23), compared with those in control rats. In the present study, we found that the plasma ET-1 level was unchanged or tended to be lower than normal, suggesting that increased sequestration of ET-1 in different local tissues might prevent excessive accumulation of ET-1 in the plasma of rats with DM. After diabetic rats were treated with a dual ET antagonist, the plasma ET-1 level increased remarkably. ET antagonism has been reported to decrease urinary excretion of ET-1 in rats with STZ-induced DM (24). In contrast to the present findings regarding plasma levels of ET-1, ACE activities are elevated and associated with reduction of nitric oxide and cGMP levels in the systemic and cavernous blood of patients with ED (25). In the present study, the plasma ET-1 level did not increase but the penile ET-1 level did increase in rats with DM, and this upregulation was normalized by ET antagonism. Indeed, the local tissue ET system can be affected independently of the systemic ET-1 system. Dual ET antagonism decreased the upregulation of the gene encoding ET-1 in renal tissues in diabetic rats (26). The adrenergic agonist norepinephrine (27) and ET-1 (28, 29) are two vasoconstrictors believed to play important roles in regulating the penile blood flow. Thus, the reduction in the penile ET-1 level may partly improve the blood flow in penile tissues, and the improvement in blood flow might normalize the alterations in several angiogenic and apoptotic factors implicated in the pathogenesis of ED. It is difficult to speculate about the mechanism of the recovery of downregulated Bcl-2 expression in the DM penis due to ET antagonism, because ET-1 has been shown to reduce apoptosis (30). ET-1 does not reverse the reduction in the Bcl-2 level in irradiated human melanocytes (31).

In conclusion, the present study demonstrates that the expression of the antiapoptotic molecule Bcl-2 is significantly reduced in the penile tissues of rats with early stage DM and that dual ET antagonism is able to ameliorate this downregulation. However, the present study does not shed light on the mechanism concerning the reversal of Bcl-2 downregulation in the diabetic penis due to ET antagonism. A similar experimental protocol should be used in the future to investigate nondiabetic control rats.

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