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Erection induced by Tx2-6 toxin of *Phoneutria nigriventer* spider: Expression profile of genes in the nitric oxide pathway of penile tissue of mice

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ABSTRACT

The peptides Tx2-5 and Tx2-6, isolated from the whole venom of "armed-spider" *Phoneutria nigriventer* venom, are directly linked with the induction of persistent and painful erection in the penis of mammals. The erection induced by Tx2-6 has been associated with the activation of nitric oxide synthases. There is a scarcity of studies focusing on the outcome of Tx2-6 at the molecular level, by this reason we evaluated the gene profile activity of this toxin at the nitric oxide (NO) pathway. After microarray analyses on cavernous tissue of mice inoculated with Tx2-6 we found that only 10.4% (10/96) of these genes were differentially expressed, showing a limited effect of the toxin on the NO pathway. We found the genes *sparc*, *ednrb*, *junb*, *cdkn1a*, *bcl2*, *ccl5*, *abcc1* over-expressed and the genes *sod1*, *s100a10* and *fth1* under-expressed after inoculation of Tx2-6. The differential expressions of *sparc* and *ednrb* genes were further confirmed using real-time PCR. Interestingly, *ednrb* activates the L-arginine/NO/cGMP pathway that is involved in the relaxation of the cavernous body. Therefore the priapism induced by Tx2-6 is a consequence of a highly specific interference of this neurotoxin with the NO pathway.

1. Introduction

The armed-spider *Phoneutria nigriventer*, of the Ctenidae family, inhabits South American countries; it hunts by wandering the soil searching for preys and has an aggressive behavior against humans (Brazil, 1925). Typically, signs observed from bites of the *Phoneutria* spider include pain, hyperemia and edema of the affected region. However, young and elderly individuals bitten by the *Phoneutria* spider can also present intense tachycardia, hypertension, pulmonary edema and priapism (Bucaretchi et al., 2000, 2008). The whole venom of *P. nigriventer* is composed of distinct polypeptides and biologically active molecules, most of them neurotoxins (Richardson et al., 2006) and interferes with the function of ionic channels (Araujo et al., 1993; Corzo and Escoubas, 2003; Corzo et al., 2005; Dos Santos et al., 2002; Kalapothakis et al., 1998; Martin-Moutot et al., 2006; Richardson et al., 2006; Troncone et al., 2003); it also causes vascular damage in the central nervous system of rats that ultimately disrupts the hematoencephalic barrier (de Paula Le Sueur et al., 2003; Le Sueur et al.,

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2004). Purified toxins from the PhTx3 fraction of the *Phoneutria* venom block specifically calcium channels, thus inhibiting glutamate release, calcium uptake and glutamate uptake in synaptosoma (Cordeiro Mdo et al., 1993; Pinheiro et al., 2006). On the other hand, the PhTx2 fraction affects the voltage-dependent gating of sodium channels (Matavel et al., 2002, 2009; Richardson et al., 2006). Interestingly, neurotoxins isolated from the PhTx2 fraction induce painful and persistent penile erection (priapism), most notably in pediatric patients (Cordeiro Mdo et al., 1992; Diaz, 2004) although it may also occurs in adults alike (Bucaretchi et al., 2008). Penile erection is a complex neurovascular event controlled by chemical mediators released from the cavernous nerve terminals and the sinusoidal endothelium of erectile tissues. Nerves and endothelial cells directly release nitric oxide (NO) in the penis, where it stimulates guanylyl cyclase to produce cGMP (cyclic guanosine monophosphate) that subsequently lowers intracellular calcium levels. These events trigger the relaxation of arterial and trabecular smooth muscle cells, leading to arterial dilatation and penile erection (Agarwal et al., 2006; Napoli and Ignarro, 2003). On the other hand, phosphodiesterase 5 (PDE5) hydrolyzes cGMP and this abrogates the erection mediated by the NO/cGMP smooth muscle relaxation. Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS), it has three isoforms: inducible (iNOS); neuronal (nNOS) which is expressed in neuronal cells in the cavernous body; and endothelial (eNOS) expressed equally in smooth muscle cells in the cavernous body and endothelium (Burnett, 1997; Musicki and Burnett, 2006).

Among the different peptides already identified in the PhTx2 fraction from the venom of *Phoneutria* only Tx2-5 and Tx2-6, which share 89% of similarity in the amino acid sequence, have been observed to stimulate relaxation of the smooth muscles of the cavernous body of rabbits, rats and mice, thereby inducing the penile erection (Andrade et al., 2008; Nunes et al., 2008; Rego et al., 1996; Yonamine et al., 2004). The priapism induced by these neurotoxins has been directly linked with nitric oxide pathway in rats and mice (Nunes et al., 2008; Yonamine et al., 2004). Interestingly, priapism induced by Tx2-5 can be partially suppressed in mice pretreated with a non-selective nitric oxide synthase inhibitor (L-NAME, N[omega]-Nitro-L-arginine methyl ester hydrochloride) (Yonamine et al., 2004).

Pretreatment of rats with L-NAME inhibited penile erection after inoculation of Tx2-6, and nitric oxide levels in cells of the cavernous body are increased after the administration of the toxin (Nunes et al., 2008). Since Tx2-6 seems to be highly linked to nitric oxide production, priapism induced by this neurotoxin could involve the activity of several genes of nitric oxide pathway. In order to investigate this hypothesis, we analyzed the mRNA profile expression of genes involved in the nitric oxide pathway in mouse erectile tissue after Tx2-6 treatment. Our findings could give new insights regarding the mechanisms of Tx2-6-induced priapism, and also provide a nitric oxide pathway-guided therapy to be used in the treatment of erectile dysfunction.

2. Material and methods

2.1. Toxin

Whole venom from the "armed-spider" was obtained by electrical stimulation. The toxin was then dried and separated by chromatography on a Sephadex G50 column. The active peak was further purified by high-pressure liquid chromatography. The identity of the Tx2-6 peptide was confirmed by MALDI-TOF mass spectrometry (Ettan–Amersham Biosciences) and sequenced by Edman degradation (Edman, 1949).

2.2. Animals

Fourteen male Swiss adult mice (*Mus musculus*, Rodentia, Mammalia) between 18 and 24 weeks of age and weighing 30–35 g were provided by the Central Bioterism of the University of São Paulo Medical School (São Paulo, Brazil). The mice were maintained at our animal care facility for 1 week prior to experimental use. The experiments were approved by the local ethical board council no. 759/06 (University of São Paulo, Medical School, Brazil).

The animals were separated into two groups with each group receiving intracavernosal inoculation (icv) with the following reagents:

(i) Control group ($n = 7$) injected with 20 μl of saline solution (0.9% NaCl);
(ii) Treated group ($n = 7$) injected with 1 μg/kg of the Tx2-6 toxin diluted in 20 μl of saline solution.

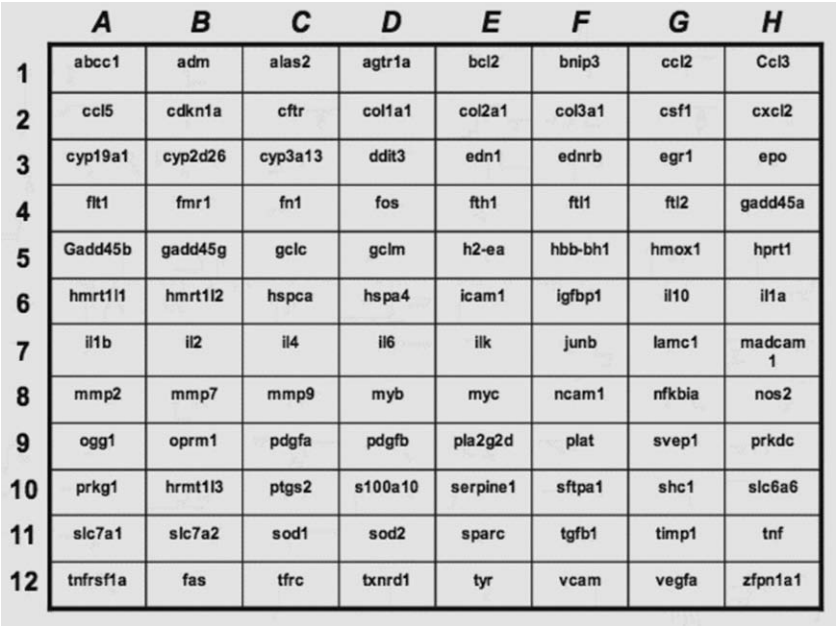
One hour after inoculation of the Tx2-6 penile erection was observed in the treated group. All animals were sacrificed after 1 h after the initiation of erection and the penile organ was removed. Each sample was immediately frozen in liquid nitrogen and stored at −80 °C until processing (usually next day).

2.3. RNA extraction

Total RNA from both the control and test groups was extracted with Trizol® (Invitrogen, CA, USA) and purified on RNeasy columns (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purity and concentration of the samples were determined by the absorbance readings at 260 and 280 nm with a spectrophotometer (Nanodrop ND-1000-Thermo Fisher Scientific, MA, USA). In order to evaluate the integrity of the extracted RNA, all samples were analyzed in agarose gel electrophoresis with formaldehyde under denaturing conditions. 18S and 28S bands were observed for each sample. The RNA samples were quantified and pooled into two groups: (1) RNA from all mice injected with Tx2-6 and (2) RNA from all mice injected with saline. In the end each group contained 5 μg of total RNA.

2.4. Synthesis of labelled probes

The cDNA probes were prepared from the 5 μg of purified RNA from each pool. Complementary DNA (cDNA) was



	A	B	C	D	E	F	G	H
1	abcc1	adm	alas2	agtr1a	bcl2	bnip3	ccl2	Ccl3
2	ccl5	cdkn1a	cftr	col1a1	col2a1	col3a1	csf1	cxcl2
3	cyp19a1	cyp2d26	cyp3a13	ddit3	edn1	ednrb	egr1	epo
4	flt1	fmr1	fn1	fos	fth1	ftl1	ftl2	gadd45a
5	Gadd45b	gadd45g	gclc	gclm	h2-ea	hbb-bh1	hmox1	hprt1
6	hmrt1l1	hmrt1l2	hspca	hspa4	icam1	igfbp1	il10	il1a
7	il1b	il2	il4	il6	ilk	junb	lamc1	madcam 1
8	mmp2	mmp7	mmp9	myb	myc	ncam1	nfkbia	nos2
9	ogg1	oprm1	pdgfa	pdgfb	pla2g2d	plat	svep1	prkdc
10	prkg1	hrmt1l3	ptgs2	s100a10	serpine1	sftpa1	shc1	slc6a6
11	slc7a1	slc7a2	sod1	sod2	sparc	tgfb1	timp1	tnf
12	tnfrsf1a	fas	tfrc	txnrd1	tyr	vcam	vegfa	zfpn1a1

Fig. 1. Location of the genes involved in the nitric oxide pathway. Position of mouse genes spotted on the surface of nylon membranes of the Mouse Nitric Oxide Signaling Pathway. For additional details see Methods.

synthesized using the RevertAid™ H Minus M-First Strand cDNA Synthesis Kit (MBI Fermentas, ON, Canada) with 5 μg of the pooled RNA and 500 pmol/ml oligo dT. The reaction was incubated at 70 °C for 5 min and immediately transferred to ice. Then, 4 μL of 5× first strand buffer, 10 mM of each dNTP, 1 μL DNAse-free water and 200 U reverse transcriptase were added to the reaction mixture. The reaction was incubated at 42 °C for 60 min. Finally, the enzyme was heated at 70 °C for 15 min to stop the reaction. The synthesized cDNA was then used as a template for linear amplification by DNA polymerase (LPR cocktail, Superarray Bioscience Corporation, MD, USA) with Biotin-16-dUTP (Roche, Mannheim, Germany) and specific primers according to the manufacturer's instructions (Superarray Bioscience Corporation, MD, USA).

2.5. Microarray membrane

The effect of Tx2-6 on gene expression was analyzed using commercially available cDNA arrays corresponding to the murine NO pathway (GEArray Q series-MM034 Mouse Nitric Oxide Signaling Pathways, Superarray Bioscience Corporation, MD, USA). Each array consisted of a nylon membrane with 450 base pairs (bp) cDNA sequences from 96 genes spotted in quadruplicate (tetraspots). These genes are involved in the mouse NO pathway, and the locations of these genes on the membrane are depicted in Fig. 1.

2.6. Hybridization and microarray analysis

The membranes were pre-hybridized for 6 h at 60 °C and then hybridized overnight at 60 °C with the denatured and biotinylated cDNA probes. The membranes were then washed twice with 2× SSC, 1% SDS and twice with 0.1× SSC, 0.5% SDS for 15 min at 60 °C. The membranes were blocked for 40 min and incubated with alkaline phosphatase-conjugated streptavidin (1:8000) for 10 min. The membranes were washed with 1× Buffer F (Superarray, MD, USA) for 20 min and the hybridized biotinylated probes were detected by chemiluminescence using the CDP-*Star* alkaline phosphatase substrate (Superarray, MD, USA). The membranes were exposed to Kodak BioMax films (KODAK, NY, USA) for periods between 1 and 20 min. Finally, the films were scanned and the images were digitized for further analysis.

2.7. Quantitative PCR

The altered gene expressions of *ednrb*, *sparc* and *sod1* were verified by real-time quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) analysis. RNA samples used in qRT-PCR were the same used to perform the microarray experiments. cDNA was synthesized using 1.5 μg of RNA. Reverse transcription was made using oligo dT 12–18 primer and SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) as recommended by the manufacturer. Each cDNA mixture was diluted 1:5 in distilled H_2O for subsequent PCR amplification.

The following primers were used: *sparc* (NCBI accession No. NM_009242) sense 5′-ACC ACA CGT TTC TTT GAG AC-3′, antisense 5′-CAT CAG AGG GAG AGA GTT CA-3′, covering a 159-bp sequence; *ednrb* (NCBI accession No. NM 007904) sense 5′-ATT GGT ATC AAC ATG GCT TC-3′, antisense 5′-TTG GCT TTG AAC TTC AGG-3′, covering a 170-bp sequence; *sod1* (NCBI accession No. NM 011434.1) sense 5′-CCA GTG

CAG GAC CTC ATT TT-3′, antisense 5′-TCC CAG CAT TTC CAG TCT TT-3′, covering a 251-bp sequence; and the control reference gene β-actin (NCBI accession No. NM 007393.3) sense 5′-CTG TGG CAT CCA CGA AAC TA-3′, antisense 5′-AGT ACT TGC GCT CAG GAG GA-3′, covering a 199-bp region. The efficiency of amplification for all primers was determined by standard dilution curves.

Cycling and fluorescence detection were performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). The reaction mixture contained 4 μl of RealMasterMix SYBR ROX (5′ Prime, Berlin, Germany), 1 μmol/μl of forward and reverse primers and 1 μl of diluted cDNA in a total volume of 10 μL. The cycling conditions were: 95 °C for 2 min and 40 cycles of [95 °C/15 s; 60 °C/15 s; 68 °C/30 s]. No template controls (NTCs = water added instead of cDNA) were conducted. Each reaction was amplified in triplicates. For normalization of the data, the mRNA expression of β-actin (housekeeping gene) was chosen as a reference gene expression level The gene expression levels in treated samples relative to the controls were calculated using the comparative C_t method ($\Delta\Delta C_t$ method) with the following formulas: $\Delta C_t = C_t$ (target) $- C_t$ (normalized, β-actin); $\Delta\Delta C_t = \Delta C_t$ test $- \Delta C_t$ control. Fold increase in the expression of specific mRNA in the treated group compared to normal controls was calculated as $2^{(-\Delta\Delta C_t)}$ (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Densitometric quantification of microarray images was performed using the quantitative software, ArrayLab (Diracom Bioinformática, São Paulo, Brazil). In order to quantify spot intensities, we used a grid tool from the software and quantified the signal and background intensities for each tetraspot on the microarray, and calculated the sum of the pixel intensities within each spot. This value represented the total amount of cDNA hybridized to the target DNA on the membrane. The four local background samples surrounding the spot were measured, and the average background was subtracted from the average signal of the tetraspots. Values less than zero were discarded (background > spot signal). In order to normalize the signal intensity between the two arrays, a global normalization method was used (Cao et al., 2002; Sasaki et al., 2001). The data filtering criteria were as follows: at least one of the spot intensities to be compared had to be more than twice the background intensity, and the spot intensity ratios had to be higher than 2.0 [$-1 < \log_2$(Control) for over-expression] or lower than -2.0 [$\log_2$(Treated) > 1 for sub-expression]. A dispersion plot containing the $\log_2$ (Control) and $\log_2$ (Treated) values was constructed using the Excel program (Excel 2007 – Microsoft) in order to visualize the distribution of the genes affected by Tx2-6 treatment.

The Mann–Whitney test was used to verify the relative quantification of the expression differences for the selected genes (*sparc*, *ednrb* and *sod1*) between the control and the Tx2-6 treated group. Differences were considered significant at *P*-value of less than 5% ($P \leq 0.05$). The MedCalc 9.3 statistical software (Mariakerke, Belgium) was used to construct the bar graphs.

3. Results

3.1. Erection induced by Tx2-6

After 35–45 min of Tx2-6 injection we observed that penile erection was present in all mice. This effect lasted for 120–140 min after inoculation. On the other hand, mice inoculated only with saline did not present any sign of penile erection.

3.2. Gene expression profile

In order to identify differentially expressed genes, we compared the images of arrays hybridized to biotin-16-UTP DNA probes that were generated from the total RNA of the control and treated groups (Fig. 2). Analyses of the control and treated membranes revealed that 32 of the 96 genes (33.3%) on the array had a detectable hybridization signal while 64 of the 96 genes (66.7%) had either only trace levels of hybridization deemed to be background or no signal at all (Fig. 2). Genes that exhibited a 2-fold or greater change in mRNA expression level in either direction between mice with Tx2-6 inoculation and control mice were considered to be differentially expressed. Following this criterion, we identified 10.4% (10/96) with differential expression. Seven of the genes were over-expressed (black dots in Fig. 3) and three of the genes were under-expressed (grey dots in Fig. 3) by the Tx2-6 treated mice compared to controls.

3.3. Characteristics of genes differentially expressed by Tx2-6

Genes identified to be differentially expressed in the inoculated mice compared to the control mice were involved in cell cycle control, apoptosis, immune system activation, free radical metabolism and signal transduction (Table 1). Among the over-expressed genes, many of them have specific cellular functions including interference with the cell cycle progression (*cdkn1a*, cyclin-dependent kinase inhibitor 1A and *junb*, jun-B oncogene), signal transduction (*ednrb*, endothelin receptor type B), activation of the immune system (*ccl5*, chemokine C–C motif ligand 5), cell adhesion and extracellular matrix remodeling (*sparc*, secreted acidic cysteine-rich glycoprotein gene), and inhibition of apoptosis (*bcl2*, B-cell leukemia). In addition, we also found that the *abcc* (ATP-binding cassette 1) gene was over-expressed in Tx2-6 treated mice, an interesting finding considering that this gene was associated with defense mechanisms against toxic compounds.

Conversely, the under-expressed genes also participated in numerous cellular functions including iron homeostasis (*fth* – ferritin heavy chain 1), dimerization of anxa2/p36 *s100a10* (S100 calcium binding protein A10) and protection against oxidative stress (*sod1* – superoxide dismutase 1).

3.4. PCR quantification

We next performed real-time PCR to validate the distinct expression levels of the *sparc*, *ednrb* and *sod1* genes as observed on the cDNA microarrays. The efficiency of amplification for the various PCR reactions as determined by the standard dilution curves (data not shown) was close

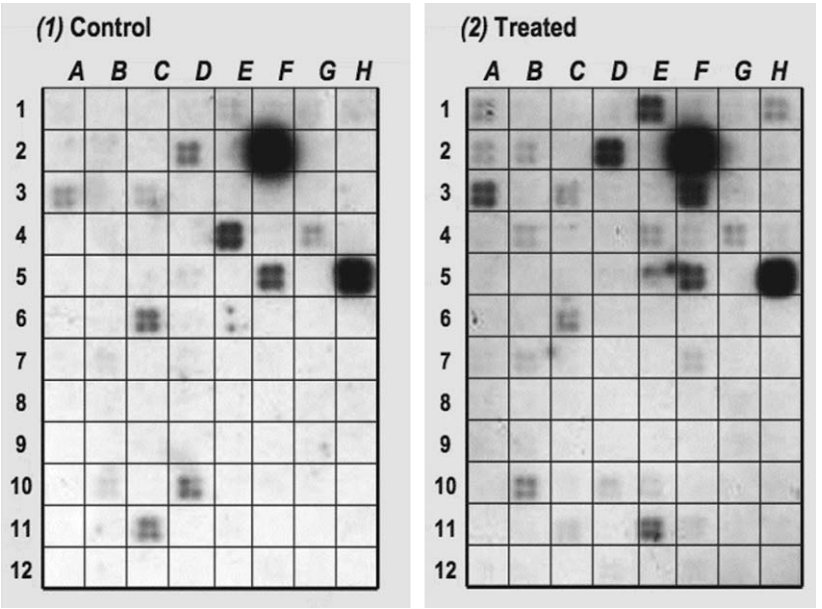


Fig. 2. Membrane microarrays containing the 96 genes. Genes involved in the mouse nitric oxide (NO) pathway were spotted in quadruplicate. Panel (1) depicts the hybridized cDNA probe of pooled RNA obtained from seven mice of the control group inoculated with saline alone. Panel (2) depicts the hybridized cDNA probe of pooled RNA obtained from seven mice (treated group) inoculated with Tx2-6. Columns (A–H) and lines (1–12) indicate the location of distinct genes involved in the NO pathway of mice according to Fig. 1.

to 100%, thereby allowing further analysis using the comparative ΔC_t method. Fig. 4 shows the normalized mRNA expressions of *sparc*, *ednrb* and *sod1* in the Tx2-6 treated group, compared with those in the control group. Values were represented as mean $\pm$ SE of at least five samples for each experimental group in triplicate wells per point. The mRNA expressions of *sparc* ($P \leq 0.001$, 1.75-fold over-expressed) and *ednrb* ($P \leq 0.001$, 1.8-fold over-expressed) were significantly higher in the treated group than those in the control group. However, the mRNA expression level of *sod1* as measured in animals treated with Tx2-6 was not statistically different than the expression of *sod1* in control animals ($P \geq 0.05$).

4. Discussion

During sexual stimulation, the NO synthesized from L-arginine by neuronal NO synthase (nNOS) is released from non-adrenergic non-cholinergic (NANC) nerve endings or from NO synthases of endothelial cells (eNOS) that were stimulated by acetylcholine released from cholinergic nerve endings (Fig. 5). NO activates, in turn, a soluble guanylyl cyclase of the smooth muscle cell that forms cyclic guanosine monophosphate (cGMP). Subsequently, in response to activation of prostaglandin receptor (EP) by prostaglandins, cyclic adenosine monophosphate (cAMP) is also produced in the smooth muscle cell by adenylyl ciclase. Both, cGMP and cAMP activate their respective protein kinases G (*i.e.* PKG and PKA, respectively) which then phosphorylate certain proteins and ion channels. As a consequence, the intake of calcium ions is decreased. Myosin light chain phosphatase (MLC-phosp) is activated by this fall in the myoplasmic calcium levels. Myosin light chain (MLC) is dephosphorylated by activated MLC-phosp, and detaches from the actin filaments causing relaxation of smooth muscle (Fig. 5). This relaxation allows the inflow of blood into the corpus cavernosum of penis

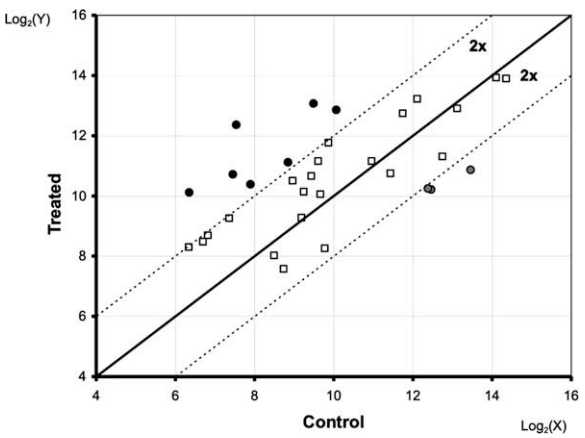


Fig. 3. Logarithmic scatter plots of the normalized fluorescence intensity values from the control and Tx2-6 treated microarrays. The position of each dot on the scatter plot corresponds to the normalized average signal intensity (log scale) of a single gene. Both the *X* and *Y*-axes indicate the normalized log-intensity signal of the spots detected on the control and Tx2-6 treated microarray membranes. The middle line indicates values that represent a treated/control ratio of 1.0 (similar levels of expression in both tissues). The upper line represents a treated/control ratio of 2.0 (2-fold greater expression in the Tx2-6 treated erectile tissue compared to control); over-expressed genes after Tx2-6 treatment are indicated as black circles. The lower line represents a treated/control ratio of 0.5 (2-fold lower expression in treated mice compared to control); the under-expressed genes are shown as grey circles.

Table 1
Differentially expressed genes in mouse erectile tissue after inoculation of Tx2-6.

Gene	Location	Fold change[a]	Gene description
Over-expressed genes			
sparc	E11	+4.24	Secreted acidic cysteine-rich glycoprotein; matricellular protein with roles in adhesion, fibrosis, angiogenesis, and ECM remodeling
junb	F7	+3.68	Jun-B oncogene; transcription factor
ednrb	F3	+3.50	Endothelin receptor type B; G protein coupled receptor for endothelins
cdkn1a	B2	+3.18	Cyclin-dependent kinase inhibitor 1A; blocks cell cycle progression
bcl2	E1	+2.71	B-cell leukemia/lymphoma 2; anti-apoptotic signaling
ccl5	A2	+2.40	Chemokine (C–C motif) ligand 5; involved in the inflammatory response
abcc1	A1	+2.19	ATP-binding cassette, sub-family C (CFTR/MRP), member 1; involved in multi-drug resistance; protection against toxic compounds
Under-expressed genes			
fth	E4	−2.67	Ferritin heavy chain 1; involved in iron homeostasis; stores iron in a soluble, non-toxic, readily available form
s100a10	D10	−2.33	S100 calcium binding protein A10; induces the dimerization of anxa2/p36
sod1	C11	−2.22	Superoxide dismutase 1, soluble; protects against oxidative stress

[a] Logarithmic base 2 expression of the genes in the treated samples relative to expression in the control samples, positive values indicate a relative increase in expression, and the negative values indicate a relative reduction in gene expression.

thus leading to erection (for review see Dean and Lue, 2005; Jin and Burnett, 2006; Maas et al., 2002).

It has been proposed that Tx2-5 and Tx2-6 induce erection by activation of NO synthases (dashed arrows in Fig. 5) (Nunes et al., 2008; Yonamine et al., 2004). This is based on the observation that half of mice treated prior to Tx2-5 inoculation with an L-NAME, a non-selective NOS inhibitor, did not present erection. Likewise, 7-nitro-indazole, a specific inhibitor of nNOS, completely reverted the effects of Tx2-5 in treated mice (Yonamine et al., 2004). In the same way, pretreatment of rats with L-NAME abolished the effect of erection induced by Tx2-6 (Nunes et al., 2008). Alternatively, Tx2-6 also could spur the potential of erection by increasing the mRNA levels of the *ednrb* and *sparc* genes that could result in increased protein products, as will be discussed below.

We analyzed the gene expression profile of erectile tissue after Tx2-6 treatment using the microarray technology. Our results showed that only 10% of the genes involved in the NO pathway were differentially expressed. Additionally, differential expressions of *sparc* and *ednrb* genes were further supported by real-time PCR results.

Then, we focused our attention on the *sparc* gene because it encodes a multi-functional protein that mediates cell–matrix interactions and cellular functions (Bradshaw et al., 1999; Schiemann et al., 2003). *Sparc* also influences a number of diverse biological processes such as vascular function. It has been shown that in osteoblasts, *sparc* promotes G protein (Gs) coupled to adenylyl cyclase to generate cyclic AMP (cAMP) (Kessler and Delany, 2007) and cAMP induce relaxation of trabecular smooth muscle (Dean and Lue, 2005). *Sparc* also interacts with the actinomyosin complex to potentially modulate the machinery involved in smooth muscle relaxation and contraction (Barker et al., 2005; Shi et al., 2007) (Fig. 5). Interestingly, erectile tissue of diabetic rats, a disease that can induce erectile dysfunction, revealed that after electrical induction of penile erection the *sparc* gene was under-expressed (Sullivan et al., 2005). This finding contrasts with our experiments that detected over-expression of *sparc* gene in penile erection induced by Tx2-6. Thus it is tempting to speculate that *sparc* gene may have an important role in penile erection.

Moreover, over-expression of *ednrb* (*endothelin receptor type B*) was also confirmed by real-time PCR. This receptor has been found on the endothelium and smooth muscle cells in both humans and animal models (Christ et al., 1995; Parkkisenniemi and Klinge, 1996). As is depicted in Fig. 5, *ednrb* is activated by endothelins (ET), a family of peptides mainly synthesized in the endothelial cell layer with potent vasoconstrictor and vasodilator properties (Filippi et al., 2003; Granchi et al., 2002). Activation of *ednrb* on endothelial cells mediates an increase in endothelial nitric oxide synthase (NOS) activity, and this effect results in the release of NO that in turn mediates relaxation of the smooth muscle responsible for erection (Christ et al., 1995; Parkkisenniemi and Klinge, 1996).

Conversely, *sod1* (*superoxide dismutase 1*) was an under-expressed gene in the erectile tissue after Tx2-6 injection. This gene encodes the superoxide dismutase enzyme, SOD

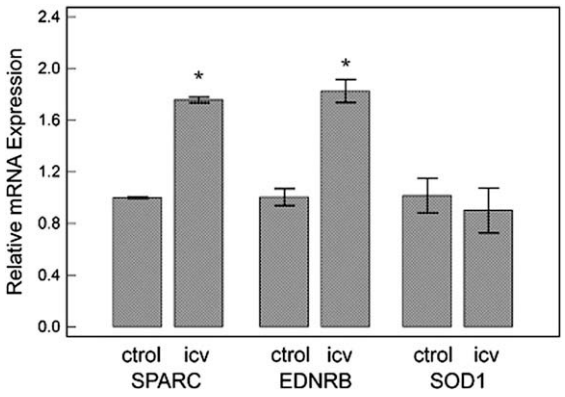


Fig. 4. Relative mRNA expression of *sparc*, *ednrb* and *sod1* genes. The intensity was measured by real-time PCR in mice inoculated with Tx2-6 and control animals inoculated with saline. Values on the *y*-axis represent mRNA levels in arbitrary units, with all results normalized to the expression of the housekeeping gene (β-actin) of the same sample. Values are means ± SE for five experiments by group and triplicate wells per point. *Sparc* and *ednrb* gene expressions were significantly higher in the treated group compared to those in the control animals. *Sod1* gene was not differentially expressed. A probability level of $^{*}P \leq 0.05$ was considered significant; ctrol: control group; icv: treated group injected intracavernously with Tx2-6 1 μg/kg.

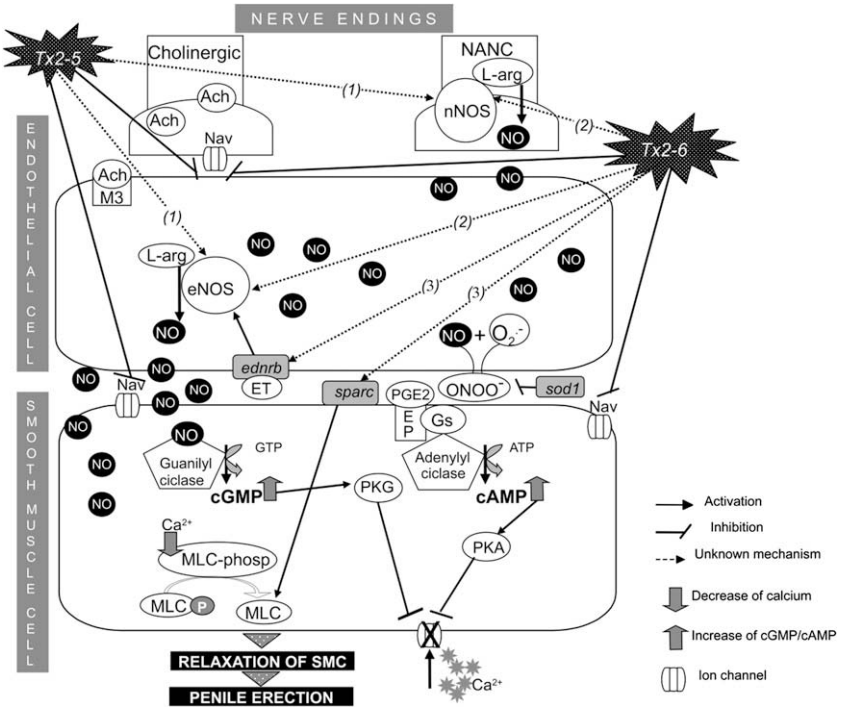


Fig. 5. Effect of Tx2-5 and Tx2-6 on the penile erection mechanism. The key molecular events associated with nitric oxide (NO) production in the corpus cavernosum are depicted in the diagram; the possible interference of Tx2-5 and Tx2-6 with specific events of the NO pathway to induce priapism is also shown. Recent studies indicate that Tx2-5 and Tx2-6 affect the penile erection mechanism: (1) Tx2-5 has a direct action on voltage-gated sodium channels (Nav) and it may also affects, by unknown mechanisms, nitric oxide synthases – nNOS and eNOS (Yonamine et al., 2004). (2) In addition to the interference with Nav channels, Tx2-6 increases nitric oxide levels, possibly through activation of nNOS and eNOS (Nunes et al., 2008). (3) Since Tx2-6 deregulates mRNA expression of *ednrb*, *sod1* and *sparc* genes, we propose that *ednrb* and *sparc* induce priapism due to the important functions they have in the mechanism of erection. Ach: acetylcholine; cAMP: cyclic guanosine monophosphate; ATP: adenosine triphosphate; Ca^{2+}: calcium ions; Cholinergic: cholinergic nerve ending; NANC: non-adrenergic, non-cholinergic nerve ending; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; nNOS: neuronal nitric oxide synthase; *ednrb*: endothelin receptor type B; *sod*: superoxide dismutase; *sparc*: secreted acidic cysteine-rich glycoprotein; EP: prostaglandin receptor; cGMP: cyclic guanosine monophosphate; GTP: guanosine triphosphate; Gs: G protein; L-arg: L-arginine; MLC: myosin light chain; MLC-phosp: myosin light chain phosphatase; M3: muscarinic receptor 3 for acetylcholine; Nav: voltage-gated sodium channel; P: phosphate group; PGE2: prostaglandin E2; PKA: protein kinase A; PKG: protein kinase G; O_2^-: superoxide; $ONOO^-$: peroxynitrite; Tx2-5 and Tx2-6: peptides from the *Phoneutria nigriventer* spider venom.

(Fig. 5), that plays a vital role in protecting the cell against oxidant agents, such as peroxynitrite ($ONOO^-$), a by-product of reaction between nitric oxide and superoxide ($O_2^{\cdot-}$) (Agarwal et al., 2006). Peroxynitrite is a highly oxidative substance, and can damage DNA, lipids and a diverse range of proteins. Thus, under-expression of the protective factor *sod1* in the erectile tissue after Tx2-6 injection would be predicted to have a detrimental effect on the cells. However, real-time PCR did not indicate that *sod1* was differentially expressed after Tx2-6 treatment. Given the specificity of real-time PCR compared to microarray analysis, we believe that PCR results provided a more accurate data and thus concluded that the mRNA levels of the *sod1* gene remained unchanged in the penile tissue in both groups.

Interestingly, among the 96 genes involved in the NO pathway of mouse that were analyzed in our study, only a small fraction of them were affected by Tx2-6. Although, penile erection as mediated by Tx2-6 may rely primarily on the NO pathway, the activity of Tx2-6 was highly specific to only a few genes in this pathway. Notably, the *ednrb* gene may have pivotal function in the penile erection induced by Tx2-6 (Fig. 5). Thus, more specific experiments should be performed in order to pinpoint the mechanism by which Tx2-6 induces penile erection and to map the repertoire of genes that may be regulated by this spider toxin.

In summary, neurotoxins Tx2-5 and Tx2-6, isolated from the venom of the armed-spider *P. nigriventer*, induce priapism in mammals (Andrade et al., 2008; Nunes et al., 2008; Yonamine et al., 2004) and the use of inhibitors of nitric oxide synthases curbs this effect (Yonamine et al., 2004). By this reason we focused specifically on genes involved on the nitric oxide pathway in mouse erectile tissue after inoculation of Tx2-6. We detected a small fraction of genes of nitric oxide pathway differentially expressed. In addition, we propose a molecular model to explain the interference of *sparc*, *sod1* and *ednrb* genes with the mechanism of the penile erection (Fig. 5). These genes have not been previously identified in the context of induction of erection by Tx2-6, and therefore represented novel candidates for further studies on the physiology of erection. A detailed understanding of the gene expression profile during penile erection induced by Tx2-6 could also provide therapeutic insights of erectile dysfunction.

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Conflicts of interest

All authors declare that there are no conflicts of interest.

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