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Diabetes-Induced Changes in the Alternative Splicing of the *Slo* Gene in Corporal Tissue

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Abstract

Objectives— Erectile dysfunction is a common diabetic complication. Preclinical studies have documented that the *Slo* gene (encoding the BK or Maxi-K channel α -subunit) plays a critical role in erectile function. Therefore, we determined whether diabetes induces changes in the splicing of the *Slo* gene relevant to erectile function.

Methods— Reverse transcriptase-polymerase chain reaction was used to compare *Slo* splice variant expression in corporal tissue excised from control and streptozotocin (STZ)-induced diabetic Fischer F-344 rats. Splice variants were sequenced, characterized by patch clamping, and fused to green fluorescent protein to determine cellular localization. The impact of altered *Slo* expression on erectile function was further evaluated in vivo.

Results— A novel *Slo* splice variant (SV_{cyt}, with a cytoplasmic location) was predominantly expressed in corporal tissue from control rats. STZ-diabetes caused upregulation of a channel-forming transcript SV₀. Preliminary results suggest that SV₀ was also more prevalent in the corporal tissue of human diabetic compared with nondiabetic patients. The change in isoform expression in STZ-treated rats was partially reversed by insulin treatment. Intracorporal injection of a plasmid expressing the SV₀ transcript, but not SV_{cyt}, restored erectile function in STZ-diabetic rats.

Conclusions— Alternative splicing of the *Slo* transcript may represent an important compensatory mechanism to increase the ease with which relaxation of corporal tissue may be triggered as a result of a diabetes-related decline in erectile capacity.

Keywords

Diabetes; Erectile dysfunction; Maxi-K channel; *Slo* gene; Splicing

1. Introduction

Increasing evidence suggests that altered smooth muscle (SM) cell function contributes to the progression of diabetic complications, particularly with respect to vascular disease [1–3]. Erectile dysfunction (ED) is a common vascular disease with an increased prevalence among

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Conflicts of interest

The authors have nothing to disclose.

diabetic patients [4]. From a pathophysiologic standpoint, ED results from impaired relaxation of corporal and arterial SM cells [5–7].

Changes in the cytosolic $[Ca^{2+}]$ provide the main stimulus for altered SM contraction, and transmembrane Ca^{2+} flux through L-type, voltage-dependent Ca^{2+} channels is a primary factor in this process [8]. Ca^{2+} channels, in turn, are regulated by hyperpolarizing currents generated by K channels [4,9–13]. Several lines of experimental evidence suggest that the Maxi-K channel (the large-conductance, calcium-sensitive K channel, or BK channel) plays a key role in erectile physiology [6,14,15].

Previous work has shown that 8 wk of streptozotocin (STZ)-induced diabetes in Fischer-344 (F-344) rats produced demonstrable changes in erectile capacity [16]. The importance of the *Slo* gene in regulating corporal SM tone and its restorative effects after gene transfer in aged and STZ-diabetic animals has been recently established [6,15,17], as well as its potential use in human gene therapy [18].

The α -subunit of the Maxi-K channel is encoded by the *Slo* gene, which can undergo alternative splicing to generate several isoforms [19]. Alternative splicing of the *Slo* transcript is known to be a dynamic process, responding to various stimuli, including hormones [20–22]. However, we are unaware of any studies documenting diabetes-related changes in *Slo* transcript expression. Therefore, we investigated the impact of STZ-diabetes on *Slo* splice variant expression in corporal tissue from F-344 rats.

2. Methods

2.1. Animals

Forty-one F-344 rats (Taconic Farms, Germantown, NY) aged 8–10 wk (200–240 g) were used. The number of replicates in each experiment is given in the figure legends. Rats were fed Purina laboratory rodent chow *ad libitum* and housed individually with a 0700–1900 light cycle. Two or 8 wk of STZ-diabetes was induced in 18 animals via a single intraperitoneal injection of STZ (35 mg/kg) dissolved in citrate buffer (60 ml of 0.1 mol/l citric acid and 40 ml of 0.2 mol/l Na_2HPO_4 , pH 4.6). Age-matched control animals received an injection of vehicle only [23]. One group of 8-wk diabetic animals was treated daily with 2 units insulin sc (Eli Lilly, IN, USA) for 1 wk. Tail blood glucose was determined 6–8 h after each insulin injection. Blood glucose prior to insulin treatment was > 300 mg/dl in diabetic rats; after treatment this value fell to < 100 mg/dl. All rats were euthanized by placement within a CO_2 gas chamber. Corpus cavernosum was harvested, flash frozen in liquid nitrogen, and stored at $-70^\circ C$.

2.2. Human tissue

Corporal tissue was procured during penile prosthetic implant surgery as approved by the AECOM/Montefiore Hospital IRB. Samples were flash frozen in liquid nitrogen and stored at $-70^\circ C$.

2.3. Reverse transcriptase-polymerase chain reaction, cloning, and sequencing of splice variants

Total RNA was extracted from frozen tissue with the use of the TRIzol (Invitrogen, CA, USA) method according to the manufacturer's instructions. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the use of RedTaq (Invitrogen) with the following combination of primers: the housekeeping gene ribosomal protein, large subunit, RPL19: RPL19R – CCTCATTCTCCTCATCC, RPL19F – CGCCAATGCAACTCCCG; for the *Slo* pore region (Fig. 1A): KmPF – ACAACCAGGCTCTCACCTAC, KmPR –

TTTCTTCCACTAACCGCAC; and for the region of *Slo*-spanning splice sites I through III (Fig. 1A): BKF1 – GAGGAGACACATGGCAG, KmV5R – ATAGACCCACA-AACACAATG (GIBCO/Invitrogen).

Bands were excised from the gel and were subcloned into pCRII-TOPO (Invitrogen) for sequencing. A restriction enzyme map of the *Slo* gene was created with the use of an online tool (the ExPASy [Expert Protein Analysis System] proteomics server of the Swiss Institute of Bioinformatics). The restriction enzyme sites BlnI and BsrGI flank the known splice sites I–III of the *Slo* gene but are absent from the pVAX vector, enabling the subcloning of the splice variant SVcyt from pCR-TOPO into pVAX-hSlo [24].

2.4. Expression of splice variants in human cell lines

Stably transfected human embryonic kidney fibroblasts (HEK293; Clontech, CA, USA) were generated by transfecting cells with pVAX-SVcyt and pVAX-SV0 followed by G418 selection (400 µg/ml).

2.5. Protein extraction and Western blot analysis

Total protein was prepared from cells and extracts subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis using commercial rabbit anti-GAPDH antibody or rabbit anti-Maxi-K- α antibody (Calbiochem, CA, USA). The visualized proteins were quantified with the use of model GS-700 imaging densitometer and molecular analyst (Bio-Rad, CA, USA).

2.6. Intracellular localization

The *hSlo* splice variants SVcyt and SV0 were subcloned from pVAX into pEGFP (Invitrogen) with the use of XhoI x SmaI. This procedure results in in-frame replacement of the last 33 amino acids at the N-terminal of *hSlo* with green fluorescent protein (GFP). These plasmids were used to transfect HEK293 cells. After 48 h, cells were plated onto cover slips. After a further 24 h, cells were fixed with the use of 4% paraformaldehyde and examined by fluorescence microscopy.

2.7. Electrophysiology

For channel analysis in HEK cells, the conventional tight-seal patch clamp method was used to examine currents with the use of a patch 1D amplifier with all current and voltage recordings digitized using a Neurocorder, as previously described [25]. Seal resistance was typically 10–50 G Ω . Bath and pipette solutions are described in Fig. 2. Experimental protocols were run with the use of pCLAMP software, version 6.4 (Axon Instruments, Inc, Foster City, CA, USA) running on a Dell Pentium PC (Dell Inc, Round Rock, TX, USA).

2.8. Determination of intracavernosal pressure

Animals were anesthetized by pentobarbital sodium 35 mg/kg ip and surgically prepared for direct neurostimulation of the cavernous, as previously described [6], at the following current increments: 0.5, 1, 2, and 6 mA. Naked plasmid DNA (100 µg of pVAX-SV0 or pVAX-SVcyt) or the carrier alone was injected as a single 150-µl bolus into the corpora of STZ-diabetic animals with the use of a 29-G needle. Age-matched control rats were also studied. A one-way analysis of variance (ANOVA) was used to determine treatment effects. Changes in intracavernosal pressure (ICP) and systemic blood pressure (BP) were recorded at each level of neurostimulation. Results were expressed as the mean $\pm$ SEM.

3. Results

3.1. STZ diabetes alters *Slo* splice variant expression in rats

RT-PCR identified two major transcripts of the *Slo* gene in age-matched control rats (≈ 0.72 and 0.46 kb; Fig. 1B). PCR products (< 0.46 kb) were shown by sequence analysis to be artifacts, and all other bands accounted for less than 5% of the major transcripts (determined by densitometry). Subcloning and sequencing indicated that the larger-molecular-weight band corresponded to a *Slo* transcript with sequence between sites I and III (SV0; depicted in Fig. 1A) and the smaller-molecular-weight band to a product without exons between sites I and III (SVcyt; Fig. 1D). As far as we are aware, the splice variant SVcyt (so named because the protein product has a cytoplasmic location; Fig. 3) has not been described previously.

Densitometric analysis of PCR products demonstrated that, in age-matched control rats, SV0 was the minor transcript, occurring at an SV0-to-SVcyt ratio of 0.03 ± 0.033 (Fig. 1B, Table 1; $N = 3$ rats). Comparison of the product generated by primers against the conserved pore region of *Slo* with that of a housekeeping gene (RPL19; ribosomal protein L19) demonstrated that transcription was not altered after 2 or 8 wk of STZ-diabetes. After 2 wk of STZ diabetes the SV0/SVcyt ratios increased by approximately 39-fold, despite little change in erectile capacity (Fig. 4). After 8 wk of STZ-diabetes, the SV0/SVcyt ratio further increased to 2.4 ± 0.15 , representing ≈ 80 -fold increase in SV0 expression.

RT-PCR analysis of splice variants expression showed that the steady-state rate of transcription of the *Slo* gene in diabetic and insulin-treated diabetic animals was similar (Fig. 1C, Table 1). However, there was a reduction in the corporal tissue SV0/SVcyt ratio in insulin-treated diabetic animals. Insulin was administered after 8 wk of STZ-diabetes, suggesting that insulin reverses the STZ-induced changes in splicing. This observation is consistent with previous work documenting that insulin can reverse STZ-diabetes induced ED in rats [16,26,27].

3.2. Human diabetic patients exhibit alternative splicing of the *Slo* transcript

Our initial observations revealed qualitatively similar observations on *Slo* transcript expression in human corporal tissue. Sequencing of the PCR products demonstrated that SVcyt and SV0 transcripts also are present in the human corporal tissue (Fig. 1C). As in the STZ diabetic rat, the ratio of SV0/SVcyt (Table 2) was less in corporal tissue excised from nondiabetic compared with diabetic patients. Specifically, the mean SV0/SVcyt ratio in diabetic patients was 2.41 and that for nondiabetic patients was 0.086 ($p < 0.001$, Student t test for unpaired samples).

3.3. SVcyt and SV0 demonstrate different electrophysiological properties in HEK cells

Functional studies to elucidate the electrophysiologic properties of SVcyt and SV0 were conducted in HEK293 cells with little or no endogenous Maxi-K channel. *Slo* expression plasmids (pVAX-SV0 and pVAX-SVcyt) were constructed from the splice variants (SVcyt and SV0). HEK293 cells were transformed with the plasmids, and protein expression was analyzed by Western blotting followed by densitometry. There was no significant difference in SV0 or SVcyt protein expression levels when normalized to the housekeeping gene GAPDH (Fig. 2A). Patch-clamp experiments in HEK293 cells stably transfected with pVAX-SV0 demonstrated the presence of single-channel events consistent with the presence of the Maxi-K channel, whereas transfection with pVAX-SVcyt did not (Fig. 2B). Specifically, electrophysiologic characterization of the cloned channel SV0 in HEK cells confirmed responsiveness to calcium (Fig. 2C), voltage sensitivity (Fig. 2D), and blockade by iberiotoxin (Fig. 2E). The corresponding I/V (current/voltage) curve had a slope conductance of 201 pS and reversal potential near the E_K (approximately $\mu 75$ mV; Fig. 2F).

3.4. SVcyt- and SV0-derived proteins have different intracellular localizations

Alternative splice forms of Maxi-K can be targeted to different cellular compartments [28]. Therefore, we determined the site of expression of the gene products of SVcyt and SV0 by fusing them to GFP and transforming HEK293 cells. As illustrated in Fig. 3, GFP alone was distributed evenly throughout the cell and GFP fused to SV0 was localized to the plasma membrane. In contrast, GFP fused to SVcyt demonstrated punctate staining within the cytoplasm. The failure of SVcyt to reach the plasma membrane is consistent with the lack of detectable membrane currents associated with expression of SVcyt in HEK293 cells.

3.5. Increased SV0 transcript may compensate for the detrimental effects of STZ diabetes on erectile capacity at early time points

As shown in Fig. 4, at the 2-wk time point, there was no significant difference between the ICP response at both submaximal (0.75 mA) and maximal (6 mA) levels of current stimulation in the diabetic and age-matched control rats, that is, they both had normal erectile function. At the 2-wk time point, there was an increase in the SV0/SVcyt ratio (Fig. 4, Table 1) to $\approx 70\%$ of the value seen at the 8-wk time point when erectile capacity was significantly diminished.

3.6. Slo splice variant SV0, but not SVcyt, can restore erectile function in STZ-diabetic rats

The lack of activity of SVcyt in the HEK293 cells may result from the absence of important cofactors in these in vitro test systems. Therefore, gene transfer studies were performed in an in vivo bioassay system [17]. Briefly, we examined the ability of plasmids expressing either SV0 or SVcyt to restore the diminished erectile capacity produced by 8 wk of STZ diabetes. A one-way ANOVA at each stimulation level revealed that SV0-treated rats had significantly higher ICP/BP ratios than the SVcyt-treated rats at all levels of stimulation, and a significantly higher mean ICP/BP value than both SVcyt and untreated diabetic rats at all but the lowest level of stimulation (ie, 0.5 mA; $p < 0.05$ in all cases; Fig. 5). In addition, coinjection of plasmids expressing both SVcyt and SV0 restored erectile function to a level indistinguishable from SV0 alone.

4. Discussion

This study reports the presence of a novel *Slo* splice variant (SVcyt) expressed in corporal tissue of nondiabetic rats. Although the splice variant SVcyt is the predominant splice form of *Slo* found in nondiabetic (ie, age-matched control) corpora, STZ-diabetes was associated with a progressive upregulation of an alternative spliceform: SV0. After 2 wk of diabetes, there was approximately a 40-fold increase, and after 8 wk, there was an 80-fold increase in SV0 compared with SVcyt expression. Our initial observations revealed qualitatively similar observations on *Slo* transcript expression in human corporal tissue. Treatment of STZ-diabetic animals with insulin restored erectile function and increased expression of SVcyt.

Furthermore, these data demonstrate that SV0 forms a functional channel in vitro and plays a physiologic role in erectile function in vivo, whereas SVcyt does not form a function channel in vitro, and its role in nondiabetic corporal tissue in vivo remains to be determined. Although the SV0 transcript is expressed at low levels in normal animals, SV0 provides a potent repolarizing current, and the amount of protein product probably accounts for the high channel activity in corporal SM cells.

Heightened contractility or impaired relaxation of corporal SM, or both, are thought to be major components of diabetes-related alterations in erectile capacity in diabetic rats and humans [4, 16,17]. This report suggests that increased SV0 transcripts may mitigate diabetes-related effects on erectile capacity at early time points in the disease process (Table 1, Fig. 4), and moreover that a plasmid expressing the SV0 transcript ameliorates the decline of erectile

capacity in the 8-wk STZ-diabetic rat. Previous studies showing an age-related decline in erectile capacity observed in the retired breeder male rat imply that altered ionic mechanisms (such as impaired K-channel-mediated hyperpolarization) may be generally involved in ED [6]. Consistent with this suggestion are recent pharmacologic studies demonstrating decreased sensitivity of human corporal tissue strips obtained from impotent diabetic patients to relaxation with K-channel modulators (ie, pinacidil [7]), and furthermore that *slo*⁻/*slo*⁻ knockout mice exhibit diminished erectile function [14].

Interestingly, STZ-diabetes-induced changes in *Slo* transcript splicing were not accompanied by alterations in the rate of *Slo* transcription. Nonetheless, exogenous transgene overexpression with an active *Slo* isoform restores erectile function (Fig. 5). These observations have stimulated the development of *hSlo*-derived gene transfer therapy for ED (hMaxi-K, which encodes the SV0 splice variant). In fact, a recently completed phase 1 clinical trial [24] suggests that the relatively long-term safety and efficacy of *Slo* gene transfer observed in preclinical studies (ie, 4–6 mo [15,17]) may have similar potential to restore erectile function in patients with organic ED (Melman et al, 2006, in press; [15]).

The mechanism by which diabetes mellitus alters *Slo* gene splicing in corporal SM cells remains to be determined. However, *Slo* transcript expression is modulated by various hormonal mechanisms [20–22], and in STZ-induced diabetic rats, glucocorticoid levels as well as glucocorticoid receptor messenger RNA (mRNA) are increased [29]. In addition reduced testosterone plasma levels and hypogonadal status have also been evidenced in STZ-diabetic rats [30]. Changes in the levels of these hormones possibly may be causally related to the alternative splicing of the *Slo* gene in corporal tissue described in this report.

Our preliminary observations (Table 2) indicate that qualitatively similar alterations in *Slo* gene splicing appear to occur in human corporal tissue. Clearly, further work on human corporal tissue is required to better substantiate the importance of these findings to the corresponding human erectile disease. Given the ubiquitous distribution and diverse physiologic roles of the Maxi-K channel, it is conceivable that altered *Slo* splicing may represent a more general tissue/cellular response observed with other medical conditions. The validation of this latter possibility will remain the province of future investigations.

5. Conclusions

Despite the diabetes-related switch in *Slo* mRNA from an inactive isoform (SVcyt) to an active isoform (SV0), there appears to be a relatively immutable upper limit on the endogenous transcription of *Slo*. We hypothesize that the switch to the functionally active splice form of the *Slo* channel that occurs within the first 2 wk of diabetes is sufficient to maintain physiologic function of corporal tissue, at relatively early stages in the diabetic disease process. However, the rate of transcription of the *Slo* gene does not change with the duration of diabetes. As such, at the 8-wk time point, we suggest that the maximally increased hyperpolarizing ability provided by switching to the SV0 splice form is no longer sufficient to compensate for other progressive pathophysiologic changes occurring with diabetes. The end result is insufficient endogenous Maxi-K channel expressed in corporal SM to maintain erectile capacity in the STZ-diabetic rat. Supplementation of the functional Maxi-K channel isoform using naked DNA delivery (ie, gene transfer) of SV0, but not SVcyt, leads to recovery of erectile function. Such observations may also have important implications in the understanding and potential treatment of the end-organ complications of diabetes mellitus in male patients.

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Fig. 1.

(A) Graphic depiction of the *Slo* gene SV0. The six commonly reported sites of alternative splicing are in roman numerals, and the sites of restriction enzymes BlnI and BsrG1 are shown relative to the primers (as boxes) amplifying the pore region or splice sites I through III. (B) An example of the analysis of splice variants expressed in the smooth muscle tissue from the corpora of age-matched control and 2-wk and 2-mo diabetic rats. Polymerase chain reaction (PCR) products were run on a 1.5% agarose gel and were visualized with ethidium bromide under ultraviolet illumination. (A total of six animals were used for each time point; 2-wk diabetic [$N = 3$], 2-wk AMC [$N = 3$], 2-mo diabetic [$N = 3$], and 2-mo AMC [$N = 3$]). (C) Example of the *Slo* splicing that occurs in the corpora cavernosa of 2-mo diabetic rats treated with insulin ($N = 2$) compared with untreated 2-mo diabetic rats ($N = 2$). (D) Sequence of the cloned PCR products in human diabetic and nondiabetic patients (bold letters). Sequencing was performed at the Albert Einstein College of Medicine Core Sequencing Facility.

Fig. 2.

(A) Western blot analysis of the expression of *Slo* splice variants in HEK293 cell lines. (B) Patch clamping to detect channels in stably transfected HEK293 cells. The bath solution was as follows: 136 mmol/l NaCl, 6 mmol/l KCl, 1 mmol/l MgCl₂, 10 mmol/l HEPES, pH 7.2; the pipette solution was as follows: 140 mmol/l KCl, 10 mmol/l NaCl, 1 mmol/l MgCl₂, 10 mmol/l HEPES, 5 mmol/l EGTA. (C–E) Electrophysiological characterization of HEK293 cell line transfected with SV0. (C) Left panel, a 20-s segment of 60-s recording of inside-out configuration at 0 potential and 0 calcium. Right panel, when 50 nmol/l Ca was introduced into the bath, the probability of two active open channels being observed increased. The dashed line indicates the closed and two open-channel current levels. (D) The voltage sensitivity of the channel. Left panel, there are few channels open at 0 voltage. Right panel, open channel activities are detected when a 50-mV potential is applied. (E) The channel was sensitive to 100 nmol/l iberiotoxin in the outside-out configuration. (F) The channel I/V (current voltage) plot.

Fig. 3. Visualization of proteins tagged with green fluorescent protein (GFP-SVcyt and GFP-SV0) or with vector alone (GFP) in HEK293 cells.

Fig. 4. Comparison of the normalized intracavernosal pressure/blood pressure ratios in streptozotocin (STZ)-induced diabetic and age-matched control rats at 2 wk and 2 mo after establishment of diabetes.

Fig. 5.

Intracavernosal pressure response to cavernous nerve stimulation in 8-wk streptozotocin (STZ)-induced diabetic F-344 rats treated with plasmid expressing different splice variants (pVAX-SV0 [$N = 5$] or pVAX-SVCyt [$N = 5$]) or with injection of equal amounts of both plasmids (pVAX-SV0 and pVAX-Svcyt [$N = 5$]) as well as untreated STZ-induced diabetic [$N = 5$] and age-matched control F-344 rats [$N = 5$].

Densitometric analysis of polymerase chain reaction products from the corpora of diabetic and nondiabetic rats, and insulin-treated diabetic rats

Table 1

	2 weeks		2 months	
	AMC	Diabetic	AMC	Diabetic + Insulin
Pore/RPL19	0.44 ± 0.12	0.47 ± 0.15	0.35 ± 0.04	0.38 ± 0.013
SV0/Svct	0.041 ± 0.03	1.61 ± 0.3	0.03 ± 0.033	2.4 ± 0.15 0.6 ± 0.16

Note: A total of 6 animals were used for each time point (2-wk diabetic [N = 3], 2-wk AMC [N = 3], and 2-mo diabetic [N = 3], and 2-mo AMC [N = 3]). In diabetic animals treated with insulin, two animals were used. Each experiment was performed in duplicate; the polymerase chain reaction products were visualized in agarose gel stained with ethidium bromide and quantitated with the use of densitometry. Densitometric readings were averaged for the six readings and the expression of the pore region normalized to the housekeeping gene RPL19.

AMC = age-matched control.

Table 2

Summary of the predominant splice variants detected in corporal tissue excised from patients

Patient no.	Age (yr)	Condition	SV0/SV1
Nondiabetic patients			
1	51	Prostate cancer/HTN	0.046
2	32	Transsexual	0.088
3	51	Peyronie's	0.035
4	66	Nondiabetic	0.032
5	75	Nondiabetic	0.230
Diabetic patients			
6	61	NIDDM	0.09
7	60	DM/HTN	5.25
8	60	NIDDM	2.7
9	46	DM	2.5
10	51	DM	2.3
11	81	NIDDM/DM/Peyronie's	2.1
12	61	DM	2.4
13	60	NIDDM/DM	2.3
14	69	DM	2.1
15	67	NIDDM	2.7
16	56	DM	2.1

DM = diabetes mellitus; HTN = hypertension; NIDDM = non-insulin-dependent diabetes mellitus.