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ORIGINAL ARTICLE

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Increased expression of TRPC4 channels associated with erectile dysfunction in diabetes

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SUMMARY

In recent reports, an association between altered TRPC channel function and the development of various diabetic complications has drawn the attention of many investigators. The aim of this study was to investigate the expression of TRPC4 channels of corpus smooth muscle (CSM) cells in diabetes, and to evaluate the association between erectile dysfunction (ED) and altered TRPC4 channel function. The expression of TRPC4 in the penile tissue of human, normal and diabetic rat was investigated using RT-PCR, western blotting and immunohistochemistry (IHC). In vivo gene transfer of dominant negative (DN) TRPC4 into the CSM of rat was conducted. In vivo pelvic nerve stimulation was performed to measure erectile function. Expression of TRPC1, TRPC3, TRPC4 and TRPC6 in human and rat CSM tissues was confirmed by RT-PCR, western blot and IHC. In the diabetic rat, the expression levels of mRNA and protein of the TRPC4, and TRPC6 were significantly increased compared to control rats ($p < 0.05$). The change in TRPC4 expression in the diabetic rats was higher than those of the other TRPC subunits ($p < 0.05$). The IHC showed that only TRPC4 expression had a higher intensity in the diabetes compared to normal rats ($p < 0.05$). Gene transfection with $TRPC4^{DN}$ into the diabetic rats restored erectile function to levels similar to that of normal controls. Gene expression of $TRPC4^{DN}$ in CSM tissue was confirmed by RT-PCR 2 weeks after transfection. This study demonstrated that TRPC4 channel expression increased in the penile CSM cells of diabetic rats. The down-regulation of TRPC4 with DN form restored erectile function in the diabetic rats. The alteration of TRPC4 channel is one of pathophysiology of ED and could be a target for drug development for ED.

INTRODUCTION

As PDE5 inhibitors were introduced as the first line treatment of erectile dysfunction (ED), more than 50 million patients with any cause of ED have been successfully treated with PDE5 inhibitors (Hatzimouratidis *et al.*, 2010). However, approximately 30–40% of patients with ED are not responsive to PDE5 inhibitors and require additional invasive treatments (Hatzimouratidis & Hatzichristou, 2005; Eardley *et al.*, 2010). Given these outcomes, other pathophysiology, such as the RhoA/ROCK pathway, maxi-K channel or transient receptor potential (TRP) channel, may also be involved in ED (Sung *et al.*, 2012; Hannan *et al.*, 2013). Research is continuing on compounds that may benefit those men who do not experience satisfactory results from oral PDE5 inhibitors (Albersen *et al.*, 2010).

The TRP family is a large group of ion channel genes that is divided into six subfamilies: TRPC (canonical), TRPV (vallinoid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin) and TRPML (mucolipin). In particular, TRPCs are non-selective, Ca^{2+}-permeable cation channels that include seven members; TRPC1-7. TRPC channels can be activated by G-protein-coupled receptor (GPCR)-Gαq-Phospholipase C (PLC) pathway. The activation mechanisms of TRPC4 have been proposed GPCR of the $G\alpha_{q/11}$-PLCβ and receptor tyrosine kinases-PLCγ (Jeon *et al.*, 2013). TRPC channels are expressed in smooth muscle cells in various-sized vessels and organs such as the uterus and the gastrointestinal tract (Inoue *et al.*, 2006). TRPC channels have recently emerged as important players in the control of Ca^{2+} homeostasis and smooth muscle function (Gonzalez-Cobos & Trebak, 2010; Selvaraj *et al.*, 2012).

In recent reports, an association between altered TRPC channel function and the development of various diabetic complications has drawn the attention of many investigators (Graham

et al., 2012). TRPC4 channels have been known to contribute to the regulation of endothelial function (Freichel *et al.*, 2001), which is one of the key factors in ED development. There have also been reports of increased levels of TRPC4 channel mRNA and protein in diabetes (Chung *et al.*, 2009; Evans *et al.*, 2009). Accordingly, the role and level of TRPC4 channels in ED in diabetes is critical to discovering the molecular pathophysiology of ED. However, there are no data regarding ED in diabetes and the level of TRPC4 channels in the penile corpus smooth muscle (CSM). We already performed the patch clamp study to verify the role of TRPC4 using transfection of TRPC4 and/or negative form of TRPC4 in the HEK cells. In this article, the dominant negative (DN) of TRPC4 inhibited the function of wild-type (WT) channel activity of TRPC4 in HEK 293 cells (Jeon *et al.*, 2013). The aim of this study was to evaluate the level of TRPC4 channels in CSM cells in diabetes and to investigate the association between ED and the dysfunction of TRPC4 channels using gene transfer of DN form of TRPC4 and an in vivo cavernous nerve stimulation model.

MATERIALS AND METHODS

All studies were performed according to a protocol approved by the Internal Review Board of the Samsung Medical Center/Sungkyunkwan University School of Medicine. Informed consent was obtained from patients providing penile cavernosal tissues. Human tissue was obtained from the corpus cavernosum of patients with organic ED who were undergoing the implantation of penile prostheses. All the rats utilized in these studies were treated according to the guidelines of the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Institute. A total of 72 adult male Sprague–Dawley® rats were used in these studies.

Corpus smooth muscle cell culture

Homogeneous explant cell cultures of human CSM cells were prepared as previously described (Palmer *et al.*, 1994; Zhao & Christ, 1995). Human CSM cells were cultured in 10% foetal bovine serum-Dulbecco's modified Eagle's medium at 37 °C in 5% CO_2, and the medium was changed every 2–3 days. Primary cultured cells were used between passages 2 and 4.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis for measuring TRPC mRNA

Total RNA was extracted from cultured human CSM cells, frozen tissues and rat corporal tissues using the TRIzol (Invitrogen, Carlsbad, CA, USA) method according to the manufacturer's instructions. RNA was used to synthesize first-strand cDNA using random hexamer primers and a SuperScript III First-Strand Synthesis System (Invitrogen). PCR was performed with Taq DNA polymerase (Roche Applied Science, Mannheim, Germany) to confirm the expression of TRPC mRNA. The combination of primers employed in the reaction is outlined in Table 1. PCR products were separated by 1.8% agarose gel electrophoresis. DNA bands were visualized by ethidium bromide staining under ultraviolet light.

Quantitative real-time RT-PCR

To quantify the changes in the expression of TRPC subtypes in diabetic rats, quantitative real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene-specific TaqMan primers and probes for rat TRPC1 (Rn00585625_m1), TRPC3 (Rn00572928_m1), TRPC4 (Rn00584835_m1), TRPC6 (Rn00677559_m1) and β-actin (actin-Rn00667869_m1) were purchased from Applied Biosystems. Real-time PCR assays were performed using ABI TaqMan Universal Master Mix in a final volume of 10 μL. Triplicate measurements were performed for all samples. The relative mRNA levels were calculated using the relative standard curve method.

Western blotting

Specific antibody were used with a mouse monoclonal anti-TRPC1 antibody (1 : 2000 dilution; Santa Cruz, CA, USA), a mouse monoclonal anti-TRPC4 antibody (1 : 2000; Neuromab, Davis, CA, USA), a rabbit polyclonal anti-TRPC6 antibody (1 : 500; Alomone Laboratories, Jerusalem, Israel) or a monoclonal anti-β-actin antibody (1 : 5000; Abcam, Cambridge, MA, USA). For quantification, films were scanned on a flatbed scanner, and the relative band intensities were digitalized and analysed using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). Western blots were repeated at least six times using different rat tissues. The relative intensities of the bands were quantified using the ImageJ 1.34 image analyser system (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry (IHC)

Double immunofluorescence labelling of TRPC and smooth muscle α-actin was performed for control and diabetic rat tissues. The specimens were embedded in Optimum Cutting Temperature Compound (Sakura Finetek, Torrance, CA, USA), rapidly frozen in liquid nitrogen, and then stored at −70 °C. Cryostat sections (10 μm) were rinsed with PBS four times for

Table 1 Primers for human TRPC1, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7 mRNA and rat TRPC1, TRPC3, TRPC4 and TRPC6 mRNA

Gene	Forward primer	Reverse primer	Size (bp)
hTRPC1	CAAGATTTTGGAAAATTTCTTG	TTTGTCTTCATGATTTGCTAT	372
hTRPC3	TGACTTCCGTTGTGCTCAAATATG	CCTTCTGAAGCCTTCTCCTTCTGC	318
hTRPC4	TCTGCAAATATCTCTGGGAAGAATGC	AAGCTTTGTTCGTGCAAATTTCCATTC	415
hTRPC5	GTGGAGTGTGTGTCTAGTTCAG	AGACAGCATGGGAAACAGGAAC	502
hTRPC6	CTCAAGAAAGAAAACCTCTC	ATGACTGGATCTTCACTAGAC	428
hTRPC7	GGATGCAGATGTGGAATGGAAG	CGTCATTTTCTCTGTCCACCTG	365
hGADPH	CTTTGGTATCGTGGAAGGACTC	TCTTCCTCTTGTGCTCTTGCTG	550
rTRPC1	GAT TTT GGG AAA TTT CTA GGA ATG	CTC ATG ATT TGC TAT CAG CTG G	363
rTRPC3	CCT GAG CGA AGT CAC ACT CCC AC	CCA CTC TAC ATC ACT GTC ATC C	529
rTRPC4	CTG CAG ATA TCT CTG GGA AG	GCT TTG TTC GAG CAA ATT TCC	412
rTRPC6	ACC TCT GCA GAT TTC ACT TG	CCT GGA TAA GCT CAG ACA TC	506
rGADPH	ATA GAC AAG ATG GTG AAG GTC	TAC TCC TTG GAG GCC ATG TAG	1000

15 min and then incubated with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature to reduce non-specific staining by the secondary antibody. The tissue sections were then incubated overnight at 4 °C with a mixture of primary antibodies (anti-TRPC and anti-smooth muscle α-actin) at the appropriate dilution in PBS containing 1% BSA and 0.5% Triton X-100. The following antibodies were used: mouse monoclonal anti-TRPC1 (1 : 50; Santa Cruz) and anti-TRPC4 (1 : 50; Neuromab), rabbit polyclonal anti-TRPC6 (1 : 50; Abcam), and mouse monoclonal or rabbit polyclonal anti-smooth muscle α-actin (1 : 500; Abcam). To evaluate TRPC4 expression in endothelial cells, the sections were incubated with mouse monoclonal anti-TRPC4 (1 : 50; Neuromab) and rabbit polyclonal anti-von Willebrand factor (VWF, 1 : 100; Millipore, Billerica, MA, USA). Negative controls were generated by omitting the primary antibody. After washing three times with PBS-Triton, the sections were incubated with a mixture of Alexa Fluor 488- and 594-conjugated secondary antibodies (1 : 1000; Invitrogen) for 1 h at room temperature. The sections were rinsed and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Immunofluorescent images were observed using a laser scanning confocal imaging system (Zeiss LSM 700; Oberkochen, Germany). The immunofluorescence intensity of the TRPC Ab was quantitatively analysed with the ImageJ 1.34 image analyser system (National Institutes of Health) for six preparations per group.

Induction of diabetes in rats

A 4-month period of diabetes mellitus was induced in experimental animals (8 weeks old at the time of injection) via a single intraperitoneal injection of streptozocin (60 mg/kg) dissolved in citrate buffer (60 mL of 100 mM citric acid and 40 mL of 200 mM disodium phosphate, pH 4.6), as previously described (Rehman *et al.*, 1997). An age-matched control group received an injection of the vehicle alone. Diabetic rats were enrolled in the study when they were confirmed to be diabetic for 1 week (defined as exhibiting blood glucose levels greater than 300 mg/dL).

DNA constructs

The DN form of TRPC4β was produced by PCR-mediated mutagenesis that replaced the three highly conserved residues LFW571-573 with AAA571-573 using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA), and the mutation was confirmed by DNA sequencing. The DN effect of the mutant was confirmed based on WT channel activity in HEK 293 cells transfected with WT TRPC4β by patch-clamp electrophysiology (data not shown).

In vivo gene transfer of TRPC4^{DN} into CSM cells

To evaluate whether diabetes-induced increases in CSM tone are associated with an increase in the expression of TRPC4, we examined the effect of inhibiting TRPC4 activity via gene transfer of the DN TRPC4 mutant on the erectile function of diabetic rats.

Four months after the induction of diabetes, rats were anaesthetized intraperitoneally with ketamine (50 mg/kg). The intracorporal injection was accomplished with 200 μg of pcDNA3 vector only or pcDNA-TRPC4^{DN} in 100 μL of PBS with 20% sucrose solution. The penile ligature was removed 2 min after the injection.

In vivo erectile function test using pelvic nerve stimulation

In vivo pelvic nerve stimulation was performed to investigate erectile function 2 weeks after intracorporal injection of vector alone into age-matched normal rats (normal control), vector alone (diabetic control) or TRPC4^{DN} into diabetic rats. The detailed methodology of measuring the erectile response has been described previously (Rehman *et al.*, 1997). The electrical field stimulation parameters were as follows: 6 V, 5 ms, 12 Hz, sp and a duration of 60 sec using an electric stimulator (model PG 721A; Austin Electronic Specialties Inc., Palo Alto, CA, USA). Systemic arterial blood pressure (BP) was monitored via carotid artery cannulation with polyethylene-50 tubing. The ratio of maximal (intracavernosal pressure) ICP to arterial BP obtained at the peak of the erectile response was determined to control for variations in arterial BP.

RT-PCR to confirm TRPC4^{DN} gene expression

At the end of the pelvic nerve stimulation, the corporal tissue was quickly extracted and fresh-frozen in liquid N_2 to confirm TRPC4DN gene expression using RT-PCR. The cDNA reverse transcription product was amplified with gene-specific primers

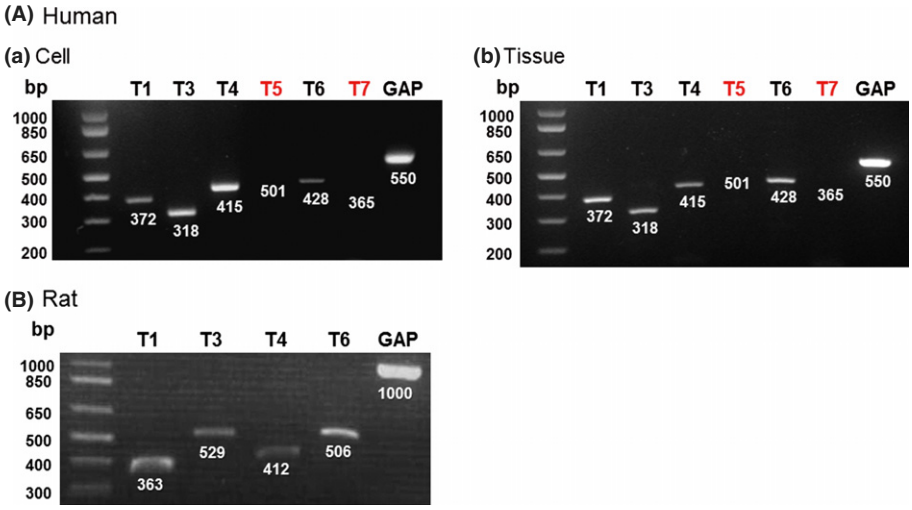
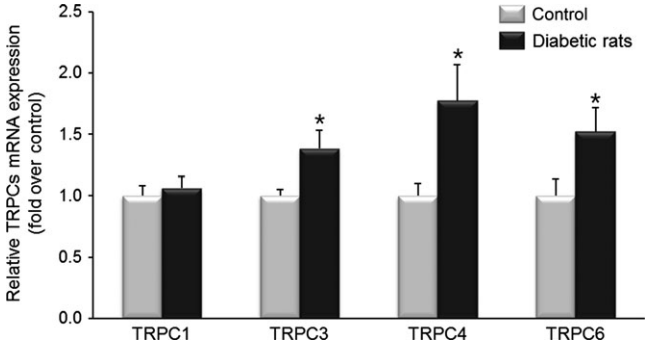


Figure 1 RT-PCR analysis of TRPC mRNA in corporal smooth muscle of human (A; a: cell and b: tissue) and rat (B). TRPC1, TRPC3, TRPC4 and TRPC6 mRNA were expressed in human penile tissues, cultured human corpus smooth muscle (CSM) cells and rat CSM tissues, whereas the mRNA of the TRPC5 and TRPC7 isoforms were not. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control and RNA input internal control.

Figure 2 Comparison of relative level of TRPC mRNA expression between normal and diabetic rats. The number of rats is the same for diabetic and control tissues. TRPC3: 1.4 ± 0.15-fold, TRPC4: 1.8 ± 0.29 and TRPC6: 1.5 ± 0.20; $n = 7$; *$p < 0.05$ vs. normal controls.



for the region between the cytomegalovirus promoter region and TRPC4 using two-stage nested PCR. The nested primers for the first PCR were 5′-ATCGCTATTACCATGGTGATG-3′ and 5′-TG GGCACTGAGACACCTTTC-3′. The primers for the second PCR were 5′-AGTTCTATTACAAAAGAAATGTC-3′ and 5′-GAACTGTTT ATCAAGGAGAATG-3′. The reaction occurred in a Perkin-Elmer Thermal Cycler (Perkin-Elmer Corp., Foster City, CA, USA) under the following conditions: an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 sec, 50 °C for 30 sec and 72 °C for 30 sec, with a final extension step at 72 °C for 7 min.

Statistical analysis

The data are expressed as the means ± standard error and were analysed using the IBM SPSS 20.0 statistical package (IBM, Armonk, NY, USA). A two-tailed Student's *t*-test was employed, when appropriate, for the comparison of group means. All the differences were considered significant if $p < 0.05$.

RESULTS

Expression of TRPC isoforms in human and rat CSM cells

RT-PCR analyses showed that among the seven known TRPC isoforms, TRPC1, TRPC3, TRPC4 and TRPC6 mRNA were endogenously expressed in human penile tissues, cultured human CSM cells and rat CSM tissues, whereas the mRNA of the TRPC5 and TRPC7 isoforms was not (Fig. 1).

Diabetes-induced changes in mRNA and protein expression of TRPC4

Real-time PCR showed that the relative expression levels of TRPC3, TRPC4 and TRPC6 mRNA were significantly increased in the diabetic rat compared with normal controls (TRPC3: 1.38 ± 0.15-fold, TRPC4: 1.77 ± 0.29-fold, and TRPC6: 1.52 ± 0.20-fold; $n = 7$; $p < 0.05$ vs. normal control; Fig. 2), whereas there was no difference in TRPC1 expression (1.06 ± 0.09-fold; $n = 7$; $p > 0.05$ vs. normal controls). The change in TRPC4 expression was higher than those of the other TRPC subunits. Consistent with the real-time PCR results, western blot analysis indicated that both TRPC4 and TRPC6 protein levels were significantly increased in diabetic rats compared with normal controls, whereas the changes in the expression of TRPC1 were not statistically significant. The difference in TRPC4 expression between the controls and the diabetic model was more prominent by western blotting. (TRPC4: 3.21 ± 0.24-fold and TRPC6: 2.24 ± 0.37; $n = 8$; $p < 0.05$ vs. normal control; Fig. 3).

To examine the cellular localization and changes in the expression levels of TRPC channels, immunohistochemical experiments were performed on penile tissues. In normal control rats, immunohistochemical staining confirmed the expression of TRPC1, TRPC4 and TRPC6 in penile tissues. Immunoreactivity for TRPC channels was evenly detected in all regions analysed. Double labelling with anti-TRPC antibodies and antibodies directed against α-SMA revealed that TRPC1, TRPC4 and TRPC6 staining overlapped with α-SMA staining, indicating that these proteins localized to the smooth muscle (Fig. 4A). Importantly, similar to the results of the real-time PCR analysis and western blotting, the CSM tissue of diabetic rats had a higher intensity of TRPC4 expression compared with normal rats. More intense TRPC4 staining was observed in the tissue of penile sinus, vein and artery (1.77 ± 0.03-fold vs. control; $n = 6$; $p = 0.03$; Fig. 4B). TRPC4 also co-localized with α-SMA in diabetic rats. Although TRPC6 immunofluorescence was slightly higher in diabetic rats than in normal controls, there was no statistically significant difference between the two groups, as for TRPC1 (Fig. 5). Only background staining was observed in the

Figure 3 Protein levels of TRPCs using western blotting (A). The protein level of TRPC4 was higher in the diabetic rats than in the normal controls. The number of rats is the same for diabetic and control tissues. TRPC1: 1.1 ± 0.08-fold over control, TRPC4: 3.2 ± 0.24 and TRPC6: 2.24 ± 0.37; $n = 8$; *$p < 0.05$ vs. normal controls (B).

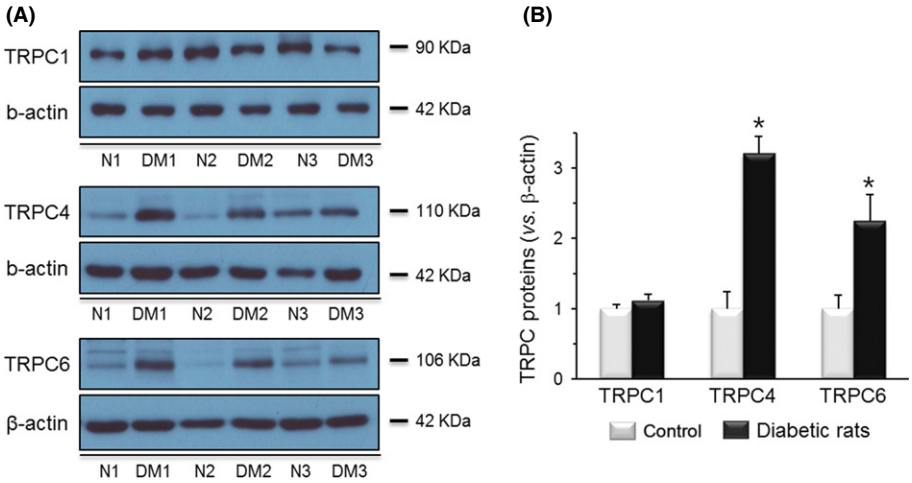
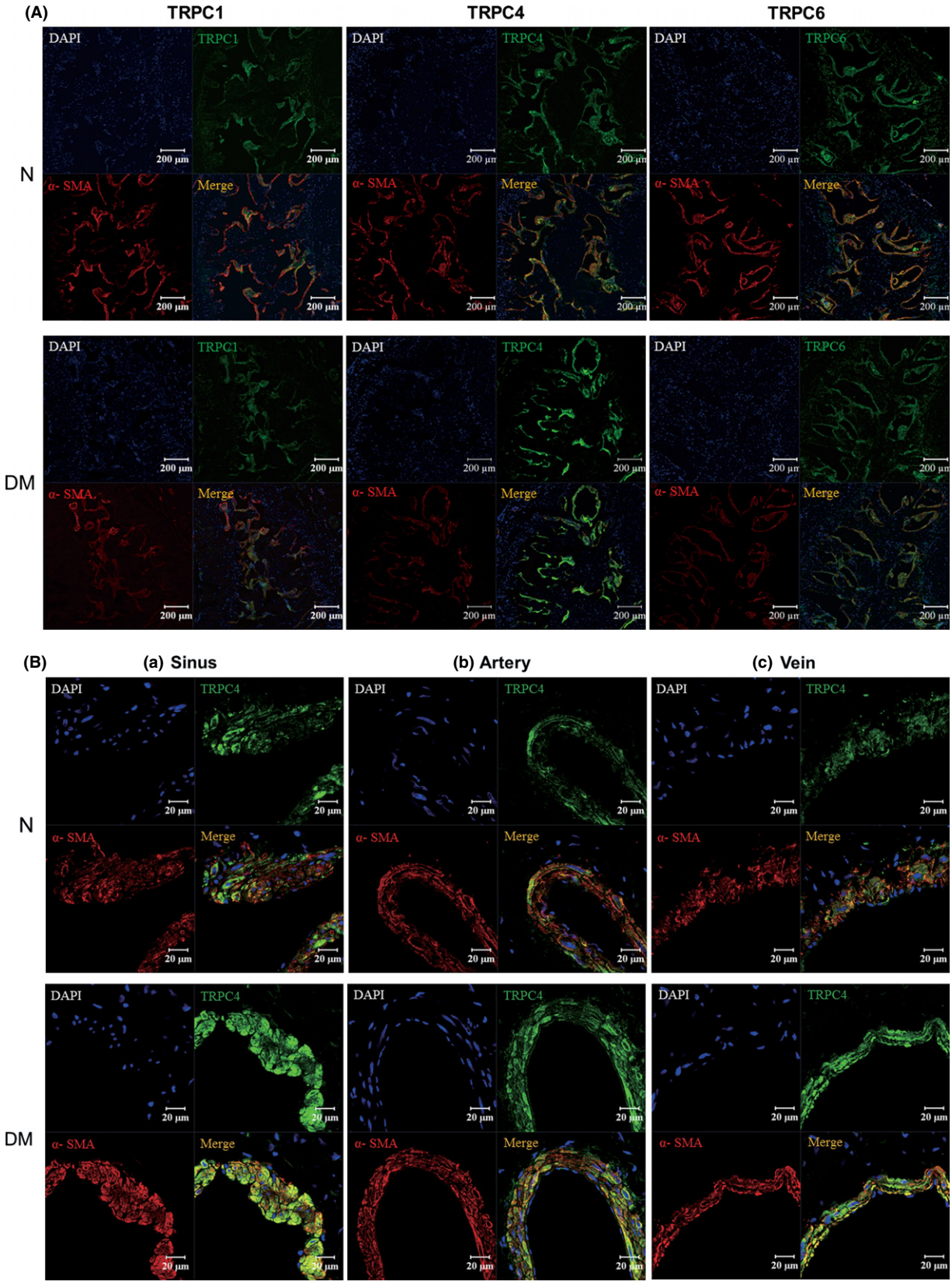


Figure 4 Immunohistochemical studies of the expression of TRPC1, TRPC4 and TRPC6 in the rat penile tissues (A, ×50). Increased intensity of TRPC4 in the penile sinus, vein and artery (B, ×400). The blue, green and red colour indicate 4′,6-diamidino-2-phenylindole (DAPI), TRPC and α-actin respectively.



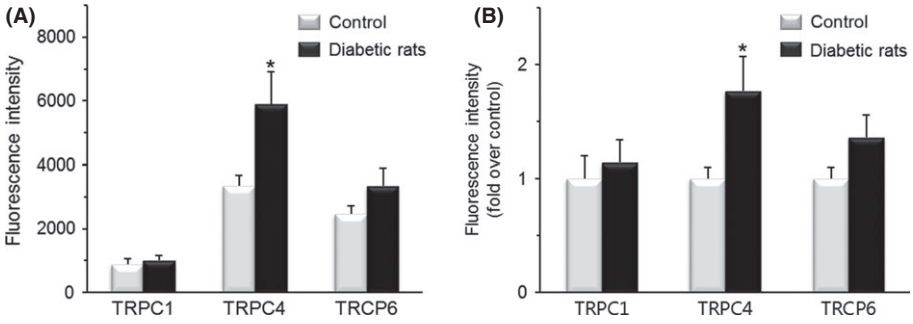


Figure 5 Fluorescence intensity of TRPC1, TRPC4 and TRPC6 immunohistochemical staining in the rat penile tissue (absolute values, A) and comparison with normal controls (fold over control, B). Intensity of TRPC4 in the diabetic rat was significantly higher than the normal control (1.77 ± 0.03-fold, $n = 6$, $p = 0.03$). The number of rats is the same for diabetic and control tissues. *$p < 0.05$ vs. normal controls.

negative controls, which were incubated in the absence of primary antibody (data not shown). Taken together, our findings obtained from RT-PCR, western blotting and IHC strongly suggest up-regulated expression of TRPC4 channels in the CSM of diabetic rats on the mRNA and protein levels.

Expression of TRPC4 in penile endothelial cells of rat

Immunohistochemical studies showed that the expression of TRPC4 was also confirmed in the penile endothelial cells of normal and diabetic rats (Fig. 6A, ×50, and Fig. 6B, ×400). The VWF seemed to be decreased in the diabetic rat penile tissue, but the immunofluorescence intensity of the VWF and TRPC4 in the endothelial cells could not be quantitatively measured (Fig. 6B).

In vivo pelvic nerve stimulation to compare erectile function between normal control, diabetic control and TRPC4^{DN}-transfected diabetic rats

The erectile response to electrical stimulation in normal rats and diabetic controls are shown in Fig. 7A and B. Diabetic rats (ICP/BP ratio, 50.3 ± 2.4%, $n = 8$, Fig. 7B) had a significantly lower ICP/ABP ratio than normal controls (83.7 ± 5.6%, $n = 10$, $p < 0.05$, Fig. 7A). Gene transfection with TRPC4^{DN} (76.5 ± 2.0%, $n = 10$, Fig. 7C) restored the erectile function of diabetic rats to a level similar to that of normal controls (Fig. 7D). All responses were reproducible 30 min after initial stimulation.

Confirmation of transfected TRPC4^{DN} gene in in vivo experimental model by RT-PCR

Gene expression of TRPC4^{DN} in CSM tissue was confirmed by RT-PCR 2 weeks after transfection (Fig. 8). In the diabetic rats, restored erectile function and an increased ICP/BP ratio was induced by the gene transfer of TRPC4^{DN} and was maintained for at least 2 weeks.

DISCUSSION

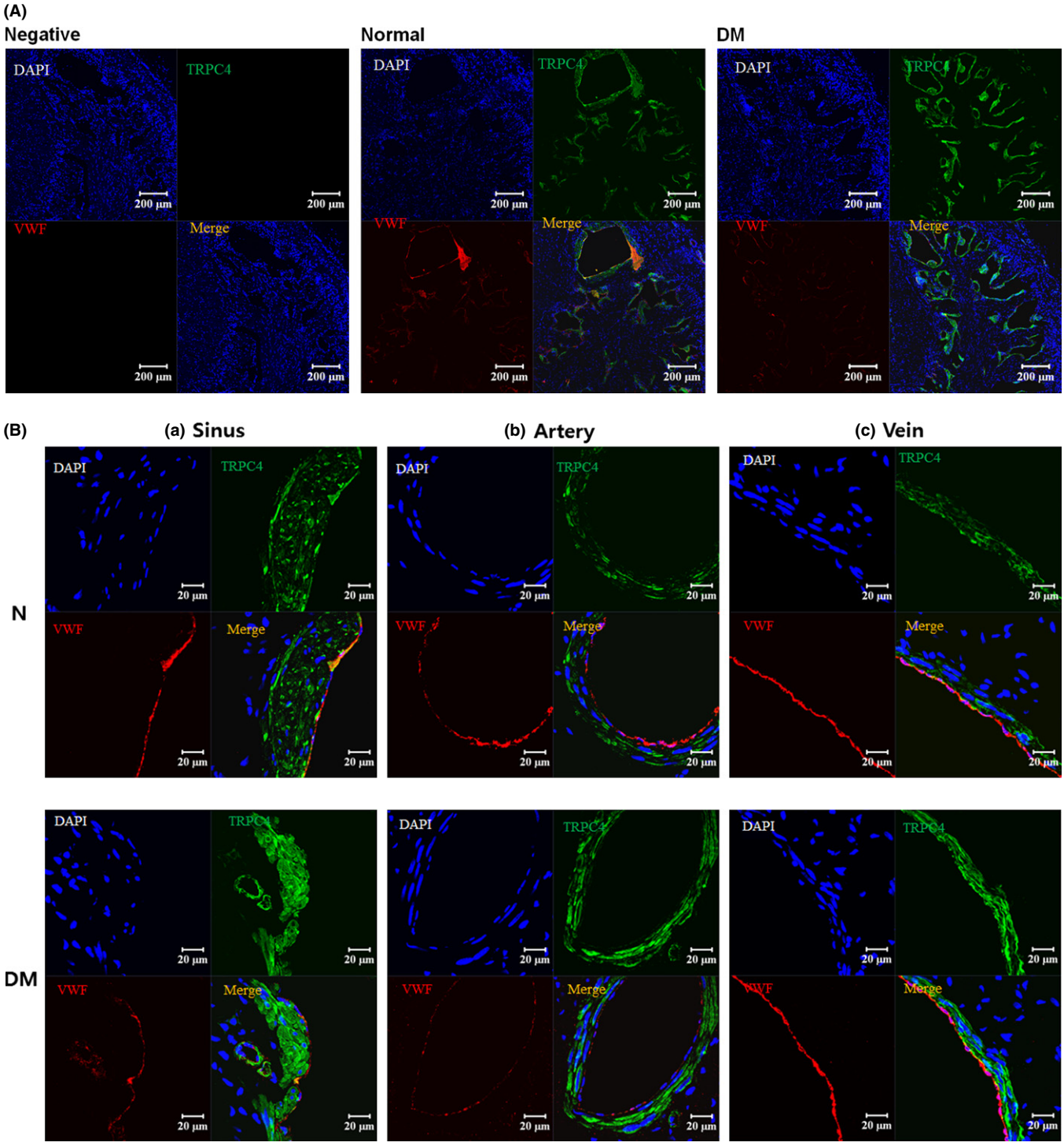
As TRPC4 expression is widely accepted in blood vessels, the description of its expression in penile vessels could be anticipated. In this study, the expression of TRPC4 in rat penile CSM tissues was verified in the level of mRNA, protein and tissue expression. In the diabetic rat model, the levels of TRPC4 expression were significantly increased compared with normal rats, and down-regulation by gene transfection with the DN form of TRPC4 restored erectile function similar to the level of normal rats. To our knowledge, this study is the first to confirm the expression of TRPC4 channels in the penile CSM and increased expression in several types of vessels in diabetic rats, and to investigate whether the dysfunction of TRPC4 channels could be an important pathophysiology regarding ED.

One of the most important roles of TRPC4 channels is the regulation of endothelial cell function. TRPC4 channels have been involved in the regulation of Ca^{2+} entry into various cells, and TRPC4 mRNA is expressed in mouse, human and bovine vascular endothelial cells (Groschner *et al.*, 1998; Freichel *et al.*, 1999; Kamouchi *et al.*, 1999). Increased levels of TRPC4 are also associated with hypoxia-induced smooth muscle cell proliferation. Chronic hypoxic exposure activates transcription factors, leading to the production of growth factors that stimulate smooth muscle cell proliferation, resulting in vascular remodelling and constriction (Stenmark *et al.*, 2006). One of the transcription factors involved in this process is AP-1, which regulates Ca^{2+}-sensitive genes. Culturing human pulmonary artery endothelial cells under hypoxic conditions results in increased TRPC4 mRNA and protein expression, enhanced store-operating channel (SOC) entry, and a Ca^{2+} release-activated Ca^{2+} current (CRAC) (Fantozzi *et al.*, 2003). These phenomena are accompanied by the enhanced binding of AP-1 to a number of AP-1-responsive genes involved in proliferation. The expression of siRNA against TRPC4 in endothelial cells prevents hypoxia-induced increases in TRPC4 expression and AP-1 binding. Thus, TRPC4 appears to be involved in mediating several aspects of hypoxia-induced gene expression and cell proliferation (Abramowitz & Birnbaumer, 2009).

The altered function of TRPC4 channels is also implicated in the proliferation of vascular smooth muscle cells. Although TRPC4 is widely expressed in vascular smooth muscle cells and endothelial cells from human vascular beds and different-sized arteries, the channel's contribution to smooth muscle cell physiology might be more complex (Yip *et al.*, 2004; Watanabe *et al.*, 2008). TRPC channel is proposed to coordinate endothelium-dependent vascular smooth muscle regulation (Tiruppathi *et al.*, 2002). Treatments of subarachnoid haemorrhage arteries with antibodies targeting either TRPC1 or TRPC4 inhibited endothelin-1-induced Ca^{2+} entry and vasoconstriction (Xie *et al.*, 2007). Zhang *et al.* (2004) demonstrated that enhanced proliferation was associated with an increase in TRPC4 expression and an increase in SOC entry induced by cyclopiazonic acid in human pulmonary artery smooth muscle cells. The treatment of these cells with siRNA against TRPC4 inhibited purinergic-induced proliferation, the increase in TRPC4 expression, and enhanced SOC entry, suggesting a potential role for TRPC4 in vascular remodelling during pathophysiological responses (Zhang *et al.*, 2004; Gonzalez-Cobos & Trebak, 2010).

Abnormalities of vascular reactivity and function are common in diabetes (Cooper *et al.*, 2001; Graham *et al.*, 2012). Recent reports showed that altered functions of TRPC channels are associated with the pathophysiology of diabetic vasculopathy by

Figure 6 Immunohistochemical studies of the expression of TRPC4 in the penile endothelial cells of normal and diabetic rats (A, ×50 and B, ×400). The blue, green and red colour indicate DAPI, TRPC and von Willebrand factor (VWF) respectively.



altering Ca^{2+} signalling. Chung *et al.* (2009) demonstrated that the level of TRPC4 mRNA was increased in saphenous veins from type 2 diabetic patients. The authors concluded that diabetes modulates the capacitative calcium entry, likely through the SOC channel, and specifically via the regulation of TRPC. TRPC1, TRPC4 and TRPC6 channels were detected at a significantly greater level in diabetic caudal artery smooth muscle from the Goto-Kakizaki (GK) rats compared with non-diabetic Wistar rats (Mita *et al.*, 2010). In this study, Mita *et al.* suggested that changes in TRPC channel expression may be partly responsible for the dysfunction of receptor-mediated Ca^{2+} entry in the caudal artery smooth muscle of diabetic rats. Evans *et al.* (2009) demonstrated that the TRPC4 protein level was significantly increased in cultured aortic smooth muscle cells from diabetic GK rats. Angiotensin II-induced Ca^{2+} influx was significantly enhanced in diabetic rats, and this response was decreased by a

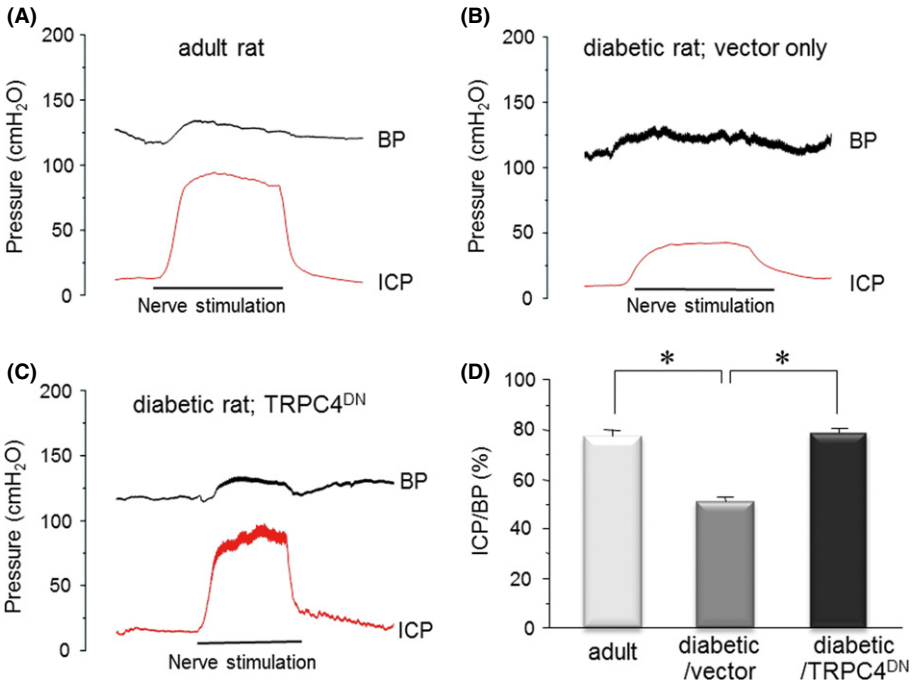


Figure 7 In vivo pelvic nerve stimulation to compare erectile function between normal control (ICP/BP ratio, 50.3 ± 2.4%, $n = 10$, A), diabetic control (83.7 ± 5.6%, $n = 8$, B) and TRPC4^{DN}-transfected diabetic rats (76.5 ± 2.0%, $n = 10$, C). Diabetic rats had a significantly lower ICP/ABP ratio than normal controls ($p < 0.05$). The ratio of diabetic rats with gene transfection of TRPC4^{DN} was higher than diabetic control ($p < 0.05$), and was not different from that of normal controls (D). *$p < 0.05$

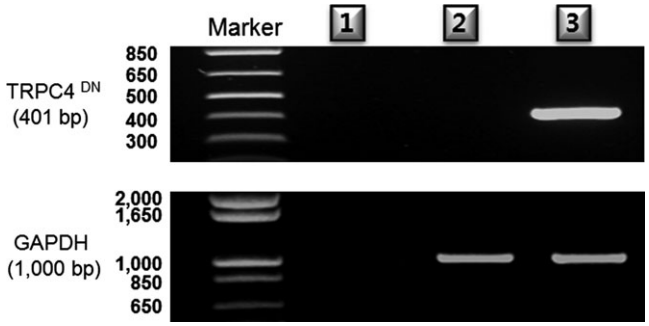


Figure 8 Gene expression of TRPC4^{DN} in the CSM tissue of rat 2 weeks after transfection. Lane 1, negative control without RT. Lane 2, transfected with vector only. Lane 3, transfected with pcDNA encoding dominant negative (DN) mutant genes.

diacylglycerol analogue, an activator of TRPC3/6/7 channels, and an inhibitor of TRPC4/5. The authors concluded that the 1/4/5 subgroup of TRPC proteins plays a significant role in elevated Ca^{2+} influx in diabetic GK aortic vascular smooth muscle cells.

In the penile tissue of diabetes, the elevated expression of TRPC4 induces abnormal regulation of the vascular smooth muscle, which plays a significant role in the pathophysiology of ED. Possible mechanisms of diabetic vasculopathy related to TRPC channels might be mediated through abnormally increased SOC entry and the decrease in vascular smooth muscle cells content itself. In diabetes, an elevation of TRPC channel-mediated Ca^{2+} entry consequently augments vascular smooth muscle contraction and proliferation, and then impairs endothelial function. Taken together, these data suggest that the abnormal function of TRPC4 channels in diabetic patients might be an important pathophysiology of ED and a potential target for new pharmacological interventions for ED.

This study had several limitations. We did not confirm whether forced expression of TRPC4^{DN} restores the content of vascular smooth muscle cells. It would be very meaningful to know the change in content in vascular smooth muscle cells following the gene transfer of TRPC4^{DN}. In addition, the mechanism of increased expression of TRPC4 in the penile cavernosal tissue of diabetic rats remains unknown. Further study should be followed regarding that abnormal expression of TRPC4 channel affect the structure and/or number of vascular smooth muscle cells.

CONCLUSIONS

This study demonstrated that TRPC4 channel expression increased in the penile CSM cells of rats with diabetes. The gene transfer of TRPC4^{DN} into the penile tissue restored erectile function in the diabetic rats. The alteration of TRPC4 channel might be one of pathophysiology of ED and could be a target for drug development for ED.

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AUTHOR'S CONTRIBUTIONS

HHS, SHC and SWL conceived and designed the idea for the article. HHS and SHC wrote the article. HHS, SHC, MK, MRC and SCK acquired the data. HHS, SHC, MRC, DHH, IS and SWL analysed and interpreted the data. IS and SWL provided guidance and critically revised the article.

FINANCIAL DISCLOSURES

There is no conflict of interest to disclose.

REFERENCES

Abramowitz J & Birnbaumer L. (2009) Physiology and pathophysiology of canonical transient receptor potential channels. *FASEB J* 23, 297–328.

Albersen M, Shindel AW, Mwamukonda KB & Lue TF. (2010) The future is today: emerging drugs for the treatment of erectile dysfunction. *Expert Opin Emerg Drugs* 15, 467–480.

Chung AW, Au Yeung K, Chum E, Okon EB & van Breemen C. (2009) Diabetes modulates capacitative calcium entry and expression of transient receptor potential canonical channels in human saphenous vein. *Eur J Pharmacol* 613, 114–118.

Cooper ME, Bonnet F, Oldfield M & Jandeleit-Dahm K. (2001) Mechanisms of diabetic vasculopathy: an overview. *Am J Hypertens* 14, 475–486.

Eardley I, Donatucci C, Corbin J, El-Meliegy A, Hatzimouratidis K, McVary K, McVary K, Munarriz R & Lee SW. (2010) Pharmacotherapy for erectile dysfunction. *J Sex Med* 7, 524–540.

Evans JF, Lee JH & Ragolia L. (2009) Ang-II-induced Ca(2+) influx is mediated by the 1/4/5 subgroup of the transient receptor potential proteins in cultured aortic smooth muscle cells from diabetic Goto-Kakizaki rats. *Mol Cell Endocrinol* 302, 49–57.

Fantozzi I, Zhang S, Platoshyn O, Remillard CV, Cowling RT & Yuan JX. (2003) Hypoxia increases AP-1 binding activity by enhancing capacitative Ca^{2+} entry in human pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 285, L1233–L1245.

Freichel M, Schweig U, Stauffenberger S, Freise D, Schorb W & Flockerzi V. (1999) Store-operated cation channels in the heart and cells of the cardiovascular system. *Cell Physiol Biochem* 9, 270–283.

Freichel M, Suh SH, Pfeifer A, Schweig U, Trost C, Weissgerber P, Biel M, Philipp S, Freise D, Droogmans G, Hofmann F, Flockerzi V & Nilius B. (2001) Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in $TRP4^{-/-}$ mice. *Nat Cell Biol* 3, 121–127.

Gonzalez-Cobos JC & Trebak M. (2010) TRPC channels in smooth muscle cells. *Front Biosci* 15, 1023–1039.

Graham S, Yuan JP & Ma R. (2012) Canonical transient receptor potential channels in diabetes. *Exp Biol Med (Maywood)* 237, 111–118.

Groschner K, Hingel S, Lintschinger B, Balzer M, Romanin C, Zhu X & Schreibmayer W. (1998) Trp proteins form store-operated cation channels in human vascular endothelial cells. *FEBS Lett* 437, 101–106.

Hannan JL, Albersen M, Kutlu O, Gratzke C, Stief CG, Burnett AL, Lysiak JJ, Hedlund P & Bivalacqua TJ. (2013) Inhibition of Rho-kinase improves erectile function, increases nitric oxide signaling and decreases penile apoptosis in a rat model of cavernous nerve injury. *J Urol* 189, 1155–1161.

Hatzimouratidis K & Hatzichristou DG. (2005) A comparative review of the options for treatment of erectile dysfunction: which treatment for which patient? *Drugs* 65, 1621–1650.

Hatzimouratidis K, Amar E, Eardley I, Giuliano F, Hatzichristou D, Montorsi F, Vardi Y & Wespes E. (2010) Guidelines on male sexual dysfunction: erectile dysfunction and premature ejaculation. *Eur Urol* 57, 804–814.

Inoue R, Jensen LJ, Shi J, Morita H, Nishida M, Honda A & Ito Y. (2006) Transient receptor potential channels in cardiovascular function and disease. *Circ Res* 99, 119–131.

Jeon JP, Roh SE, Wie J, Kim J, Kim H, Lee KP, Yang D, Jeon JH, Cho NH, Kim IG, Kang DE, Kim HJ & So I. (2013) Activation of TRPC4beta by Galphai subunit increases Ca^{2+} selectivity and controls neurite morphogenesis in cultured hippocampal neuron. *Cell Calcium* 54, 307–319.

Kamouchi M, Philipp S, Flockerzi V, Wissenbach U, Mamin A, Raeymaekers L, Eggermont J, Droogmans G & Nilius B. (1999) Properties of heterologously expressed hTRP3 channels in bovine pulmonary artery endothelial cells. *J Physiol* 518(Pt 2), 345–358.

Mita M, Ito K, Taira K, Nakagawa J, Walsh MP & Shoji M. (2010) Attenuation of store-operated Ca^{2+} entry and enhanced expression of TRPC channels in caudal artery smooth muscle from Type 2 diabetic Goto-Kakizaki rats. *Clin Exp Pharmacol Physiol* 37, 670–678.

Palmer LS, Valcic M, Melman A, Giraldi A, Wagner G & Christ GJ. (1994) Characterization of cyclic AMP accumulation in cultured human corpus cavernosum smooth muscle cells. *J Urol* 152, 1308–1314.

Rehman J, Chenven E, Brink P, Peterson B, Walcott B, Wen YP, Melman A & Christ G. (1997) Diminished neurogenic but not pharmacological erections in the 2- to 3-month experimentally diabetic F-344 rat. *Am J Physiol* 272, H1960–H1971.

Selvaraj S, Sun Y, Watt JA, Wang S, Lei S, Birnbaumer L & Singh BB. (2012) Neurotoxin-induced ER stress in mouse dopaminergic neurons involves downregulation of TRPC1 and inhibition of AKT/mTOR signaling. *J Clin Invest* 122, 1354–1367.

Stenmark KR, Fagan KA & Frid MG. (2006) Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ Res* 99, 675–691.

Sung HH, Kam SC, Lee JH, Chae MR, Hong C, Ko M, Han DH, So I & Lee SW. (2012) Molecular and functional characterization of ORAI and STIM in human corporeal smooth muscle cells and effects of the transfer of their dominant-negative mutant genes into diabetic rats. *J Urol* 187, 1903–1910.

Tiruppathi C, Freichel M, Vogel SM, Paria BC, Mehta D, Flockerzi V & Malik AB. (2002) Impairment of store-operated Ca^{2+} entry in TRPC4 (−/−) mice interferes with increase in lung microvascular permeability. *Circ Res* 91, 70–76.

Watanabe H, Murakami M, Ohba T, Takahashi Y & Ito H. (2008) TRP channel and cardiovascular disease. *Pharmacol Ther* 118, 337–351.

Xie A, Aihara Y, Bouryi VA, Nikitina E, Jahromi BS, Zhang ZD, Takahashi M & Macdonald RL. (2007) Novel mechanism of endothelin-1-induced vasospasm after subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 27, 1692–1701.

Yip H, Chan WY, Leung PC, Kwan HY, Liu C, Huang Y, Michel V, Yew DT & Yao X. (2004) Expression of TRPC homologs in endothelial cells and smooth muscle layers of human arteries. *Histochem Cell Biol* 122, 553–561.

Zhang S, Remillard CV, Fantozzi I & Yuan JX. (2004) ATP-induced mitogenesis is mediated by cyclic AMP response element-binding protein-enhanced TRPC4 expression and activity in human pulmonary artery smooth muscle cells. *Am J Physiol Cell Physiol* 287, C1192–C1201.

Zhao W & Christ GJ. (1995) Endothelin-1 as a putative modulator of erectile dysfunction. II. Calcium mobilization in cultured human corporal smooth muscle cells. *J Urol* 154, 1571–1579.

