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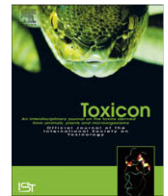

# Erection induced by Tx2-6 toxin of *Phoneutria nigriventer* spider: Expression profile of genes in the nitric oxide pathway of penile tissue of mice

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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.

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## 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

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