Toxicon:X 19 (2023) 100159

Contents lists available at ScienceDirect

Toxicon: X

journal homepage: www.journals.elsevier.com/toxicon-x

Short Communication

Vasodilator activity of *Poecilotheria ornata* venom involves activation of the NO/cGMP pathway and inhibition of calcium influx to vascular smooth muscle cells

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ARTICLE INFO

Handling Editor: Denise Tambourgi

Keywords:
Tarantula venom
Poecilotheria ornata
Vasodilation
Rat aorta
Nitric oxide
Calcium

ABSTRACT

Tarantula venoms may be a natural source of new vasodilator components useful in pharmacological research. Moreover, biological function data of the venoms are important to enhance the knowledge about the biodiversity and evolution of these species. The present study aims to describe the vasodilatory activity induced by the venom of *Poecilotheria ornata* on isolated rat aortic rings. This venom induced a vasodilator activity that was significantly reduced after incubation with L-NAME or ODQ. Measurements of nitrite concentrations on rat aorta homogenates showed that the venom significantly increased the basal levels. Moreover, the venom attenuates the contraction induced by calcium. These results suggest that *P. ornata* venom contains a mixture of vasodilator components that act through the activation of the nitric oxide/cGMP pathway, as well as, through an endothelium-independent mechanism that involves the calcium influx into vascular smooth muscle cells.

1. Introduction

Spiders belonging to the Theraphosidae family, also known as tarantulas, produce venoms composed mainly of peptide toxins that target ion channels of the nervous system (Escoubas and Rash, 2004; Klint et al., 2012). However, some studies suggest that spider venoms also contain vasodilator compounds, most of them acting by endothelium-dependent mechanisms that involve activation of nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) as the primary pathway (Weinberg et al., 2002; Lee et al., 2007; Rattmann et al., 2008; Horta et al., 2013; Ma et al., 2018).

In a previous study, it was found that the venom of the tarantula *Poecilotheria regalis* induces a vasodilatory activity on isolated rat aortic rings, probably triggered by the activation of the nitric oxide (NO)/cGMP pathway (Díaz-Peña et al., 2019). In a subsequent study, it was described a subfraction obtained from the venom of *P. regalis* that contains inhibitor cystine knot (ICK) peptides and induce vasodilation through the suppression of calcium influx into the vascular smooth muscle (Díaz-Peña et al., 2023).

Tarantula venoms of the genus *Poecilotheria* are considered as theraphosids of medical importance (Ahmed et al., 2009; Fuchs et al., 2014; Alsultan et al., 2023), and may be an interesting source in the bio-prospecting of new vasoactive compounds useful in pharmacological research. In addition, biological function data of the venoms are important to enhance the knowledge about the biodiversity and evolution of these species. Therefore, this short communication aims to describe the vasodilatory activity induced by the venom of *Poecilotheria ornata* on isolated rat aortic rings.

Abbreviations: Ach, acetylcholine; ADP, adenosine diphosphate; BCA, bicinchoninic acid; cGMP, cyclic guanosine monophosphate; EC_{50}, half-maximal effective concentration; EDTA, ethylenediaminetetraacetic acid; eNOS, endothelial nitric oxide synthase; E_{max}, maximum effect; HPLC, high-performance liquid chromatography; L-NAME, Nω-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1*H*-[1,2,4] oxadiazolo[4,3-*a*]quinoxalin-1-one; PBS, phosphate-buffered saline; Pov, *Poecilotheria ornata* venom; TEA, tetraethylammonium.

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https://doi.org/10.1016/j.toxcx.2023.100159
Received 9 March 2023; Received in revised form 1 May 2023; Accepted 15 May 2023
Available online 20 May 2023

2. Methods

2.1. Animal handling

Male Wistar rats, weighting 200–250 g, were obtained from Bioterio of the Universidad Nacional Autónoma de México (UNAM). All experiments were performed according to the Mexican Official Standard NOM-062-ZOO-1999 for the production, care, and use of laboratory animals. Additionally, care and use of the animals were also approved by the bioethics committee of Facultad de Química of the Universidad Autónoma de Querétaro, registered as CBQ20/029.

2.2. Venom extraction

Adult female spiders used in the present study were captive-bred at the facilities of the Arachnidarium of the Universidad Autónoma de Querétaro (UAQ), licensed as INE/CITES/DGVS-CR-IN-0619-QRO/00). Venom was obtained by electrical stimulation in the chelicerae of anesthetized animals, according to a previously reported method (Rocha-e-Silva et al., 2009) with some modifications (Díaz-Peña et al., 2019). The venom from various specimens was pooled and the protein content was determined by means of bicinchoninic acid (BCA) assay (Smith et al., 1985) using a QuantiPro BCA assay kit (Sigma, St. Louis, Missouri, USA; Cat #QPBCA).

2.3. Vasodilatory activity

Vasodilatory activity was assayed in rat aortic ring preparations via organ bath tension measurements by a Grass FT03 force-displacement transducer, connected to a Grass 7D polygraph, according to a previous report (Díaz-Peña et al., 2019). After the equilibration time, a contraction was then induced using 1 μM phenylephrine hydrochloride (Sigma, St. Louis, Missouri, USA; Cat # PHR1017). Once this contraction had become a sustained action, different increasing cumulative concentrations of the venom were added to the organ bath (0.1, 0.316, 1, 3.16, 10, 31.6, 100, and 200 μg protein/ml). The tension responses were expressed as percentage of vasodilation of the initial contraction achieved by phenylephrine.

To determine endothelium dependence of vasodilatory activity, the endothelium of the rat aorta was removed by an *in-situ* perfusion with 5 ml saline solution containing 0.2% deoxycholic acid. The absence of endothelium was confirmed by the dilatory response (≤10%) induced by acetylcholine (1 μM) in phenylephrine-precontracted aortic rings.

To determine the influence of the NO/cGMP pathway, intact endothelium aortic rings were incubated 20 min before contraction, with 1 μM Nω-nitro-L-arginine methyl ester (L-NAME) (Sigma, St. Louis, Missouri, USA; Cat #N5751) or 1 μM 1*H*-[1,2,4] oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (Sigma, St. Louis, Missouri, USA; Cat #O3636) in separate assays.

Measurements of the concentrations of nitrite in rat aorta homogenates were made to determine the activity of nitric oxide synthase (NOS). First, the aorta segments were incubated with the venom of *P. ornata* (40 μg/ml) for 30 min, acetylcholine (50 μg/ml) was used as a positive control in this assay. Then, the aorta segments were homogenized in phosphate buffer solution 100 Mm (pH 7.4) in the presence of a protease inhibitor (Sigmafast protease inhibitor cocktail tablets, EDTA free). The homogenates were centrifuged at 16,945×*g* for 10 min at 4 °C. The supernatants were recovered, and their protein concentration was determined by the Bradford method (Bradford, 1976). Nitrite concentrations of the supernatants were measured employing a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical. Item: 780001. Batch: 0584154). Results were expressed as μmol/mg of homogenate protein.

The influence of extracellular Ca^{2+} in the vasodilatory activity of the venom was determined using calcium-depleted Krebs-Henseleit solution in non-precontracted aortic rings. Then, the contraction induced by $CaCl_2$ (10 mM), in the presence of *P. ornata* venom (100 μg protein/ml) or verapamil (1 μM) (Sigma, St. Louis, Missouri, USA; Cat #V4629), was evaluated.

To determine the role of different potassium channels in the venom-induced vasodilation, aortic rings were preincubated with 1 mM tetraethylammonium iodide (TEA) (Sigma, St. Louis, Missouri, USA; Cat # 235938) 20 min before phenylephrine contraction; and then was evaluated the cumulative concentration-response curve of the *P. ornata* venom.

In addition, some experiments were performed to obtain information on the stability and biochemical characteristics of the vasodilatory components of the venom. For these experiments, 20 min before its evaluation, the venom (10 μg protein/ml) was incubated in a boiling bath solution in the presence (reduced venom) or absence (denatured venom) of 0.05% β-mercaptoethanol (Bio-Rad, Hercules, CA, USA; Cat # 161–0710). The same concentration of β -mercaptoethanol was also evaluated as a control.

2.4. Data analysis and statistics

All experiments were assayed using rat aortic rings obtained from different animals (n = 3 6), all data follows a normal distribution. Data analysis and statistics were performed in Prism 5.0 (GraphPad Software, Inc., USA) by fitting log-concentration response curves using non-linear regression analysis. Half-maximal effective concentration (EC_{50}) and maximum effect (E_{max}) values were estimated the curves using non-linear regression analysis and expressed as the mean with 95% confidence and ±standard error of the mean (S.E.M.), respectively. Statistical comparisons of the curves were done using two-way ANOVA, followed by a Bonferroni post-hoc test. Multiple comparisons between three or more groups were analyzed by a one-way ANOVA, followed by a Dunnett's test. In all experiments, statistical significance is set at $p < 0.05$. Cohen's d was calculated as a measure of the effect size, a value of at least 0.8 was considered to be a large effect, 0.5 was medium, and 0.2 was small (Streiner and Norman, 2004).

3. Results and discussion

P. ornata venom induced a rapid and long-lasting concentration-dependent vasodilatory activity. The EC_{50} value was 3.60 (1.64–7.90) μg protein/ml with an E_{max} value = 72.54 ± 4.36% on phenylephrine-precontracted rat aortic rings (Fig. 1A). This vasodilator activity was slightly reduced in experiments carried out with endothelium-denuded aortic rings [EC_{50} = 5.47 (1.97–15.12) μg protein/ml with an E_{max} value = 59.76 ± 4.95%], statistical comparison of the curves, using raw data, showed significant differences between the treatment (two-way ANOVA: p = 0.0049, F = 8.394), Bonferroni post-test did not show differences at any concentration ($p > 0.05$); the effect sizes were calculated using Cohen's d statistic and showed small effect size at a concentration of 0.1 μg/ml (d = 0.26), medium effect sizes at concentrations of 0.316–3.1 μg/ml (d = 0.56, 0.65, and 0.79), and large effect sizes at concentrations of 10–200 μg/ml (d = 0.95, d = 1.11, d = 1.20, and d = 0.89). These results suggests that vascular endothelium is partially required for the vasodilatory effect. It is important to mention that under control conditions, the vehicle did not reduce the contraction induced by phenylephrine. Acetylcholine (Ach) was used as a positive control in endothelium-intact [EC_{50} = 0.025 (0.019–0.032) ng/ml with an E_{max} value = 60.29 ± 0.87%] or endothelium-denuded rat aortic rings (Fig. 1B).

Evaluation of the vasodilatory activity of the venom in the presence of L-NAME or ODQ showed an uncompetitive antagonistic effect in both cases (Fig. 1C), experiments with L-NAME showed a EC_{50} value = 16.31 (6.78–39.28) μg protein/ml with an E_{max} value = 40.69 ± 4.55%, whereas the experiments with ODQ showed a EC_{50} value = 6.70 (1.74–25.83) μg protein/ml with an E_{max} value = 47.77 ± 5.43%. Statistical comparison of the curves, using raw data, showed significant differences between the treatment with both L-NAME and ODQ (two-

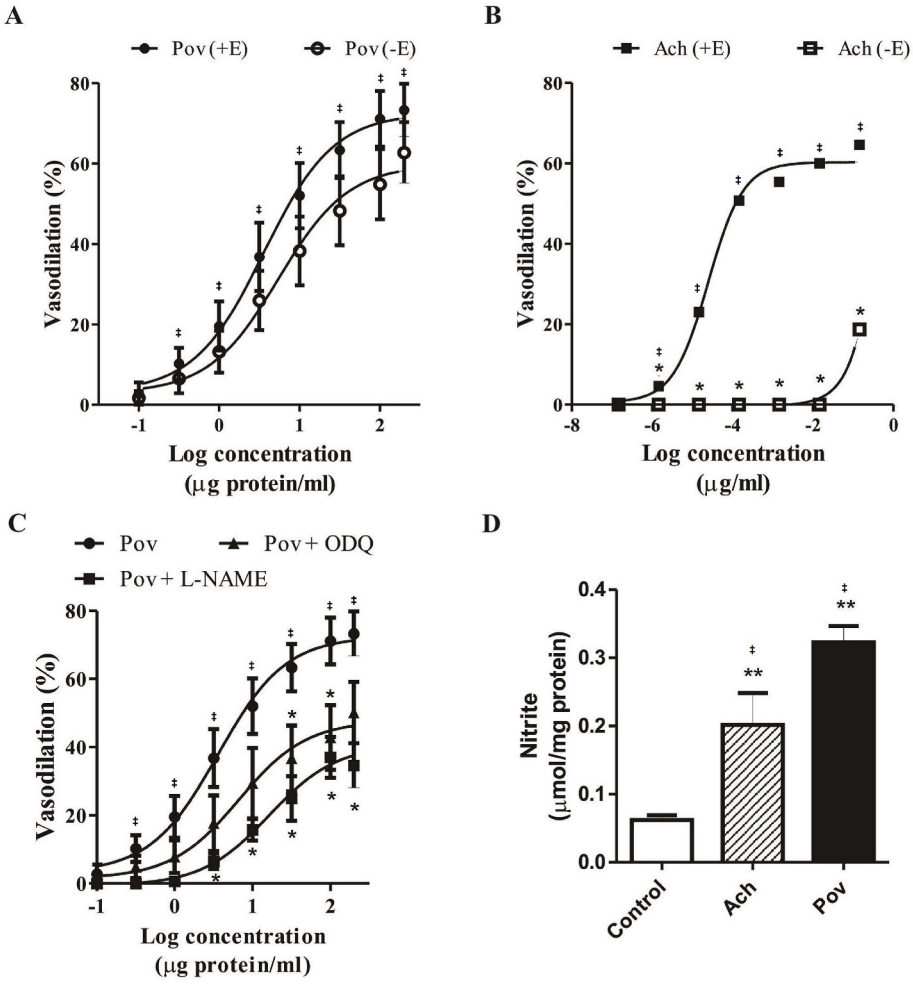

Fig. 1. A) Concentration-response curves showing the vasodilator activity of *P. ornata* venom (Pov) in endothelium-intact (E+) or endothelium-denuded (E−) rat aortic rings (n = 6). **B)** Vasodilator activity induced by acetylcholine (Ach), used as a positive control (n = 3). **C)** Concentration-response curves showing the vasodilator activity of Pov in the presence of 1 µM L-NAME (n = 5) or 1 µM ODQ (n = 3). **D)** Total nitrite concentration in rat aortic homogenates (n = 4) in the absence (control) or presence of Pov (40 µg/ml) or Ach (50 µg/ml). Values are expressed as means ± S.E.M. *Statistical significance ($p < 0.05$), two-way ANOVA, followed by a Bonferroni post-hoc test. ** Statistical significance ($p < 0.05$), one-way ANOVA, followed by a Dunnett's test. ‡ Cohen's $d > 0.2$.

way ANOVA: $p < 0.0001$, $F = 33.35$); Bonferroni post-test showed significant differences at concentrations of 3.16–200 µg/ml ($p < 0.01$) in the case of L-NAME, and at concentrations of 31.6 and 100 µg/ml ($p < 0.05$) in the case of ODQ; the effect sizes were large at all concentrations in both cases (Cohen's $d > 0.8$). L-NAME avoids NO production by inhibiting nitric oxide synthase (eNOS) in vascular endothelium, while ODQ prevents cGMP increase by inhibiting guanylate cyclase in vascular smooth muscle cells (Abdallah et al., 2020), these results suggest that the venom from *P. ornata* induces vasodilation at least partially through a NO/cGMP pathway. Nitrite concentration significantly increased with respect to basal nitrite levels when aortic segments were incubated in the presence of the venom of *P. ornata* (One-way ANOVA: $p = 0.0007$, $F = 18.16$; Dunnett's test: $q = 6.02$; Cohen's $d = 10.4$), these levels were higher than that induced by acetylcholine (Fig. 1D). Total nitrites are the stable end products of the reaction of NO with molecular oxygen (Nagpure and Bian, 2016), thus, these results shown that the venom of *P. ornata* contains components that increase the production of NO through the activation of NO/cGMP pathway.

On the other hand, *P. ornata* venom significantly reduced calcium-induced contractions of endothelium-intact rat aortic rings, Ca^{2+} contractions were reduced by 70% in the presence of the venom (one-way ANOVA: $p < 0.0001$, $F = 36.02$; Cohen's $d = 7.08$). Verapamil, a L-type Ca^{2+} channels inhibitor (Martinez et al., 2018) was used as a positive control (Fig. 2A). These results suggest that the vasodilatory activity of the venom also involves the suppression of calcium influx to vascular smooth muscle cells. Ion channels provide the main source of Ca^{2+} that determines vascular tone (Tykocki et al., 2017), the presence of toxins that inhibit L-type voltage-gated calcium channels in tarantula venoms has been reported (Dutra et al., 2008; Ono et al., 2011; Klint et al., 2014). In addition, vasodilatory concentration-response curve of the venom was slightly modified in the presence of TEA (Fig. 2B) [EC_{50} = 9.37 (5.50–15.99) µg protein/ml with an E_{max} value = 77.93 ± 3.95%], a Ca^{2+}-activated and voltage-activated potassium channels blocker (Abdallah et al., 2020); statistical comparison of the curves using two-way ANOVA showed significant differences between the treatment ($p = 0.0462$, $F = 4.15$), Bonferroni post-test did not show differences at any concentration ($p > 0.05$); the effect sizes calculated using Cohen's d statistic showed large effect sizes at concentrations of 0.1–31.6 µg/ml ($d = 0.82, 2.12, 1.90, 1.33, 0.87$, and 0.88), small effect size at a concentration of 100 µg/ml ($d = 0.30$) and large effect size at concentration of 200 µg/ml ($d = 0.95$). These results suggest that the venom of *P. ornata* also contains toxins that modulate potassium channels, the presence of potassium channel toxins in tarantula venoms has been reported (Escoubas and Rash, 2004; Swartz, 2007).

Finally, incubation of the venom of *P. ornata* in a boiling bath for 20 min, resulted in a slight significant change in its vasodilatory action (One-way ANOVA: $p = 0.0067$, $F = 7.426$; Dunnett's test: $q = 2.04$; Cohen's $d = 2.22$), suggesting that this activity mainly involves thermostable components. Moreover, incubation of the venom in a boiling bath solution with β-mercaptoethanol resulted in a significantly reduced vasodilatory activity (One-way ANOVA: $p = 0.0067$, $F = 7.426$; Dunnett's test: $q = 3.68$; Cohen's $d = 4.22$) (Fig. 2C). These results suggests that disulfide rich peptides might be responsible for the vasodilatory action of the venom. Most peptide neurotoxins isolated from venoms of tarantulas exhibit a specific folding comprised of stabilizing disulfide bonds that confer them stability at high temperatures but not in

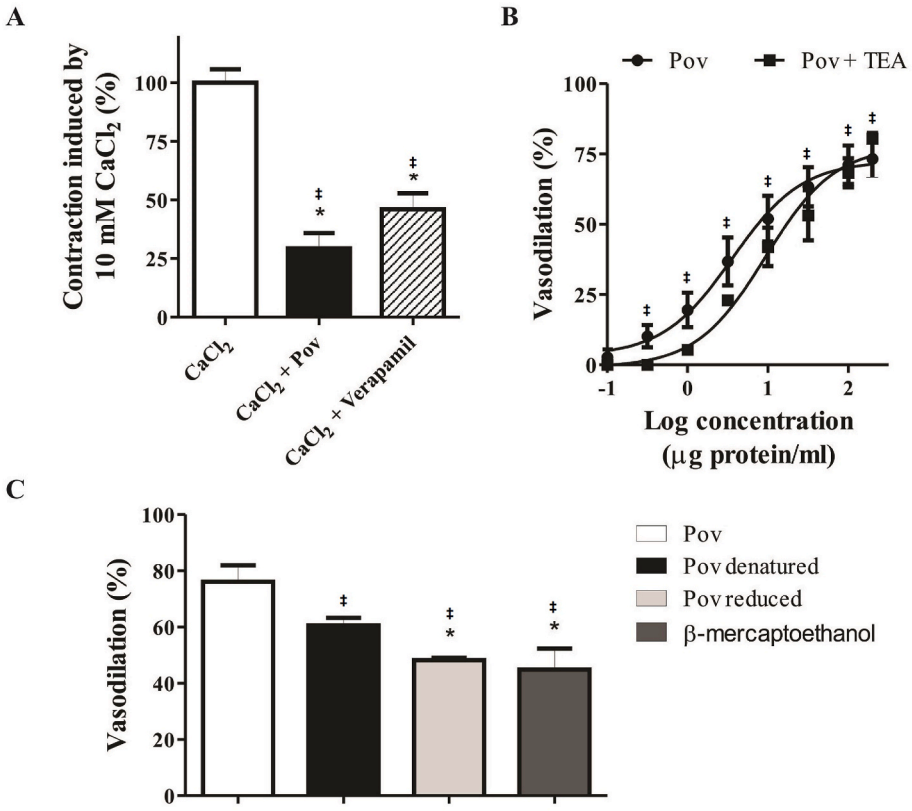

Fig. 2. A) Reduction of calcium-induced contraction [$CaCl_2$ (10 mM) = 100% contraction (n = 7)] by 100 µg protein/ml of *P. ornata* venom (Pov, n = 4) or 1 µM of verapamil (n = 5) in endothelium-intact rat aortic rings. **B)** Concentration-response curves showing vasodilator activity of Pov (n = 6) in the presence of 1 mM tetraethylammonium iodide (TEA, n = 3). **C).** Vasodilator activity induced by 100 µg protein/ml of Pov under the following experimental treatments: denatured by incubation in a boiling bath for 20 min (Pov denatured, n = 3) and reduced by incubation in a boiling bath for 20 min in the presence of 0.05% β-mercaptoethanol (Pov reduced, n = 3). β-mercaptoethanol without venom was evaluated as a control (n = 3). * Statistical significance ($p < 0.05$), one-way ANOVA, followed by a Dunnett's test. ‡ Cohen's d > 0.2.

reductive conditions (Herzig and King, 2015).

In summary, the results of the present study suggest that the venom of *P. ornata* contains a mixture of vasodilator components that act via different mechanisms. Some components of the venom induce vasodilation by modulation of calcium influx on vascular smooth muscle cells, and some other components act via the activation of the NO/cGMP pathway. These results enhance the knowledge about the biological function of the venoms of *Poecilotheria* spiders and are useful for future comparative studies.

Funding

This research did not receive any specific grant funding agencies in the public, commercial, or not-for-profit sectors.

Ethical statement

All experiments were performed according to the Mexican Official Standard NOM-062-ZOO-1999 for the production, care, and use of laboratory animals. Additionally, care and use of the animals were also approved by the bioethics committee of Facultad de Química of the Universidad Autónoma de Querétaro, registered as CBQ20/029.

CRediT authorship contribution statement

Enrique de Jesus-López: Data curation. **Luis Cuéllar-Balleza:** Methodology. **Luis Fernando Díaz-Peña:** Writing – original draft. **Francisco J. Luna-Vázquez:** Data curation, Methodology. **César Ibarra-Alvarado:** Methodology. **Alejandro García-Arredondo J:** Conceptualization, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

de Jesús-López E. and Díaz-Peña L.F. acknowledge scholarships from the National Council of Science and Technology (CONACYT), Mexico.

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