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Blockade of neuronal nitric oxide synthase abolishes the toxic effects of Tx2-5, a lethal *Phoneutria nigriventer* spider toxin

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Abstract

The primary goal of this study was to determine whether Tx2-5, a sodium channel selective toxin obtained from the venom of the spider *Phoneutria nigriventer*, produced penile erection by means of nitric oxide mechanism. Toxin identity was analyzed by MALDI-TOF, ES-MS and N-terminal amino acid sequencing. Pretreating mice with the non-selective nitric oxide synthase (NOS) inhibitor N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and the selective neuronal-NOS inhibitor 7-Nitroindazole (7-NI) prior to Tx2-5 i.p. (10 μg/25 g mouse) injection challenged the hypothesis above. Controls were injected with the D-isomer or DMSO or saline. Results demonstrated that L-NAME inhibited penile erections in about half the animals treated, while 7-NI completely abolished this effect. Interestingly 7-NI also abolished all the other symptoms of intoxication induced by Tx2-5, including salivation, respiratory distress and death. Tx2-5 killed all the animals of the control group and no one in the 7-NI-treated group. We conclude that (1) intraperitoneal injections of Tx2-5 induce a toxic syndrome that include penile erection, hypersalivation and death by respiratory distress or pulmonary edema; (2) pretreatment with the non-selective NOS inhibitor L-NAME reduces the penile erection and partially protects from the lethal effects of Tx2-5; (3) pretreatment with the nNOS-selective inhibitor 7-NI completely abolishes all the toxic effects of Tx2-5, including penile erection and death suggesting that nNOS is the major player in this intoxication; (4) toxins from other animals that affect sodium channels in the same way as Tx2-5 and induce similar toxic syndromes may have as a major common target, the activation of nitric oxide synthases.

Keywords: *Phoneutria nigriventer*; Spider venom; Neurotoxin; Nitric oxide; Sodium channels; Tx2-5

1. Introduction

The *Phoneutria nigriventer* spider is a wandering solitary spider of large proportions and endowed with very potent venom. Among the many different toxins described in this venom, one stands out for the toxicity and lethality. This toxin was first isolated, sequenced and named as Tx2-5 by Cordeiro and coworkers (1992). Several other studies employing this toxin or a fraction containing this toxin described the actions of Tx2-5 on voltage dependent sodium channels. The main actions observed in frog muscle sodium channels are the delay in inactivation. Sodium conductance and steady-state inactivation are shifted to more negative potentials and also a net reduction in current amplitude (Araujo et al., 1993; Matavel et al., 2002). Male mice injected with this toxin present a dramatic intoxication with two early signs: penile erection and hypersalivation, followed by death, with signs of severe respiratory distress. Severe human accidents involving this spider and scorpions as well, are characterized by lung edema, therefore, it seems plausible to assume that death is caused by lung edema. Since penile erection is triggered by central as well as peripheral mechanisms, a pharmacokinetic study employing iodinated toxin was performed in order to determine

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whether the toxin could penetrate the blood–brain barrier. Also, physiological penile erection involves non-adrenergic non-cholinergic transmission (NANC) and the mediation by nitric oxide (NO) have been demonstrated (Andersson and Wagner, 1995; Argiolas, 1994; Burnett, 1995; Lugg et al., 1995; Melis and Argiolas, 1997). Therefore, this study focused on the role of NO in the signs and symptoms of intoxication by Tx2-5. Our results showed that pretreatment with an inhibitor of n-NOS, 7-NI eliminated all the signs and symptoms of intoxication by Tx2-5, including the deadly lung edema.

2. Methods

2.1. Venom handling and Tx2-5 purification

P. nigriventer spider venom was obtained by routine methods employed in the arthropods laboratory of the Instituto Butantan, based on electrical milking. Venom was desiccated and kept at −20 °C until use. Desiccated venom (100 mg) was dissolved in 3 ml of cold 100 mM ammonium formiate buffer, pH 6.0 and centrifuged at 10,000*g* for 5 min to remove solids and submitted to chromatographic separation in a Sephadex G50-f column monitored by UV at 280 nm. The active peak was further purified by RP-HPLC using a micro-RPC C2-C18 Pharmacia column eluted by a gradient of 0.1% Trifluoroacetic acid and acetonitrile 25–100% in 35 min. Details of the purification procedure are similar to those described elsewhere (Troncone et al., 1995). Purification of Tx2-5 was accompanied by screening for the typical penile erection. The identity of Tx2-5 was confirmed by MALDI-TOF mass spectrometry (Ettan–Amersham Biosciences) and the typical 5116 Da was observed. Also, a sample was sequenced by regular Edman degradation in a Shimadzu PPSQ/23 sequencer and the five N-terminal amino acids corresponded to the expected sequence, except for the third residue, a cysteine in the original sequence, which is undetectable by this method.

2.2. Pretreatment with NOS inhibitors

Forty adult male Balb-C mice weighting 25 g were assigned to five groups of eight animals each. Control groups for N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) treatment received saline or 50 mg/kg of the inactive isomer N_{ω}-Nitro-D-arginine methyl ester hydrochloride (D-NAME) i.p., 24 and 0.5 h before toxin challenge. Experimental group received 50 mg/kg L-NAME in the same time schedule. A single dose of 25 mg/kg i.p. of 7-Nitroindazole (7-NI), dissolved in dimethylsulfoxide (DMSO), was injected 25 min before toxin challenge while 0.1 ml DMSO i.p. was used as control. All mice were challenged with 10 μg of Tx2-5 i.p. and observed for 60 min. Animals were checked for penile erection, salivation, survival and general behavior by three observers. Penile erection was considered positive when the penis could be exposed completely. All drugs were obtained from Sigma and reagents were of analytical or HPLC grade.

2.3. Statistics

Statistical analysis employed Graph Pad Prism software. ANOVA and contingence table evaluated groups. Significance of differences was determined by two-tailed Chi-square (X^2) test applying Yates correction.

3. Results

3.1. Blockade of NOS

Pretreatment of mice with 7-NI prevented the toxic action Tx2-5. The classical signs of intoxication like penile erection, salivation and death were not detected in this group. On the other hand, L-NAME showed a limited but significant protection with a small number of animals presenting penile erection and salivation but still four animals died. Among the control groups injected with saline, D-NAME and DMSO prior to toxin challenge, we observed 5, 8 and 8 deaths, respectively, and all the animals showed, salivation, respiratory distress and penile erection except for the saline-pretreated group where only six mice showed penile erection. Results are presented in Fig. 1.

4. Discussion

While penile erection, at a first glance seems not to be life threatening, respiratory distress and possibly, pulmonary edema are. Respiratory distress and pulmonary edema

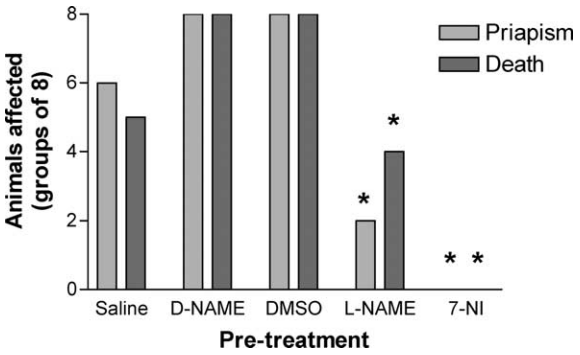

Fig. 1. Results of pretreatment with control solutions (saline, D-NAME or DMSO) and NOS inhibitors (L-NAME and 7-NI) on the toxic effects of 10 μg/25 g mouse i.p. Bars represent the number of animals presenting the symptoms of penile erection or death during the 60 min of observation. All groups had eight mice. The star represents statistical difference ($p \leq 0.05$) compared to the respective control group.

have been reported in scorpion venom intoxication but the mechanism involved in this complex and severe symptom is still obscure. Toxins acting on sodium channels seem to play a role in this symptom (Bawaskar and Bawaskar 1998; Gueron et al., 1992; Mahadevan 2000). Most of the cases of human intoxication by *Phoneutria* venom are of mild or moderate consequences involving intense local pain and edema but severe and fatal cases seem to involve pulmonary edema as well (Bucaretchi et al., 2000). The primary goal of this study was to determine whether Tx2-5, a sodium channel selective toxin, produced penile erection by means of nitric oxide mechanism. Pretreating mice with the non-selective NOS inhibitor L-NAME and the selective nNOS inhibitor 7-NI prior to Tx2-5 injection challenged this hypothesis. Results demonstrated that L-NAME inhibited penile erections in about half the animals treated while 7-NI completely abolished this effect. Much more interestingly 7-NI also abolished all the other symptoms of intoxication induced by Tx2-5, including salivation, respiratory distress and death. Tx2-5 killed all the animals of the control DMSO-treated group and no one in the 7-NI-treated group.

The participation of NO in pulmonary physiology has been clearly demonstrated and reviewed (Lee et al., 2001; Nevin and Broadley, 2002). Human and guinea-pig airway tissue seem to react in similar ways regarding NO release in response to acetylcholine, while rat airway tissue reacts to NO by inhibiting acetylcholine release. In guinea-pig lung, the NANC transmitter involved in NO triggering seems to be the vasoactive intestinal neuropeptide (VIP). Therefore, the consequences of pulmonary NO activation seem to present important species differences. The toxicity of Tx2-5 is clearly species-dependent. As reported in the early studies involving this toxin, priapism is clearly seen in mice, dogs and humans, while rats and rabbits fail to develop this symptom. Toxicity seems to go in the same fashion, with rats and rabbits much less sensitive to the crude venom than mice (Schenberg and Lima, 1966). In a previous experiment we tested Tx2-5 toxin in rats under electroencephalographic recording to detect convulsions and concentrations high enough to kill a rat failed to induce penile erection or convulsive EEG while salivation, ataxia, muscle twitches and death were observed (three rats tested—our unpublished results). Therefore, we believe that the lethality of Tx2-5 is strongly related to pulmonary NO, in the same way as that caused by scorpion sodium channel-selective toxins.

Crude *P. nigriventer* venom induced cavernosal relaxation that could be prevented by L-NAME and bradykinin B2 receptor antagonist (Antunes et al., 1993). The authors suggested that bradykinin could be the NANC agent involved in this effect. Earlier, the same authors isolated a fraction of this venom that was called Fraction XIII, responsible for increased vascular permeability in rabbit skin (Antunes et al., 1993; Lopes-Martins et al., 1994). Later, this group isolated and partially sequenced a polypeptide called PNV4 of about 17 kDa that induced rabbit cavernosal stripes relaxation (Rego et al., 1996). This peptide is different from Tx2-5. In our study N-terminal sequencing and mass spectrometry confirmed the identity of Tx2-5. A fraction of *P. nigriventer* called PhTx2 containing Tx2-5 and Tx2-6 among other peptides, was demonstrated to inhibit sodium channel inactivation (Araujo et al., 1993). Interestingly, despite the strong sequence homology among the PhTx2 toxins, these authors stated that in a preliminary test only Tx2-6 showed the sodium channel inactivation inhibition (Cordeiro et al., 1992). In a subsequent paper though, these authors stated that toxins in PhTx2 fraction, including Tx2-1, Tx2-5 and Tx2-6, had similar effects on sodium channel (Araujo et al., 1993). Yet, these authors used to inject the toxin intracerebroventricularly (i.c.v.) where Tx2-5 was the most toxic, and reported the same syndrome but failed to report penile erection. In a previous publication though, these authors did report penile erections (Rezende Junior et al., 1991).

Whether Tx2-5 induces penile erection by central or peripheral actions is still debatable. Trying to answer this question we studied the transcription of the c-fos gene as a measure of brain activation in mice intraperitoneally injected with Tx2-6 (Troncone et al., 1998). Brain areas with high c-fos transcription included the Supraoptic nucleus (SO), Paraventricular Hypothalamic nucleus (PVN), Vagal Motor nucleus, area postrema and few thalamic nuclei. Some of these brain structures are related to penile erection and, more interestingly, they are also NO-rich structures (Andersson and Wagner, 1995; Argiolas, 1994; Giuliano and Rampin, 2000; Melis and Argiolas, 1997). Whether these activations correspond to a direct action of the toxin on central nuclei or a reflexive effect attributed to the penile condition primed peripherally, is uncertain. Our results on the biodistribution of Tx2-6 (and possibly Tx2-5) suggest brain access of this toxin and support both central and peripheral effects (Yonamine et al., 2004) but a definitive conclusion demands more specific experiments.

Priapism may also be induced by scorpion toxins (Bawaskar and Bawaskar 1998; Gueron et al., 1992; Mahadevan 2000). In a recent study selective activators of sodium channels were screened in rabbit cavernosal relaxation to determine which site of the sodium channel would be related to the erectile function. The authors concluded that toxins/drugs active at sites 2, 4 and 5 are more related to cavernosal relaxation (Fernandes et al., 2003). The same group isolated and sequenced an alpha-scorpion toxin capable of relaxing rabbit cavernosal tissue. This toxin acts at site 3 of sodium channels and implicates this site in penile erection (Teixeira et al., 2003). Tx2-5 was described as a site 3 toxin, according to its effects on sodium channels (Araujo et al., 1993). Therefore, sodium channel activation seems to be the common feature among the toxins that induce NO release and are involved in penile function.

The results presented here and previous studies allow us to conclude that (1) intraperitoneal injections of Tx2-5 induce a toxic syndrome that include penile erection, hypersalivation and death by respiratory distress and

probably pulmonary edema; (2) pretreatment with the non-selective NOS inhibitor L-NAME reduces the penile erection and partially protects from the lethal effects of Tx2-5; (3) pretreatment with the nNOS-selective inhibitor 7-NI completely abolishes all the toxic effects of Tx2-5, including penile erection and death suggesting that nNOS is the major player in this intoxication; (4) toxins from other animals that affect sodium channels in the same way as Tx2-5 and induce similar toxic syndromes may have as a major common outcome, the activation of nitric oxide synthase and represent an effective therapeutic alternative for severe spider and scorpion intoxication. To have a complete picture of the NO-related effects reported here further experiments on the effects of cyclic guanosine monophosphate, phosphodiesterase-5 and guanylyl cyclase on the Tx2-5 intoxication should be performed, as well as on the inactivation of sodium channels.

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