Diminished penile expression of vascular endothelial growth factor and its receptors at the insulin-resistant stage of a type II diabetic rat model: a possible cause for erectile dysfunction in diabetes

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Abstract

Erectile dysfunction (ED) is commonly experienced in men with diabetes mellitus. Vascular endothelial growth factor (VEGF) has been extensively documented for its pathogenic significance in different complications of diabetes. We hypothesized that expressions of VEGF, its receptors and its signaling pathway Akt may be drastically altered in diabetic penile tissues and their alterations may modulate penile expression of the molecules that are believed to play a role in diabetic ED. Otsuka Long–Evans Fatty (OLETF) rats, a type II (non-insulin-dependent) diabetes mellitus, were used at the insulin-resistant stage of type II diabetes (20 weeks of age). We determined protein and mRNA expressions of VEGF, its receptors, Akt, nitric oxide synthase isoforms, and apoptosis-related molecules in the penis using immunohistochemistry, Western blotting, *in situ* hybridization, and real-time quantitative PCR analyses. The penile sections were also submitted to the Tdt-mediated dUTP nick end labeling assay for apoptosis. OLETF rats showed marked reductions in penile expression of VEGF, its two receptors and Akt. In OLETF rat penises, endothelial and neuronal nitric oxide synthase isoforms were expressed less abundantly. Furthermore, while anti-apoptotic markers, Bcl-2 and phosphorylated Bad, were down-regulated, pro-apoptotic markers, active caspase-3 and Bax, were up-regulated, resulting in the appearance of apoptotic cells in the penile tissues of OLETF rats. The VEGF signaling system would work less well in diabetic penile tissues as a result of the reduced expression, leading to diminished endothelial production of nitric oxide and apoptosis-related erectile tissue damage. We propose that the abnormalities of the VEGF signaling system in the penis may play a role in the pathophysiology of diabetic ED.

Journal of Molecular Endocrinology (2003) **31**, 401–418

Introduction

Erectile dysfunction (ED) is a common complication of diabetes mellitus, and may affect more than half of diabetic men (McCulloch *et al.* 1980). Autonomic neuropathy is still considered to be the main etiological factor in diabetic ED, and it is due to erectile failure resulting from damage to both the parasympathetic and sympathetic innervation of the corpus cavernosum (Ellenberg 1971). However,

vasculogenic factors are now recognized as having an important role in the development of ED (Sullivan *et al.* 2001), and the mechanisms by which diabetes causes ED remain under investigation.

Nitric oxide (NO) is generally believed to act as an important neuronal and non-neuronal mediator in the regulation of smooth muscle tone, blood flow and secretory function (Bredt *et al.* 1990, Bush *et al.* 1992). Because of the presence of numerous neuronal NO synthase (nNOS)-positive fibers in

the corpus cavernosum (Burnett *et al.* 1992, Tamura *et al.* 1995), nNOS is thought to be the essential origin of NO in the corpus cavernosum, and cavernosal smooth muscle relaxation is primarily due to NO released from the terminals of nNOS-containing cavernosal nerve fibers (González-Cadavid *et al.* 1999), although evidence for the expression of endothelial NO synthase (eNOS) alongside nNOS in the human corpus cavernosum has been provided (Bloch *et al.* 1998). Relaxation of cavernosal smooth muscle and the resulting synergistic increases in sinusoidal blood pressure and venous outflow resistance are generally acknowledged as the major hemodynamic events in penile erection (Andersson & Wagner 1995). Thus, penile erection is elicited principally by the release of NO in cavernosal nerve terminals of the penis as a result of sexual stimulation of central or peripheral origin (Burnett 1997, González-Cadavid *et al.* 1999). Saenz de Tejada *et al.* (1989) have shown impaired neurogenic and endothelium-dependent relaxation in isolated corpus cavernosal strips from diabetic patients with ED. Similar findings have been reported in alloxan-induced diabetic rabbits (Azadzoi & Saenz de Tejada 1992). Diminished relaxation to electrical field stimulation in cavernosal tissues taken from diabetic patients with ED has been found in association with a lack of NO production (Pickard *et al.* 1995). Consistent with these findings are the reports showing that penile expression of nNOS and eNOS are down-regulated in alloxan-induced (Akingba & Burnett 2001) and streptozotocin-induced (Cellek *et al.* 1999, el-Sakka *et al.* 1999) diabetic rats. However, no data are available on what is a key molecule leading to down-regulation of the constitutive NOS isoenzymes in diabetic penile tissues.

Vascular endothelial growth factor (VEGF) is an endothelial mitogen, angiogenic protein, and potent vasopermeability factor that mediates its effects mainly through two VEGF receptor tyrosine kinases, fetal liver kinase 1 (Flk-1) and *fms*-like tyrosine kinase 1 (Flt-1) (Ferrara 2001). VEGF is considered to be involved in the intraocular neovascularization caused by retinal ischemia, including diabetic retinopathy (Aiello *et al.* 1994). Elevated concentrations of VEGF in the intraocular fluid have been found in patients with proliferative diabetic retinopathy (Aiello *et al.* 1994). In addition, experimental and clinical studies

suggest that VEGF has a causal role in the development and progression of diabetic nephropathy (Tsuchida *et al.* 1999, Hovind *et al.* 2000). However, recent work using a variety of diabetic models suggests that extremely differential regulation of VEGF and its receptors may exist among tissues in diabetes (Chou *et al.* 2002). Up to now, changes in penile expression of VEGF and its receptors in diabetes are not known. Interestingly, intravenous injection of VEGF is reported to facilitate the recovery of erectile function in a rat model of traumatic arteriogenic ED by ligating each internal iliac artery (Lee *et al.* 2002). VEGF expression is likely to strongly affect the regulation of expression of constitutive NOS isoenzymes, which are sources of NO for penile erection as mentioned above, because VEGF has been shown to increase both eNOS mRNA and protein in endothelial cells in culture (Papapetropoulos *et al.* 1997). Furthermore, there is strong evidence that VEGF is a survival factor for endothelial cells (Dimmeler & Zeiher 2000). Consistent with its pro-survival activity, VEGF induces expression of anti-apoptotic proteins such as Bcl-2 in endothelial cells (Nör *et al.* 1999). In this regard, it should be noted that a significantly high incidence of apoptosis in the erectile tissues of streptozotocin-induced diabetic rats has been found (Alici *et al.* 2000). Accordingly, documentation of the expression of VEGF and its receptors in diabetic penile tissues could be valuable.

The present study was undertaken using Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which have been established as an animal model of congenital diabetes by selective mating (Kawano *et al.* 1992). This strain displays stable clinical and pathological features that resemble human type II diabetes mellitus, and one of its characteristics is an early manifestation of the existence of insulin resistance. Using this model, we examined mRNA and protein expressions of VEGF and its receptors (Flk-1 and Flt-1) in the penile tissues. Moreover, we investigated changes in penile expressions of constitutive NOS isoenzymes (nNOS and eNOS) as sources of NO for penile erection. We also determined whether inducible NO synthase (iNOS) is induced spontaneously in the penises of OLETF rats, because iNOS is reported to be up-regulated in diabetic human penile tissues (Seftel *et al.* 1997). Finally, to define the significance of altered VEGF expression and cell survival effect in the diabetic

ED mechanisms, cell apoptosis and expression of apoptotic regulators including several Bcl-2 family members were assessed in penile tissues from diabetic rats.

Materials and methods

Experimental animals

The experimental design was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Male OLETF rats, which have been established as an animal model of congenital diabetes by selective mating (Kawano *et al.* 1992), were obtained from the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. Male Long-Evans Tokushima Otsuka (LETO) rats, which were developed from the same colony by selective mating but did not develop diabetes, served as control animals. Animals were maintained under constant temperature (23 °C) and lighting conditions (lights on from 0600 to 1800 h) with free access to food and water. At 20 weeks of age, rats were killed by exsanguination under anesthesia with gaseous diethyl ether, and the gonadal tissues were harvested. OLETF rats exhibit the prediabetic phase characterized by postprandial hyperglycemia and insulin resistance from 10 to 20 weeks of age, the type II diabetic phase showing impaired glucose tolerance with hyperglycemia at 30 weeks of age, and the type I diabetic phase after 40 weeks of age (Kawano *et al.* 1992, Yagi *et al.* 1997). Thus, our animals were in the stage of insulin-resistant diabetes. We found that plasma glucose and insulin levels after 24 h of fasting were significantly ($P < 0.05$) higher in OLETF rats (8.3 ± 0.7 mmol/l and 202 ± 80 pmol/l, $n = 12$) than in age-matched LETO control rats (7.4 ± 0.8 mmol/l and 125 ± 55 pmol/l, $n = 12$).

Immunofluorescent staining

Immunohistochemical studies were performed with the following commercially available antibodies: anti-human VEGF rabbit polyclonal antibody (Immunological Laboratories, Fujioka, Japan), anti-human Flk-1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human Flt-1 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human eNOS rabbit

polyclonal antibody (Affinity BioReagents, Golden, CT, USA), anti-human nNOS rabbit polyclonal antibody (ZYMED Laboratories, San Francisco, CA, USA), anti-rabbit iNOS mouse monoclonal antibody (Affinity BioReagents), anti-human/mouse caspase-3 active form rabbit polyclonal antibody (R and D Systems, Minneapolis, MN, USA), anti-mouse Bax rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human Bcl-2 rabbit polyclonal antibody (Santa Cruz Biotechnology), and anti-mouse phosphorylated Bad (p-Bad) goat polyclonal antibody (Santa Cruz Biotechnology).

For immunohistochemical determination of target molecules, penile tissues were rapidly removed and frozen in liquid nitrogen. The preparations were cut in 8 µm thick sections transversely. The frozen cryostat sections were then fixed in acetone for 10 min at 4 °C and air dried. To prevent non-specific staining by the secondary antibody, the sections were blocked by non-immune serum (1% bovine serum albumin in Tris) for 30 min at room temperature. After overnight incubation at 4 °C with primary antibodies, the sections were rinsed in phosphate buffer solution and then exposed to the fluorescence secondary antibody, Cy3-conjugated AffiniPure anti-rabbit IgG or fluorescein-conjugated AffiniPure goat anti-rabbit, anti-goat or anti-mouse IgG (Jackson Immuno Research Laboratories, Westgrove, PA, USA), for 2 h according to the manufacturer's instructions. The samples processed without primary antibodies served as negative controls. The coverslips were mounted with Immunon (Thermo Shandon, Pittsburgh, PA, USA). Immunofluorescent images were observed under the laser scanning confocal imaging system (MRC-1024; Bio-Rad, Hemel Hempstead, UK).

Western blot analysis

After penile tissues had been removed and rinsed in sterilized water on ice, the tissues were minced with scissors, homogenized, and then centrifuged at 500 g for 15 min to pellet any insoluble material. The protein concentration of supernatant was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Samples were run on SDS-PAGE, using 7.5–15% polyacrylamide gel, and electrotransferred to polyvinylidene difluoride filter membrane. To reduce non-specific

binding, the membrane was blocked for 2 h at room temperature with 5% non-fat milk in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4 , 1.5 mM KH_2PO_4) containing 0.1% Tween 20. Thereafter, the membrane was incubated overnight at 4 °C with specific antibodies for VEGF, Flk-1, Flt-1, eNOS, nNOS, iNOS, caspase-3 active form, Bax, Bcl-2, p-Bad, or Akt (phospho-Akt (Ser 473) antibody; Cell Signaling Technology, Beverly, MA, USA) (1:100–1000 dilution) in PBS–Tween buffer. After washing three times with PBS–Tween buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit (Amersham International plc, Amersham, Bucks, UK), anti-mouse (Amersham International plc) or anti-goat antibody (Santa Cruz Biotechnology) diluted at 1:2000–10 000 in PBS–Tween buffer at room temperature for 60 min. The membrane was washed five times in PBS–Tween buffer. The blots were developed using the enhanced chemiluminescence detection system (Amersham International plc). The chemiluminescence was visualized using a Lumino image analyzer (LAS1000; Fuji Photo Film, Tokyo, Japan) or 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.

***In situ* hybridization protocol**

Cryostat sections, 15–20 μm in thickness, were prepared and mounted on glass slides precoated with 3-amino-propyltriethoxysilane. The *in situ* hybridization protocol used has been described in detail previously (Jesmin *et al.* 2002b). Briefly, the sections were fixed in 4% paraformaldehyde for 10 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine–HCl (pH 8.0) for 10 min. Slide-mounted sections were prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris–HCl (pH 7.5), 4 $\times$ SSC (1 $\times$ SSC: 150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% SDS, 200 $\mu\text{g}/\text{ml}$ tRNA, 1 mM EDTA, and 10% dextran sodium

sulfate. Hybridization was performed at 42 °C for 10 h in the prehybridization buffer supplemented with 10 000 c.p.m./ μl ^{35}S -labeled oligonucleotide probes. The probes were complementary to nucleotide residues 61 to 106 of VEGF cDNA (accession no. AF222779), 541 to 586 of Flk-1 cDNA (accession no. U93306), 961 to 1016 of Flt-1 cDNA (accession no. D28498), 841 to 886 of eNOS cDNA (accession no. X76309), 1 to 46 of nNOS cDNA (accession no. AF037071), 301 to 346 of Bax cDNA (accession no. S76511), and 441 to 486 of Bcl-2 cDNA (accession no. L14680). The slides were washed at room temperature for 20 min in 2 $\times$ SSC containing 0.1% sarkosyl and twice at 55 °C for 40 min in 0.1 $\times$ SSC containing 0.1% sarkosyl. The sections were either exposed to Hyperfilm- βmax (Amersham International plc) for 4 weeks or dipped in Kodak NTB2 nuclear track emulsion and exposed for 4–8 weeks. The specificity of *in situ* hybridization was confirmed by the disappearance of signals when excess doses of the corresponding non-labeled anti-sense oligonucleotides (cold) were added to the labeled (^{35}S -dATP) anti-sense oligonucleotides (hot) hybridization fluid.

RNA preparation and real-time quantitative PCR

Total RNA samples were prepared from tissues by the guanidinium thiocyanate–phenol–chloroform single-step extraction method with Isogen (Nippon Gene, Toyama, Japan) used routinely in our laboratory (Matsuda *et al.* 1999). After being isolated, treated with DNase I and quantified, RNA was reverse-transcribed to cDNA by the use of a ReverTra Ace (TOYOBO, Osaka, Japan).

The single-stranded cDNA was then used in real-time quantitative PCR for evaluation of relative expression levels of the nine genes of interest. Selected genes and primers are shown in Table 1. DNA amplification was performed in the Applied Biosystems (ABI 7900HT, Tokyo, Japan) real-time PCR machine with the GeneAmp 7900HT sequence detection system software (Perkin-Elmer Corp., Foster City, CA, USA), and the detection was made by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. The PCR reactions were set up in microtubes in a volume of 20 μl . The reaction components were 2 μl cDNA synthesized as above,

Table 1 Primers and conditions for real-time quantitative PCR analyses

Gene	Primers (5'–3')		Product size (bp)
	Sense	Antisense	
VEGF	GTACCTCCACCATGCCAAGT	GCATTAGGGGCACACAGGAC	194
Flk-1	CAGAAAAGGAGATGCCCGAC	TCCAGAGTTTTTCAGCTCTTC	300
Flt-1	AGGAGAGGACCTGGAAGTGTCTT	ATTCCTGGGCTCTGCAGGCATAG	470
eNOS	CTGCTGCCCCAGATATCTTC	CAGGTACTGCAGTCCCTCCT	230
nNOS	CCGGCTACACTTCTCCTCAC	CACGAAGCAGGGGACTACAT	210
iNOS	GCAGAATGTGACCATCATGG	ACAACCTTGGTGTGAAGGC	426
Caspase-3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC	281
Bax	CACCAGCTCTGAACAGATCATGA	TCAGCCCATCTTCTCCAGATGGT	541
Bcl-2	CACCCCTGGCATCTTCTCCTT	AGCGTCTTCAGAGACAGCCAG	519

10 µl 2 × SYBR Green master mix (Perkin-Elmer Corp.), and 0.4 µM of each pair of oligonucleotide primers (Table 1). The program was as follows: an initial step at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Regression curves were drawn for each sample and the relative amount was calculated from the threshold cycles with the instrument's software (SDS 2.0) according to the manufacturer's instructions. The PCR products were analyzed by gel electrophoresis to confirm the specificity of generated products. Relative expression levels of the target genes were normalized to the geometric mean of the two internal control genes, β -actin and gluceraldehyde-3-phosphate dehydrogenase (G3 PDH).

TUNEL assay

The Tdt-mediated dUTP nick end labeling (TUNEL) assay was carried out in the adjacent matched sections used for immunofluorescent studies by applying an *in situ* apoptosis detection kit (Takara, Otsu, Japan). The penile tissues frozen in liquid nitrogen with optimal cutting temperature compounds were cut in 8 µm thick sections transversely by cryostat. The frozen sections were then fixed in acetone for 15–30 min at room temperature and air dried. The slides were rinsed with PBS (pH 7.4) for 20–30 min, immersed in permeabilization buffer for 2–5 min over ice, and incubated with Tdt (terminal deoxynucleotidyl transferase) enzymes and labeling safe buffer for 60–90 min at 37 °C. To prevent drying during incubation at 37 °C, the slides were covered with coverslip (Takara Slide Seal for *in situ* PCR). After

being rinsed with PBS for 15 min, the coverslips were mounted with Immunon, and images were observed under the laser scanning confocal imaging system. Counts of apoptotic cells were made in cross-section in randomly selected microscopic fields at a final magnification of $\times 100$, and the average of apoptotic cell number in five fields per sample were calculated.

Statistical analysis

Values are shown as means $\pm$ s.d. Statistical analysis was performed by ANOVA with multiple comparisons by Fisher's protected least-significant difference *t*-test. Non-parametric data were analyzed by the Mann–Whitney U test or Wilcoxon signed rank test. $P < 0.05$ was considered to be statistically significant.

Results

Protein and mRNA expression of VEGF and its receptors

As can be seen in Fig. 1A–C, immunofluorescent studies showed that VEGF and its receptors, Flk-1 and Flt-1, were abundantly expressed in the corpus cavernosum of the root and body of the penis from LETO control rats. In contrast, OLETF rats exhibited modest or weak immunoreactivity for these molecules. On Western blots, we detected VEGF, Flk-1 and Flt-1 proteins in rat penile tissues as single bands migrating at 39 kDa, 200 kDa and 180 kDa respectively (Fig. 2A–C). The densitometric analysis of bands for VEGF, Flk-1 and Flt-1

Figure 1 Immunofluorescent findings for VEGF (A), Flk-1 (B) and Flt-1 (C) in the penises of LETO control (left panels) and OLETF (right panels) rats. Positive staining was shown in the corpus cavernosum of root penis. Magnification $\times 200$.

Figure 2 Immunoblot analysis for VEGF (A), Flk-1 (B) and Flt-1 (C) in the penile tissues of LETO control and OLETF rats. The upper trace of each panel shows representative blots of the respective protein in LETO (left lane) and OLETF (right lane) penises. The experiments were conducted by loading equal amounts of LETO and OLETF penile proteins in each lane. The lower panels show the bar graphs summarizing the immunoblot data. Densitometric results are expressed as a percent of each band obtained in LETO rats. Data are shown as the means $\pm$ S.D. of five experiments. * $P < 0.01$ vs LETO.

revealed a significant ($P < 0.01$) reduction in relative protein content in penile tissues from OLETF in comparison with those from LETO control rats.

In situ hybridization studies indicated that the reduction in penile expression of VEGF, Flk-1 and Flt-1 proteins in OLETF rats occurred at the level of gene expression. As depicted in Fig. 3A–C, mRNA expressions of the three molecules in corpus cavernosal tissues were evidently lower in OLETF than in LETO control rats. When we evaluated steady-state mRNA levels of VEGF and its receptors by real-time PCR (Table 2), the mRNA levels of VEGF, Flk-1 and Flt-1 were profoundly decreased in OLETF penile tissues (58%, 75% and 58% of LETO control respectively).

Protein and mRNA expression of NOS isoforms

Immunofluorescent staining for eNOS showed diffuse and marked immunoreactivity for the molecule in the corpus cavernosum of the LETO control rat penis (Fig. 4A). On the other hand, corpus cavernosal expression of eNOS was evidently faint in OLETF rats. The eNOS protein in penile tissues was also assessed using Western blotting. Bands of the predicted size (≈ 140 kDa) were detected in penile tissues, which were significantly ($P < 0.01$) reduced in OLETF com-

pared with LETO control rats (Fig. 5A). The decrease in eNOS protein in penile tissues of OLETF rats correlated with a decrease in eNOS mRNA which was obtained from *in situ* hybridization (Fig. 6A) and from real-time PCR (Table 2).

Diminished immunofluorescent staining for nNOS was detected in the corpus cavernosum (Fig. 4B) as well as in dorsal nerves (Fig. 4C) of OLETF compared with LETO control rat penile tissues. On the immunoblot illustrated in Fig. 5B, nNOS protein expression as determined by the density of 160 kDa bands relative to that of non-diabetic controls was significantly ($P < 0.01$) decreased by 40% in OLETF rat penile tissues. *In situ* hybridization signals for nNOS, which were seen in corpus cavernosal tissues, were definitely reduced in OLETF compared with LETO control rats (Fig. 6B). The striking reduction in nNOS transcripts in OLETF rat penile tissues was quantitatively confirmed by analysis using the real-time PCR method (Table 2).

As shown in Fig. 4D, iNOS protein was weakly stained in the corpus cavernosum, and its expression was apparently similar in LETO control and OLETF rats. When penile tissues were subjected to Western blot analysis for iNOS protein, the densitometric analysis revealed a slight increase in expression of 130 kDa immunostained band in OLETF rats (Fig. 5C). When penile expression of iNOS mRNA was assessed using

Figure 3 *In situ* hybridization analysis showing gene expressions of VEGF (A), Flk-1 (B) and Flt-1 (C) in the penises of LETO control (left panels) and OLETF (right panels) rats. Nuclei in cells in the corpus cavernosum of root penis were stained with hematoxylin as bluish-violet. The presence of mRNA is shown by black grains in the field. Magnification×200.

Table 2 Relative amounts of mRNAs in penile tissues from OLETF rats as compared with values in LETO control rats

mRNA from penile tissues	Relative amount of mRNA in OLETF
VEGF	0.58
Flk-1	0.75
Flt-1	0.58
eNOS	0.76
nNOS	0.41
iNOS	1.16
Caspase-3	1.61
Bax	1.33
Bcl-2	0.64

Total RNA was extracted from penile tissues and subjected to real-time PCR quantification as described under Materials and methods. Values represent the amount of mRNA relative to that in LETO control rats, which is arbitrarily defined as 1.

real-time PCR, a small increase in transcripts for iNOS was detectable in penile tissues harvested from OLETF compared with LETO control rats (Table 2).

Cell apoptosis in the corpus cavernosum

To assess whether diabetes results in apoptotic cell death in the corpus cavernosum, the tissue sections were labeled with an *in situ* TUNEL assay. As shown in Fig. 7, TUNEL-positive apoptotic cells were undetectable in corpus cavernosum from LETO control rats ($0 \pm 1/\text{field}$, $n=10$). In contrast, a significant ($P<0.01$) number of apoptotic cells was found in OLETF corpus cavernosal tissues ($3 \pm 1/\text{field}$, $n=10$).

Protein and mRNA expression of pro- and anti-apoptotic regulators

It is clear that apoptosis is regulated by the caspase family of cysteine proteases that are classified into three groups (Thornberry & Lazebnik 1998). The most prevalent caspase in the cell is caspase-3. After being processed from procaspase-3 to active caspase-3 by initiator caspases such as caspase-8, it causes the apoptotic phenotype by cleavage or degradation of several important substances. Bcl-2 acts upstream to prevent the activation of caspases (MacManus & Linnik 1997), and blocks the

pro-apoptotic actions of other Bcl-2 family members such as Bax and Bad (Merry & Korsmeyer 1997). Conversely, Bad can bind to anti-apoptotic proteins and promote apoptosis, but this ability is impaired by the mechanisms that control the state of phosphorylation of this pro-apoptotic protein (Wang *et al.* 1999).

Immunofluorescent staining for active caspase-3 revealed that its expression was enhanced in corpus cavernosal tissues from OLETF compared with those from LETO control rats (Fig. 8A). OLETF rats also showed stronger staining for Bax than LETO control rats (Fig. 8B). In contrast, diminished immunofluorescent staining for Bcl-2 was detected in corpus cavernosum of OLETF rats (Fig. 8C). Expression of pBad was less abundant in OLETF than in LETO control rats (Fig. 8D). The relative protein content of apoptotic regulators in penile tissues from LETO control and OLETF rats was identified by Western blotting. Polyclonal antibodies to apoptotic regulators detected proteins at 19 kDa for active caspase-3, 21 kDa for Bax, 26 kDa for Bcl-2 and 22 kDa for pBad (Fig. 9A–D). The densitometric analysis of bands revealed a significant ($P<0.01$) increase in relative protein content for active caspase-3 and Bax by 100% and 55% respectively, and a significant ($P<0.01$) decrease in relative protein content for Bcl-2 and pBad by 40% and 32% respectively, in OLETF rat penile tissues in comparison with non-diabetic controls.

In situ hybridization studies showed that Bax mRNA was increased and Bcl-2 mRNA was decreased in the corpus cavernosum of OLETF compared with LETO control rats (Fig. 10A and B). When real-time quantitative PCR was used for the evaluation of relative expression levels of the genes of apoptotic regulators, up-regulation of caspase-3 and Bax transcripts and down-regulation of Bcl-2 transcripts in diabetic penile tissues are presented numerically (Table 2).

VEGF activates Akt via a phosphatidylinositol 3'-kinase (PI3K)-dependent pathway, and Akt may inhibit endothelial cell apoptosis by phosphorylating pro-apoptotic proteins (Gerber *et al.* 1998, Thakker *et al.* 1999). Immunoblot analysis using the antibody that specifically reacts with Akt clearly visualized a major band with a molecular mass of 55 kDa in rat penile tissues (Fig. 9E). We found a strikingly lower level of Akt in penile tissues from OLETF rats. Quantitative analysis of immunoblots

Figure 4 Immunofluorescent findings for eNOS (A), nNOS (B and C) and iNOS (D) in the penises of LETO control (left panels) and OLETF (right panels) rats. Positive staining was shown in the corpus cavernosum (A, B and D) and dorsal nerves (C) of root penis. Magnification $\times 200$.

Figure 5 Immunoblot analysis for eNOS (A), nNOS (B) and iNOS (C) in the penile tissues of LETO control and OLETF rats. The upper trace of each panel shows representative blots of the respective protein in LETO (lane 1) and OLETF (lane 2) penises. The experiments were conducted by loading equal amounts of LETO and OLETF penile proteins in each lane. The lower panels show the bar graphs summarizing the immunoblot data. Densitometric results are expressed as a percent of each band obtained in LETO rats. Data are shown as the means $\pm$ S.D. of five experiments. * $P < 0.01$ vs LETO.

showed a decrease of 59% when compared with LETO control rat penis.

Discussion

This study using immunohistochemistry and immunoblotting has demonstrated that OLETF rats showed decreases in the protein expression of VEGF and its receptors, Flk-1 and Flt-1, in penile tissues at 20 weeks of age. There was a significant correlation between decreases in their protein and mRNA levels, as determined by real-time quantitative PCR analysis. This parallel behavior of protein and mRNA expressions implies that the decrease in the synthesis of VEGF and its receptors in penile tissues occur at the level of transcriptional regulation. At 20 weeks of age, the OLETF strain of rats exhibited significant insulin resistance and impaired glucose tolerance with hyperglycemia, as shown in our previous work (Jesmin *et al.* 2002b). The modest hyperglycemia and prominent hyperinsulinemia after 24 h of fasting observed in the present study confirmed that our animals were in the stage of insulin resistance of type II diabetes seen early in this strain.

Contrary to the present findings, we have reported that VEGF and Flk-1 expression is

enhanced in coronary vessels of the OLETF rat heart at the insulin-resistant stage of type II diabetes (Jesmin *et al.* 2002a,b). Furthermore, up-regulation of VEGF expression has been detected in the retina and renal glomeruli from the early phase of the type II diabetic model (Segawa *et al.* 1998, Tsuchida *et al.* 1999). The paradoxical changes in the expression of VEGF and its receptors suggest that local regulatory factors for their expression may differ among tissues in diabetes. In the present study, a marked reduction in Akt expression was found in penile tissues of OLETF rats. This reduced expression of Akt may be responsible for the depression of insulin-related VEGF expression in the penis from the insulin-resistant diabetic animal. We have also shown that hypoxia-inducible factor-1 α is much more highly expressed in the coronary vessels of the OLETF rat heart and the increased expression level of hypoxia-inducible factor-1 α could contribute to increased coronary expression of VEGF in this diabetic model (Jesmin *et al.* 2002a). Since hypoxia-induced expression of VEGF involves the Akt pathway (Mazure *et al.* 1997), it may be suggested that induction of VEGF by hypoxia is strongly impaired in the OLETF rat penis where Akt is down-regulated. Moreover, our recent work has indicated that

Figure 6 *In situ* hybridization analysis showing gene expressions of eNOS (A) and nNOS (B) in the penises of LETO control (left panels) and OLETF (right panels) rats. Nuclei in cells in the corpus cavernosum of root and body penis were stained with hematoxylin as bluish-violet. The presence of mRNA is shown by black grains in the field. Magnification $\times 200$.

coronary expression of VEGF and Flk-1 in the OLETF rat heart is strongly associated with prominent increases in the local generation of angiotensin II and expression of angiotensin type 1 receptors in coronary vessels (Jesmin *et al.* 2002a). However, our preliminary immunohistochemical studies showed that angiotensin II labeling was negligible and immunoreactivity for angiotensin type 1 receptors was equally weak in the penile corpus cavernosum from the two strains (LETO control and OLETF) of rats (authors' unpublished observation).

A defect of NO production in the endothelium and nitrinergic nerves in the penile corpus cavernosum has been indicated to underlie the pathophysiology of ED in diabetes (Saenz de Tejada *et al.* 1989, Azadzoi *et al.* 1992). Our study has confirmed the previous reports showing reduced penile expression of constitutive NOS isoenzymes in diabetic rats (Vernet *et al.* 1995, Cellek *et al.* 1999, el-Sakka *et al.* 1999, Akingba & Burnett 2001) and has demonstrated that penile expression levels of nNOS and eNOS in OLETF rats are regulated in a transcriptional manner.

Figure 7 Diabetes-related apoptosis in the rat penile tissues. Sections adjacent to those used for immunofluorescent studies were used to visualize cells undergoing programmed cell death by applying the TUNEL assay. Representative micrographs, with arrows pointing to TUNEL-positive apoptotic cells, for LETO control (left) and OLETF (right) rats. Magnification $\times 200$ (inset $\times 400$).

Chronic administration of an inhibitor of NOS following the establishment of diabetes with streptozotocin can protect the penile nitrinergic nerves from morphological and functional impairment (Cellek *et al.* 1999), indicating that selective nitrinergic degeneration in diabetic penises is NO dependent. The source of NO responsible for the selective nitrinergic degeneration might be iNOS. Up-regulation of iNOS has been found in diabetic patients with ED (Seftel *et al.* 1997). Furthermore, the presence of iNOS may down-regulate eNOS in the diabetic penis, since iNOS appears to have negative feedback effects on eNOS (MacNaul & Hutchinson 1993). However, there are also reports describing no changes (Escrig *et al.* 2002) or even decreases (el-Sakka *et al.* 1999) in iNOS protein content in the penile tissues of diabetic rats. In our study, a significant but small (16%) increase in iNOS gene and protein expression was observed in penile tissues of OLETF compared with LETO control rats. Nevertheless, the down-regulation of eNOS in the diabetic penis may be fully independent of the iNOS-mediated negative

feedback regulation of eNOS expression, since transcription of eNOS mRNA was not reduced even after induction of iNOS in lipopolysaccharide-treated animals despite reduced eNOS activity (Schwartz *et al.* 1997).

At the molecular level, VEGF can up-regulate eNOS expression in endothelial cells (Papapetropoulos *et al.* 1997). Furthermore, increased expression of eNOS has been reported in the rat penis after intracavernosal injection with VEGF (Lin *et al.* 2002). These findings support the importance of VEGF to eNOS induction. Thus, it would be logical to assume that the reduced expression of eNOS seen in the OLETF rat penis may be causally related to the decrease in VEGF expression in the tissue. Based on the current understanding of the roles of the eNOS isoforms in the penis, the contribution of eNOS to penile erection is considered auxiliary. However, very recent work has demonstrated that PI3K/Akt-dependent phosphorylation and activation of eNOS mediates the sustained phase of maximal penile erection (Hurt *et al.* 2002). This would

Figure 8 Immunofluorescent findings for active caspase-3 (A), Bax (B), Bcl-2 (C) and pBad (D) in the penises of LETO control (left panels) and OLETF (right panels) rats. Positive staining was shown in the corpus cavernosum of root penis. Magnification $\times 200$.

Figure 9 Immunoblot analysis for active caspase-3 (A), Bax (B), Bcl-2 (C), pBad (D) and Akt (E) in the penile tissues of LETO control and OLETF rats. The upper trace of each panel shows representative blots of the respective protein in LETO (left lane) and OLETF (right lane) penises. The experiments were conducted by loading equal amounts of LETO and OLETF penile proteins in each lane. The lower panels show the bar graphs summarizing the immunoblot data. Densitometric results are expressed as a percent of each band obtained in LETO rats. Data are shown as the means±S.D. of five experiments. * $P<0.01$ vs LETO.

suggest that Akt regulation of eNOS is important in maintaining normal penile erection. Accordingly, diminished penile expression of both eNOS and Akt observed in OLETF rats could be considered to play an important role in diabetic ED.

Unlike eNOS, nNOS does not appear to be inducible by VEGF (Sheehy *et al.* 1997). The penile expression level of nNOS has been documented to

remain unchanged in VEGF-treated rats (Lin *et al.* 2002). Thus, the VEGF-triggered biochemical events probably have no targets in the nNOS gene, which continues to produce nNOS transcripts at a steady level. However, there is one report showing that exposure to basic fibroblast growth factor (bFGF) results in a dose-dependent increase in nNOS expression in rat PC12 cells (Sheehy *et al.*

Figure 10 *In situ* hybridization analysis showing gene expressions for Bax (A) and Bcl-2 (B) in the penises of LETO control (left panels) and OLETF (right panels) rats. Nuclei in cells in the corpus cavernosum of root penis were stained with hematoxylin as bluish-violet. The presence of mRNA is shown by black grains in the field. Magnification $\times 200$.

1997). Interestingly, our Western blotting revealed that the relative content of bFGF in penile tissues of OLETF rats was decreased to 70% of LETO rats (authors' unpublished observation). This may suggest that the reduced nNOS protein content in the penile tissues of OLETF rats is, at least in part, a result of the decrease in penile expression of bFGF. However, whether the involvement of bFGF in the regulation of nNOS expression in the penis plays a critical role as a cause of the erectile failure associated with diabetes should be clarified in future studies.

There is strong evidence that VEGF is a survival factor for endothelial cells (Dimmeler & Zeiher 2000). This pro-survival activity of VEGF requires the PI3K/Akt signal transduction pathway, and activation of Akt stimulates expression of Bcl-2, an anti-apoptotic protein, and phosphorylation of Bad, a pro-apoptotic protein, thereby inhibiting apoptosis execution. As expected from the finding of the reduced expressions of VEGF, its receptors and Akt, OLETF rats exhibited decreased expression of Bcl-2 and pBad in the penile tissues. Conversely, active caspase-3, a member of caspase superfamily

which initiates apoptotic events, and Bax, a member of Bcl-2 family which causes apoptotic actions, were up-regulated, resulting in the appearance of apoptotic cells in the penile tissues of OLETF rats. The present results are in good agreement with the previous report which has shown the loss of Bcl-2 expression in cavernosal tissue from diabetic men with ED (Seftel *et al.* 1999). Also, our findings coincide with the higher number of apoptotic cells in the erectile tissues of streptozotocin-induced diabetic rats (Alici *et al.* 2000). Although further research is needed to identify the role of apoptosis in the pathophysiology of diabetic ED, we assume that apoptosis may cause some loss of the erectile tissue within the corpora, leading to degeneration of penile cavernosum with collagen replacement of the cavernosal smooth muscle.

To our knowledge, this study represents the first report that expression of VEGF, its receptors (Flk-1 and Flt-1) and its signaling pathway Akt are markedly diminished in the penile tissues from the insulin-resistant stage of type II diabetes in OLETF rats. These abnormalities may play a role in the reduction in penile expression of eNOS, and diminished eNOS-derived production of NO as well as impaired nNOS-containing cavernosal nerves seems to be involved in diabetic ED. Moreover, we suggest that the decrease in the pro-survival activity of VEGF could contribute to the loss of erectile cells by apoptosis in diabetes.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by Health Sciences Research Grants for Comprehensive Research on Aging and Health from the Ministry of Health, Welfare and Labour of Japan. SJ received a fellowship from the Ministry of Education, Science, Sports and Culture of Japan. We thank Professor T Iwanaga and Dr C N Mowa for their valuable suggestions.

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Received 23 May 2003

Accepted 28 July 2003