Asian Journal of Andrology (2009) 11: 435–442

www.nature.com/aja

Original Article

Increased expression of the nitric oxide synthase gene and protein in corpus cavernosum by repeated dosing of udenafil in a rat model of chemical diabetogenesis

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Abstract

Erectile dysfunction (ED) is a major complication of diabetes mellitus (DM). This study investigates the relationship between ED and the downregulation of constitutive nitric oxide synthase (cNOS) in the corpus cavernosum (CC) of diabetic rats. It also examines the effects of udenafil, a phosphodiesterase type 5 (PDE5) inhibitor, on ED and cNOS expression levels. After 16 weeks of daily oral treatment with udenafil in diabetic rats, the intracavernous pressure/mean arterial pressure (ICP/MAP) ratio was recorded to measure erectile function, and cNOS expression was measured using reverse transcriptase (RT)-PCR and immunoblots. Although the ICP/MAP ratio and the expression levels of endothelial NOS (eNOS) and neuronal NOS (nNOS) in the CC were markedly decreased in diabetic rats, long-term udenafil treatment improved the erectile function and increased cNOS expression compared with diabetic controls. These findings suggest that ED in DM is closely related to decreased cNOS expression in the CC and that udenafil has the ability to compensate for this pathological change by modulating cNOS expression. Udenafil also has an inhibitory role in cyclic guanosine monophosphate (cGMP) degradation.

Asian Journal of Andrology (2009) 11: 435–442. doi: 10.1038/aja.2009.27; published online 25 May 2009.

Keywords: cNOS, diabetes mellitus, erectile dysfunction, udenafil

1 Introduction

Diabetes mellitus (DM) causes erectile dysfunction (ED) through a variety of pathophysiological changes affecting psychological function, central nervous system function, androgen secretion, peripheral nerve activity, endothelial cell function and smooth muscle contraction [1].

Nitric oxide (NO) is widely recognized as a key factor in the initiation of penile erection by inducing smooth muscle relaxation in the corpus cavernosum (CC) [2]. As a neurotransmitter and vascular tone regulator, the production of NO is tightly regulated by constitutive nitric oxide synthases (cNOSs), such as neuronal NOS (nNOS), in penile neurons innervating the CC [3]. Endothelial NOS (eNOS) is active in penile vascular endothelial cells and in trabecular meshwork activated by blood flow-mediated shear stress [4, 5].

nNOS and eNOS were expressed in normal rat penile shaft at both the gene and the protein levels [6]. Although the results are conflicting regarding the effects of experimentally induced DM on NOS expression, some studies have reported a reduction in NOS activity *in vitro* and in the nNOS isozyme content in

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Received: 10 October 2008 Revised: 24 March 2009
Accepted: 9 April 2009 Published online: 25 May 2009

the CC of diabetic animals [7-9]. There has also been a report of greatly decreased corporal NO concentration in streptozotocin (STZ)-induced DM after stimulation of the cavernous nerve in rats [10]. Similarly, NO production in the penis of diabetic rats by nNOS and eNOS after chemical diabetogenesis was markedly decreased, and protein expression was correlated with NO production [11]. Among the three isoforms of NOS, inducible NOS accounts for the response to inflammatory stimuli in macrophages [12], although its role in ED is not fully understood. Otsuka Long–Evans Tokushima Fatty rats, an established animal model of congenital DM [13], exhibit lower protein expression levels of eNOS and nNOS in CC, but inducible NOS expression has been shown to be higher than that in controls [14]. On closer inspection of the published data [15], cNOS expression is definitely altered to a great extent in rat models of DM.

Phosphodiesterase type 5 (PDE5) inhibitors are effective in ED associated with DM and in obesity by inhibiting degradation of cGMP [16, 17]. However, their effects on NOS gene and protein expression levels have not been reported. Therefore, this study was designed to investigate whether chronic administration of the PDE5 inhibitor udenafil, a pyrazolopyrimidinone derivative, is able to enhance erectile function by modulating cNOS gene and protein expression levels as the underlying mechanism.

2 Materials and methods

2.1 Induction of experimental diabetes in rats

This study was carried out in accordance with the institutional 'Standard operating procedure for animal care and experiments' of Dong-A Pharmaceutical Company and the 'Guide for the care and use of laboratory animals' from the National Institutes of Health (NIH). Eight-week-old Sprague–Dawley rats were obtained from Charles River (Yokohama, Japan) and maintained under standard laboratory conditions. The rats were given food and UV-sterilized tap water *ad libitum*. DM was induced by an intravenous injection of STZ (Sigma, St. Louis, USA) dissolved in sterilized saline (55 mg per 2 mL per kg body weight). Hyperglycaemic rats with blood glucose levels > 300 mg per 100 mL were assigned to this study. The experimental groups consisted of age-matched normal control (n = 20), diabetic control (n = 20), and 5 and 20 mg kg^{-1} udenafil treatment groups (n = 20, respectively). Body weight and blood glucose levels were measured every 2 weeks after the induction of DM.

2.2 Treatments

Udenafil, a PDE5 inhibitor developed by Dong-A Pharmaceutical Company (Seoul, Korea), is distinguished by its superior pharmacokinetic profile and a low incidence of adverse effects in humans. Udenafil was dissolved in Titrisol solution (citrate sodium hydroxide buffer, pH 5.0) from Merck (Darmstadt, Germany) and orally administered daily to diabetic rats at doses of 5 or 20 mg kg^{-1} for 16 weeks. Age-matched and diabetic control rats were given vehicle only.

2.3 Evaluation of erectile function

After 16 weeks of administration, five rats from each group were anesthetized with ketamine and xylazine, and the right carotid artery was exposed and cannulated with a PE-50 tube filled with 50 IU heparinized saline to monitor mean arterial pressure (MAP). The penile crura were exposed through a perineal–scrotal and prepuce incision by spreading the overlying ischiocavernous muscle. A 26G needle connected to the PE-50 tube was inserted into the CC to measure the intracavernous pressure (ICP) and simultaneous penile erection. The lateral prostate space was exposed through a lower midline abdominal incision and the cavernous nerve was identified by removing the thin, filmy, semitransparent fascia covering the lateral prostatic space. The cavernous nerve, which usually runs from the major pelvic ganglion on the dorsolateral prostate surface, was then visualized for the erectile response. Electrostimulation was carried out for 1 min using a bipolar stainless steel electrode (Unique medical, Tokyo, Japan) by hooking the cavernous nerve 3–4 mm distal to the major pelvic ganglion. Monophasic rectangular pulses fixed at 5 ms, 3 V and 10 Hz were delivered from an electric stimulator (HSE, March-Hugstetten, Germany) during electrostimulation. MAP and ICP were recorded using a signal processor. This measurement was carried out 24 h after the last administration to eliminate the acute effects of udenafil.

2.4 eNOS and nNOS mRNA expression levels in CC

Freshly dissected CCs from 16-week-treated rats were subjected to reverse transcriptase (RT)-PCR amplification for eNOS and nNOS mRNA. Total RNA samples were isolated using Trizol (Invitrogen,

Carlsbad, CA, USA) and quantified by the following steps. The sequences of the specific PCR primers were 5′-CTGCTGCCCCAGATATCTTC-3′ and 5′-CAGGTACTGCAGTCCCTCCT-3′ for eNOS and 5′-CCGGCTACACTTCTCCTCAC-3′ and 5′-CACGAAGCAGGGGACTACAT-3′ for nNOS. Isolated RNA samples were reverse-transcribed to first strand cDNA at 48ºC for 45 min, and RNA/cDNA hybrids were denatured by 2-min incubation at 94ºC. PCR amplification, including second strand cDNA synthesis, consisted of 35 cycles of denaturation (94ºC, 30 s), template/primer annealing (eNOS: 54ºC, nNOS: 52ºC for 1 min), and extension (68ºC, 2 min). All procedures were carried out using the Access RT-PCR system (Promega, Madison, USA). One-percent agarose gel electrophoresis and ethidium bromide staining were applied to verify the 230-bp PCR products of eNOS and the 210-bp PCR products of nNOS. Densitometric analysis was carried out using the BIO-PROFIL system and BIO-1D image analysis software (Vilber Lourmat, Torcy, France). RT-PCR was carried out 24 h after the last administration to eliminate the acute effects of udenafil.

2.5 eNOS and nNOS protein expression levels in CC

The protein expression levels of eNOS and nNOS were studied using the western blot technique. Cavernosal tissue from each rat was minced and then placed into lysis buffer for 10 min. The homogenates were centrifuged at 16 000 × *g* for 20 min at 4ºC, and the protein concentration in the supernatant was determined by the Bradford method. Each sample was denatured for 10 min at 95ºC in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) and equal amounts of protein and SeeBlue plus2 size marker (Invitrogen) were loaded for each sample. The loaded samples were electrophoretically separated on 12% SDS-PAGE gels at 200 V for 35 min and transferred to nitrocellulose membranes (Invitrogen) for 1.5 h. The membranes were blocked with 5% skim milk on a shaker for 1 h. The membranes were then treated with eNOS (140 kDa)- and nNOS (155 kDa)-specific monoclonal primary antibodies (1:250 dilution, BD Bioscience, San Diego, CA, USA) for 1.5 h at room temperature and washed thrice with phosphate-buffered saline. An alkaline phosphatase-conjugated rabbit anti-mouse secondary antibody at a 1:2 000 dilution was applied for 1 h at room temperature. Specific bands for eNOS and nNOS proteins were visualized using the NBT/BCIP (Cygnus technologies, Southport, USA) substrate, and optical density was measured with the BIO-PROFIL system and BIO-1D image analysis software (Vilber Lourmat, Torcy, France). Immunoblotting was also carried out 24 h after the last administration of udenafil.

2.6 Data analysis

All statistical analyses were carried out using SigmaStat for Windows (Systat software, Point Richmond, CA, USA). The analysis of variance test was used for comparisons between experimental groups and between each test group. All data are expressed as the mean ± SD. Data from each experiment were obtained from at least 5 animals. Comparisons between the group mean values were carried out using Kruskal–Wallis ANOVA (analysis of variance) on ranks at a statistical significance level of $P < 0.05$.

3 Results

3.1 Body weight, blood glucose level and erectile function

The body weights and blood glucose levels (Table 1) of the diabetic and udenafil-treated groups were significantly different from that of normal controls at 8 weeks and at 16 weeks after the induction of DM ($P < 0.05$). Udenafil treatment has no effect on the changes caused by diabetogenesis. After completion of the 16-week treatment, erectile response was monitored to verify whether

Table 1. Changes in body weight and blood glucose level during the 16-week induction of diabetes.

Group	Body weight (g)				Blood glucose level (mg per 100 mL)			
	Normal control	Diabetic control	5 mg kg^{-1} Udenafil	20 mg kg^{-1} Udenafil	Normal control	Diabetic control	5 mg kg^{-1} Udenafil	20 mg kg^{-1} Udenafil
0 week	308.30 ± 7.42	316.00 ± 11.69	311.20 ± 7.58	312.88 ± 9.94	77.20 ± 6.78	81.00 ± 7.58	77.75 ± 7.48	80.50 ± 8.78
8 week	517.60 ± 40.14	338.86 ± 49.18*	336.63 ± 26.14*	302.94 ± 44.38***	109.50 ± 7.23	437.93 ± 61.50*	432.88 ± 48.75*	450.00 ± 71.86*
16 week	622.20 ± 52.34	355.50 ± 60.46*	345.60 ± 30.05*	318.44 ± 54.31*	105.80 ± 22.85	444.33 ± 29.58*	519.20 ± 89.31*	458.22 ± 57.36*

Data are expressed as means ± SD. $^{*}P < 0.05$ *vs*. normal control. $^{**}P < 0.05$ *vs*. diabetic control.

long-term administration of udenafil is able to maintain erectile function in DM. In the diabetic group, the maximum ICP/MAP ratio was the lowest (27.4% ± 17.0%) among all of the experimental groups and showed a significant difference with respect to the age-matched normal group (66.0% ± 8.5%), as depicted in Figure 1 ($P < 0.05$). With respect to udenafil treatment, both 5 and 20 mg kg^{-1} treatments had positive test results for erectile response, although there was no statistical significance in the low-dose group (39.4% ± 18.0%). A noticeable and significant increase in erectile response was achieved in the high-dose group (51.6% ± 9.8%) compared with the diabetic controls ($P < 0.05$). The time-course changes in ICP/MAP ratio are illustrated in Figure 1. Areas under the curve (AUCs) (ICP / MAP [%] s) from 0 to 180 s were markedly increased in the high-dose group (4252.05 ± 792.83), and the low-dose treatment produced a mild shift with respect to the diabetic control (3 414.00 ± 927.44 *vs*. 2 661.00 ± 853.59, respectively). The AUC of the normal group was 4 989.30 ± 525.74.

3.2 eNOS and nNOS mRNA expression levels in CC

As an extension to the functional study, nNOS and eNOS mRNA expression levels in CC were evaluated by RT-PCR. Figure 2 shows the representative bands and the quantified band intensities corresponding to nNOS and eNOS mRNA levels. There was a marked downregulation of nNOS mRNA expression in the CC of diabetic rats compared with the age-matched normal group. Although low-dose udenafil treatment restored nNOS mRNA expression, high-dose treatment

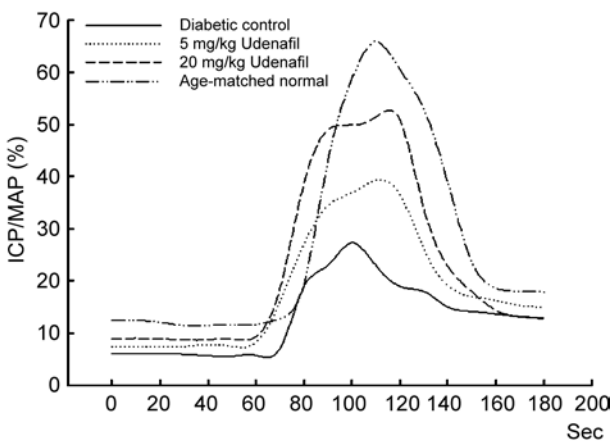


Figure 1. Time-course of changes in ICP / MAP (%). Initial baseline period (0-60 s), electrostimulation period (60–120 s), and post-stimulation period (120–180 s).

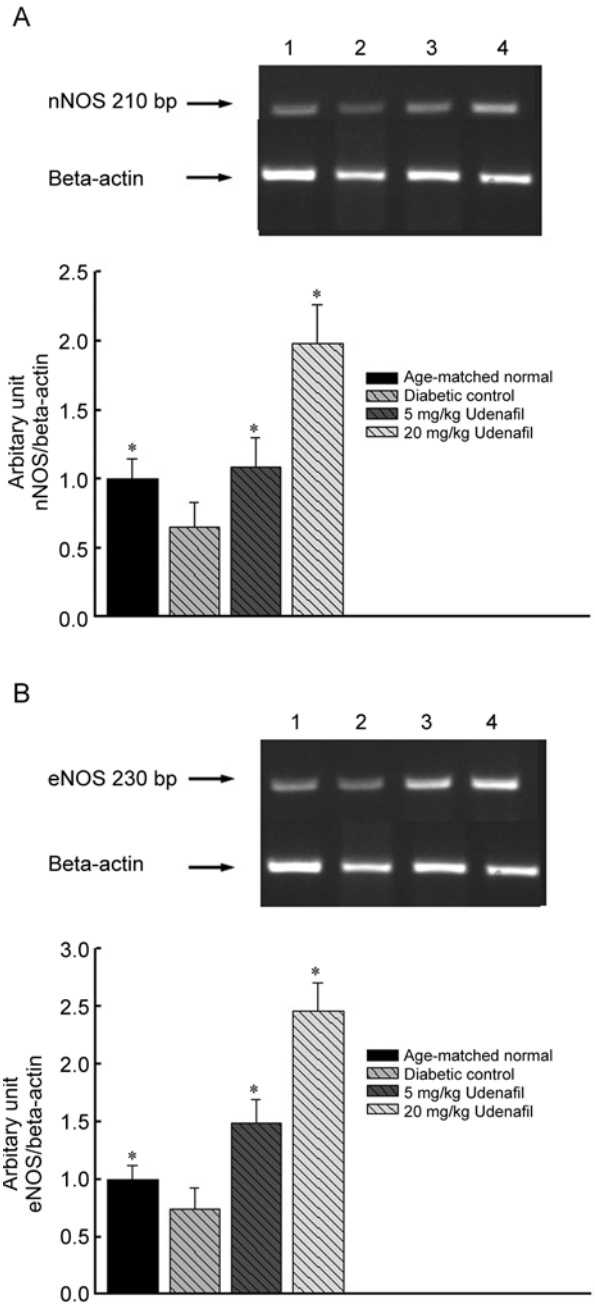


Figure 2. eNOS and nNOS mRNA expression levels in the corpus cavernosum. (A): Representative response of nNOS mRNA expression and quantified data. (B): Representative response of eNOS mRNA expression and quantified data. Lane 1: age-matched control; lane 2: diabetic control; lane 3: 5 mg kg^{-1} udenafil; and lane 4: 20 mg kg^{-1} udenafil. The densitometrically quantified data are expressed as means ± SD. $^{*}P < 0.05$ *vs*. diabetic control.

augmented this response, with significant differences with respect to the diabetic and the age-matched control groups ($P < 0.05$).

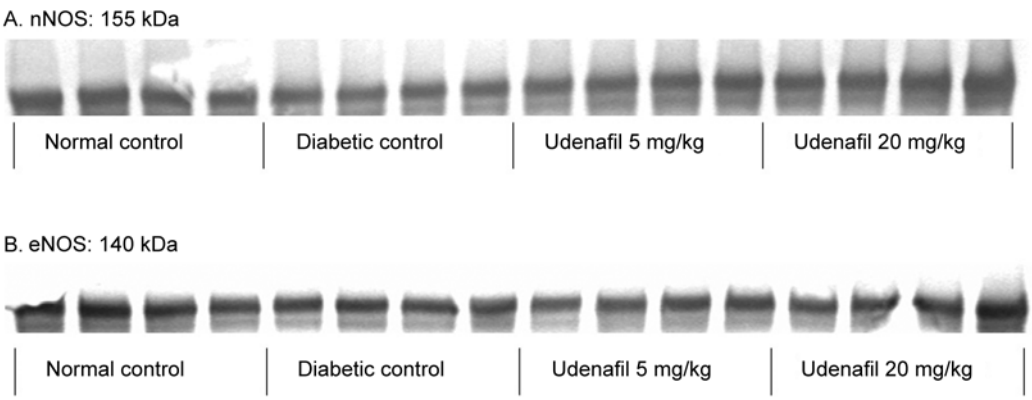
3.3 eNOS and nNOS protein expression levels in CC

Similar results were obtained for eNOS protein expression. In the diabetic control group, eNOS protein expression was markedly downregulated, and udenafil ameliorated this change. A remarkable increase in the high-dose group was in accordance with the outcomes for nNOS. The expression levels of nNOS and eNOS proteins in CC were densitometrically quantified by immunoblot band localization (Figures 3 A, B). The mean nNOS band intensity (Figure 3 C) in the diabetic control group remained at a significantly lower level (a 20.1% reduction) relative to the age-matched normal group ($P < 0.05$). Both the low- and the high-dose treatments of udenafil led to noticeable increases of 16.5% and 40.4%, respectively. These increases were statistically significant compared with the diabetic controls ($P < 0.05$). It was also obvious that nNOS protein expression in the high-dose treatment group was higher than that in the normal group. The results for eNOS protein expression were similar to those for nNOS expression. DM was characterized by a significant 21.2% reduction in eNOS expression compared with age-matched normal animals ($P < 0.05$). Low-dose udenafil treatment did not result in normal expression levels, although the 27.6% increase found with high-dose treatment was significantly higher than in the diabetic controls ($P < 0.05$), and slightly higher than in the age-matched normal animals.

4 Discussion

Recently, NO production and signal transduction in the molecular pathogenesis of ED in DM have been highlighted. However, PDE5 inhibitors have not been investigated in terms of their effects on NOS gene and protein expression levels. Therefore, this study was conducted to examine whether long-term administration of a PDE5 inhibitor, udenafil, could contribute to the preservation of cNOS gene and protein expression levels in experimental DM.

Before the molecular study, functional testing was carried out to measure erectile function in rats. A total of 5 or 20 mg kg^{-1} udenafil was administered for 16 weeks to diabetic rats *semel in die*. ICP / MAP ratios were then measured 24 h after the last dose of udenafil, which has a half-life of 3–4 h in rats. This was done to eliminate any acute effects of udenafil on erectile function. Meaningful results were obtained for erectile function in the high-dose treatment group based on the fact that two parameters of erectile function, the max-



C

	Age-matched Normal	Diabetic control	5 mg/kg Udenafil	20 mg/kg Udenafil
nNOS	100.00 ± 13.60*	79.92 ± 6.22	95.54 ± 2.78*	120.29 ± 23.25*
eNOS	100.00 ± 16.83*	78.76 ± 3.86	79.71 ± 10.18	106.34 ± 15.68*

Figure 3. Representative western blot images showing protein bands corresponding to (A) nNOS and (B) eNOS in the corpus cavernosum. (C) Intensities of densitometrically quantified immunoblot bands for nNOS and eNOS protein in the corpus cavernosum. Data are expressed as means ± SD. *$P < 0.05$ *vs*. diabetic control.

ICP/MAP ratio and AUC, were markedly increased, which implies that long-term administration of a PDE5 inhibitor in DM improves the erectile function. This also raises the possibility that these positive effects are related to upregulation of cNOS gene or protein expression levels on the basis of NO production, because NO is a key factor in the erectile response.

In DM, several theories have been proposed to explain the changes in NO production and NOS activity. In view of the mechanisms of DM-induced ED, NOS activity subsequent to the transport of *L*-arginine into the cell is not enhanced by insulin, and the plasma concentration and vascular content of *L*-arginine are reduced in diabetic rats [18]. Arginase is an enzyme that competes for the NOS substrate, *L*-arginine. The inducible form of this enzyme, arginase II, is overexpressed in CC of diabetic patients and downregulates the production of NO [19, 20]. DM also reduces the levels of NADPH (nicotinamide adenine dinucleotide phosphate), an essential cofactor for NOS, and increases the levels of calcium-elevating second messengers, such as diacylglycerol and protein kinase C, thus increasing smooth muscle contractility. Interestingly, glucose-induced elevation of protein kinase C seems to be mediated by oxidative stress in rabbit CC smooth muscle cells [21]. Furthermore, Rho-kinase protein expression is increased in diabetic corporal tissue, and the RhoA/Rho-kinase signalling pathway plays an important role in the reduction of NO synthesis through eNOS suppression [22, 23]. In the diabetic rat penile crura, ICP is markedly decreased, and the gene expression levels of apoptotic factors, such as *Bak* and *Bax,* are increased [24]. Another important factor is the formation of reactive oxygen species in DM. Reactive oxygen species from non-enzymatic glycosylation and increased NADPH oxidase activity impair endothelial function in erectile tissue and quench NO. In turn, this causes a reduction in the bioavailability of NO and peroxinitrite formation by nitrosative stress, which is highly toxic and damaging to the erectile tissue [25-27].

Whatever the mechanism, diabetic rats exhibited reduced gene and protein expression levels of cNOS in CC, and chronic treatment with udenafil induced an increase in expression, especially in the high-dose group, where overexpression of the cNOS gene was clearly observable. These results correlated well with the functional study. Therefore, it can be suggested that altered cNOS expression is, to some extent, responsible for diabetic ED, and chronic treatment with udenafil prevents or minimizes these pathophysiological changes. The overexpression of the cNOS gene does not cause concern, because the protein expression level only reached the normal level. Recently, PDE5 inhibitors have gained attention for their possible role in the alleviation of male infertility [28]. There is substantial evidence that long-term treatment with other PDE5 inhibitors has a positive effect on antioxidant capacity, relief of symptoms, and restoration of erectile function in human and animal studies. For instance, sildenafil reduces malondialdehyde levels and potentiates the total antioxidant capacity of plasma in STZ-induced diabetic rats [29], induces symptomatic improvement in pulmonary hypertension [30] and prevents myocyte hypertrophy [31]. Moreover, erectile function, protein expression of phosphorylated eNOS and NOS enzymatic activity are improved in normal rats by sildenafil treatment [32-34]. Chronic treatment with tadalafil in patients with increased cardiovascular risk improved brachial artery flow-mediated dilation, and resulted in a net increase in nitrite/nitrate levels and a decrease in the plasma endothelin-1 level [35]. Furthermore, earlier studies show that chronic treatment with udenafil does not alter blood pressure [36], and our in-house general pharmacology studies also show that chronic udenafil treatment does not affect blood pressure or heart rate (data not shown).

On the basis of the results of this study, along with the findings of earlier reports, it is important to clarify the role of chronic inhibition of PDE5 and to explore new therapeutic modalities besides 'on-demand' treatment. Diabetic ED correlates with reduced cNOS expression, activity and NO bioavailability in CC through a variety of mechanisms, including reduced substrates for NOS, increased intracellular protein kinase C levels, oxidative and nitrosative stress, endothelial cell damage and diabetic autonomic neuropathy, and RhoA/Rho-kinase signalling. Thus, increased cNOS expression owing to chronic PDE5 inhibition is of potential value in the treatment of diabetic ED, although NOS activity and NO bioavailability were not specifically tested in this study. The results of the functional study indirectly indicate that NO response and chronic PDE5 inhibition increase NOS activity and NO bioavailability in DM.

In summary, chronic inhibition of PDE5 with udenafil is a useful therapeutic strategy to prevent the progression of diabetic ED by enhancing cNOS gene and protein expression levels in diabetic CC, although the specific mechanism remains to be elucidated. These results also suggest that new treatment modalities for

udenafil, such as daily administration, may effectively improve erectile function in diabetic patients.

Acknowledgment

This study was supported in part by a Grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ2-PG4-PT01-0024).

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