Endothelium-Dependent Relaxation of Rat Mesenteric Arterial Rings by a *Phoneutria nigriventer* Venom Fraction

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ABSTRACT—*Phoneutria nigriventer* spider venom has been described as acting on several cardiovascular sites. In the present paper, a semi-purified fraction of this spider venom was studied to observe any contractile or relaxing effect in rat mesenteric arterial rings (MAR). Spider venom was first fractionated by gel filtration and subsequently by gradual isocratic steps in 0.1% trifluoroacetic acid. The first fraction of this last fractionation step is studied in the present paper and due to its main effect, it was named NORF (nitric oxide releasing fraction). No direct contractile effect was induced by NORF in relaxed MAR, suggesting no NORF-induced neurotransmitter release in this preparation. No significant influence of NORF was observed on concentration-response curves to phenylephrine on endothelium-denuded MAR, but a significant inhibitory shift of concentration-response curves was observed on endothelium-preserved MAR (EC$_{50}$/G$_{3d}$ 0.39 ± 0.07 μM for control and EC$_{50}$/G$_{3d}$ 0.68 ± 0.14 μM with NORF). NORF induced concentration-dependent relaxation in endothelium-preserved phenylephrine pre-contracted MAR but not in endothelium-denuded MAR. NORF-induced relaxation was inhibited by the nitric oxide synthase inhibitor L-NAME (N$^\text{mon}$-nitro-arginine methyl ester). Indomethacin or HOE-140 (D-Arg-[Hyp$^\text{II}$,Thi$^\text{V}$,D-Tic$^\text{V}$,Oic$^\text{VIII}$]-bradykinin) had no significant effect on NORF-induced relaxation. Acetylcholine- and NORF-induced relaxation of pre-contracted MAR were differently inhibited by atropine. The pA$_{2}$ value for atropine-acetylcholine was 9.78 ± 0.06 and that for atropine-NORF was 8.53 ± 0.30 (P<0.01). These observations suggest that NORF induces concentration-dependent liberation of nitric oxide from MAR endothelium and that a non-muscarinic mechanism might be involved in this effect. Our data suggest no involvement of prostanooids or bradykinin in the relaxing mechanism.

Keywords: *Phoneutria nigriventer* venom, Mesenteric arterial ring, Endothelium-dependent relaxation, Nitric oxide, Nitric oxide releasing fraction

*Phoneutria nigriventer* is a spider known to produce injury in animals and humans due to the toxic effects of its venom. The venom of this spider has been described as acting on nerves where it has a neurotoxic action responsible for sodium channel activation (1) and a stimulating action responsible for neurotransmitters release (2, 3). It has also been described as having direct cardiovascular actions, inducing tachycardia and arrhythmia (1, 4), contracting vascular smooth muscle (5) and increasing vascular permeability (6). Fractionation of *Phoneutria nigriventer* venom were reported by different authors as yielding different polypeptides with contracting properties on vascular smooth muscle (7, 8). Later the same group reported that the whole venom induces relaxation of rabbit corpus cavernosum through release of nitric oxide, mediated by the tissue kallikrein-kinin system (9). Such findings contrasting with responses of other isolated vascular preparations (in which always contractile and never relaxing responses have been shown) could be attributed to one of the following: a) difference on the responses whether using whole venom or its fractions or b) lack of relaxing responses, because the vascular preparation used when assaying venom fractions were de-endothelialized and no nitric oxide release would be detected.

Having the above thinking in mind, we wondered whether
intact preparations could show release of nitric oxide similar to what was shown in rabbit corpus cavernosum.

The aim of the present study was to check this hypothesis assaying the direct effect of *Phoneutria nigriventer* venom fractions on rat mesenteric arterial rings and studying any possible influence of these venom fractions on phenylephrine-induced responses of this preparation.

### MATERIALS AND METHODS

#### Venom fractionation

Venom from female and male live adult *Phoneutria nigriventer* spiders was obtained by electrical stimulation of the fangs, as described by Barrio and Vital Brazil (10).

The venom (5 – 12 µl/spider, 160 mg protein/ml) was immediately transferred to siliconized glass tubes in ice, diluted with the same volume of distilled water and centrifuged at 40,000 m/s² (4,000×g) to remove insoluble proteins and cellular debris. The supernatant was lyophilized, stored at −18°C and later fractionated by gel filtration as described in different papers (11 – 13).

The fraction P4 (a pool of the toxic material) was the first one showing any influence in drug-induced contractions of rat vascular rings. Fraction P4 was again fractionated starting with gel filtration in Superose 12 in 150 mM ammonium formate, pH 6.5, at 25°C (FPLC). Three sub-fractions (S₁, S₂ and S₃) were obtained. The first of these sub-fractions was further fractionated by gradual isotropic steps in 0.1% TFA (trifluoroacetic acid) / acetonitrile yielding 5 different sub-fractions that were assayed to observe any effect in drug-response interaction on rat arterial rings. Only one of these sub-fractions (the first of the series, a hygroscopic slightly bluish white material) showed a relevant effect that is described in the Results section of the present paper.

Due to this effect we named this sub-fraction as nitric oxide releasing fraction using the abbreviation NORF to identify it. The exact nature of this material was not yet established, but it consists of a mixture of very small peptides. NORF was lyophilized after the above described purification and stored at −18°C. Aliquots of this partially purified sub-fraction were weighed immediately before each experiment, dissolved in deionized distilled water and then added to the media bathing the biological preparation.

#### Arterial ring preparation

Most of the experiments were performed using Wistar rats weighting 250 – 350 g provided by CEBIO-UFMG (Belo Horizonte, Brazil). In some experiments (only in the below-characterized group, in which we tried to detect eventual bradykinin formation under NORF influence), Holtzman rats weighing 180 – 250 g, also provided by CEBIO-UFMG, were used. The animals were sacrificed by stunning and subsequent decapitation. A segment of the mesenteric artery was immediately excised and placed in a Petri dish containing nutritive solution at room temperature. Under a Zeiss magnifying lens, the preparation was carefully cleaned from adhering fat and connective tissues. Two or three neighbor arterial rings (2-mm-wide) were then prepared. Each arterial ring was used to perform a different experimental protocol. In some experiments, one of the rings was assayed as the control for the neighboring one used to detect the effect of venom fractions. Arterial rings were suspended in independent vertical 10-ml organ chambers filled with Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃ and 10 mM glucose) maintained at 37°C and continuously bubbled with a gas mixture of 95% O₂ and 5% CO₂. Each ring was mounted vertically between two fine stainless steel hooks. The upper one of these hooks was connected to a Grass (Quincy, MA, USA) isometric strain-gauge transducer (FT-03) and isometric contraction forces were recorded on a Grass model 7 Polygraph.

Basal tension of 1.0 g was applied to each ring at the beginning of an experiment and continuously monitored and adjusted along the whole experimental period. The rings were equilibrated in the organ chambers for 90 min with renovation of nutritive solution each 15 min before any drug addition.

In some of the arterial rings, the endothelium was mechanically rubbed off to allow the evaluation of any effect from endothelium derived substances in the responses. The integrity of the endothelium or the effectiveness of its intentional destruction was always confirmed after the equilibration period, at the beginning of an experiment, by the presence or absence of relaxation to 1 µM acetylcholine in phenylephrine pre-contracted rings.

Drugs and venom fractions were added to the muscle chamber in small volumes (50 µl or 100 µl). After adding a drug or venom fraction to the organ chamber and registering its direct action in the biological preparation or its effect on the action of another drug, the organ chamber was drained and washed. The arterial rings were then left to rest for at least 60 min, with a washing every 10 – 15 min, before any new drug assay. At the end of an experiment, the integrity of the endothelium was again confirmed.

#### Experimental protocols

**Search of any autonomic mediator release by effect of NORF:** A single dose of NORF (10 or 30 µg/ml final concentration) was added to an organ chamber containing a relaxed mesenteric arterial ring and the mechanical activity was recorded for 15 min to detect any contractile effect that could suggest an autonomic mediator release.

Effect of NORF on concentration-response curves to phenylephrine: Cumulative concentration-response curves
to phenylephrine were obtained from endothelium-preserved and endothelium-denuded mesenteric arterial rings in the absence of NORF and in the presence of this venom fraction. A first cumulative concentration-response curve was induced in an arterial ring preparation in the absence of venom using the following procedure: a sub-threshold dose of phenylephrine was added to the organ chamber (final concentration = 1.0 nM). After registering the effect of this dose, another dose was added cumulatively to the organ chamber with no washout. The same procedure was repeated each time the contractile effect of a cumulative dose reached to a plateau, until no more significant increase in the contraction force was observed upon the addition of new cumulative doses. After washing the preparation and awaiting the resting time as described above, NORF was added to the organ chamber to attain a final concentration of 30 μg/ml. After 15 min incubation time, a second concentration-response curve to phenylephrine was induced, using the same procedure.

Effect of NORF on pre-contracted preparations: Both endothelium-preserved and endothelium-denuded mesenteric arterial rings were pre-contracted with 1.0 μM phenylephrine. After reaching a contractile plateau either acetylcholine (3 μM) or NORF (30 μg/ml) was added to the organ chamber as a single dose and the effect in the contracted preparation was registered. In other experimental groups, cumulative doses (final concentrations starting from 0.03 μM and increasing up to 10 μM of acetylcholine or starting from 1.0 μg/ml and increasing up to 300 μg/ml of NORF) were added to the organ chamber instead of single doses and the effect in the contracted preparation was registered.

Aiming to find mechanisms responsible for the relaxing effect induced by NORF, a variation of the above protocol was performed. This variation consisted in adding one of the following inhibitors to the organ chamber immediately after the contractile response to phenylephrine reached a plateau, and 5 min before adding acetylcholine or NORF: a) indomethacin (10 μM); b) L-NAME (Nω-nitro-arginine methyl ester) (10 μM); c) atropine (1.0 μM); d) HOE-140 (D-Arg-[Hyp⁵,Thr³,D-Tic⁷,Oic⁹]-bradykinin) (1.0 μM). Comparison of records of relaxing effect in the presence and in absence of these inhibitors allowed conclusions about mechanisms of action.

pA₂ evaluation: Determinations of pA₂ values (defined as the negative logarithm of an antagonist concentration necessary to reduce the effect of a double concentration of agonist to the value of a single concentration) were performed according to the method described by Schild (14), with a slight modification: instead of adding the antagonist (atropine) to the nutritive solution before any agonist addition, as described by Schild, we did it only after reaching the contraction plateau for phenylephrine, always 5 min before adding a relaxing agonist, acetylcholine or NORF.

Search for bradykinin formation by effect of NORF: Relaxed Holtzman rat mesenteric arterial rings, a preparation that contracts to bradykinin (15), were exposed to a single dose of NORF (final concentration = 100 μg/ml) and mechanical activity was recorded for 15 min. The same arterial ring was subsequently challenged with bradykinin (2.0 μM) in order to certify that it contracts to this polypeptide.

Drugs
Acetylcholine chloride, phenylephrine hydrochloride, bradykinin acetate, indomethacin and Nω-nitro-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Company, St. Louis, MO, USA; Atropine sulfate was obtained from Merck Darmstadt, Germany; D-Arg-[Hyp⁵,Thr³,D-Tic⁷,Oic⁹]-bradykinin (HOE-140) was kindly supplied by Hoechst-Roussel Pharmaceutical, Frankfurt, Germany.

Data analyses
Each experimental procedure was repeated with 4 to 14 animals. Where appropriate, means, standard error of means (S.E.M.) and comparison of means through Student’s t-test or paired t-test were calculated. Concentration-response lines and EC₅₀ values were calculated using the linear and angular coefficients for the line fitted to experimental points through the minimum square method. For the comparison between EC₅₀ means (a parameter with no normal distribution) we used the corresponding negative logarithm (pD₂). For comparison between regression lines for acetylcholine- and NORF-relaxation in phenylephrine pre-contracted preparations, a parallel line analysis of variance (16) was performed. All statistical calculations were performed using computer programs elaborated by Dr. J. Weinberg. Data given in the Results section are means ± S.E.M. P-values less than 0.05 were considered to be significant.

RESULTS
Effect of NORF on relaxed mesenteric arterial rings
No contractile response was observed by the exposition of relaxed mesenteric arterial rings from Wistar rats (n = 5) to NORF (10 or 30 μg/ml).

NORF and phenylephrine-receptor interaction in mesenteric arterial rings
Concentration-response curves of control and NORF pre-incubated mesenteric arterial rings to phenylephrine are shown in Fig. 1. Left curves are for endothelium-preserved preparations and right curves are for endothelium-denuded
arterial rings. Mean threshold doses, mean maximum contraction forces and mean calculated EC₅₀’s are shown in Table 1. These three parameters, measured in presence of NORF, were significantly different from controls for endothelium-preserved rings but not for endothelium-denuded ones.

Relaxing effect of NORF on pre-contracted preparations

NORF (in concentrations of 3.0 μg/ml and higher) induced relaxation of phenylephrine pre-contracted mesenteric arterial rings (n = 14). Challenging a pre-contracted arterial ring repetitively with NORF resulted in repetitive relaxation of the same magnitude. This relaxing effect was not observed in endothelium-denuded preparations (n = 6). Relaxation induced by a single dose (30 μg/ml) of NORF was 36 ± 12% of the contractile tension induced by 1.0 μM phenylephrine in this preparation (Fig. 2). Addition of

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**Table 1.** Threshold doses, maximum contraction forces and calculated EC₅₀ values for phenylephrine in normal and endothelium-denuded rat mesenteric arterial rings

|                      | Endothelium-preserved rings | Endothelium-denuded rings |
|----------------------|-----------------------------|---------------------------|
|                      | (n = 11)                    | (n = 7)                   |
| Control in presence of NORF |                        |                           |
| Threshold dose (nM)              | 8.5 ± 2.4                   | 14.7 ± 4.1                |
| Maximum contraction (mg)         | 774 ± 52                    | 685 ± 38                  |
| EC₅₀ (μM)                  | 0.39 ± 0.07                 | 0.32 ± 0.07               |
| Control in presence of NORF |                        |                           |
| Threshold dose (nM)              | 19.6 ± 3.7                  | 7.0 ± 1.4                 |
| Maximum contraction (mg)         | 671 ± 53***                | 668 ± 43                  |
| EC₅₀ (μM)                  | 0.68 ± 0.14**              | 0.36 ± 0.08               |

30 μg/ml NORF. Values are means ± S.E.M. Significance of differences between values in the presence of NORF and respective controls were evaluated using paired t-tests. *P<0.025, **P<0.01, ***P<0.005.

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Fig. 1. Cumulative concentration-response curves to phenylephrine (PhE) in endothelium-preserved (A, n = 11) and endothelium-denuded (B, n = 7) rat mesenteric arterial rings. Each arterial ring was exposed to PhE in the absence (circles) or presence (triangles) of 30 μg/ml NORF (nitric oxide releasing fraction of Phoneutria nigriventer venom). Data points are means and vertical lines represent S.E.M.

Fig. 2. Relaxation induced by NORF (30 μg/ml) on phenylephrine (PhE) pre-contracted mesenteric arterial ring (left) and failure of same NORF concentration to induce relaxation in pre-contracted rings exposed to 10 μM l-NAME (right). The left record is representative of 14 experiments in which a single dose of NORF induced relaxation. In 5 of these experiments, the same arterial rings were subsequently incubated with l-NAME and failed to relax with NORF, as in the right record. Rings not exposed to l-NAME relaxed repeatedly to NORF.
indomethacin (10 μM) to the organ chamber prior to the venom fraction did not modify the relaxation intensity induced by acetylcholine or by NORF (n = 4 each). Incubation of pre-contracted mesenteric arterial rings with L-NAME (10 μM) led to the abolishment of the relaxing effect of NORF (n = 5, Fig. 2). HOE-140 (1.0 μM) had no effect in the relaxation intensity of acetylcholine or NORF (n = 5 each).

Relaxation induced by NORF was dependent of the dose added to the organ chamber (Fig. 3). In this particular record, both acetylcholine and NORF caused complete relaxation, but in most other records, we observed only partial relaxation, even with highest doses of the relaxing substances. Concentration-response curves to acetylcholine and to NORF with data of 5 experiments similar to the ones shown in Fig. 3 are presented in Fig. 4. A parallel line analysis of variance using data of these concentration-response curves showed no deviation of parallelism (P<0.01).

**Influence of atropine on relaxing effects of acetylcholine and NORF: pA<sub>2</sub> values**

Addition of different doses of atropine to the organ chamber when the mesenteric arterial rings were already pre-contracted with phenylephrine caused distinct degrees of inhibition of the relaxing responses to acetylcholine or NORF. As an example, relaxation induced by acetylcholine in phenylephrine pre-contracted mesenteric arterial rings was completely abolished by the presence of 1.0 μM atropine (n = 4), while NORF-induced relaxation (n = 5) was only partially inhibited (45 ± 5%) by the presence of same atropine concentration (Fig. 5). In view of such distinct relaxing responses, it became convenient to determine pA<sub>2</sub> values for atropine-acetylcholine and for atropine-NORF, as described in the Methods section. The mean pA<sub>2</sub> value for atropine-acetylcholine was 9.78 ± 0.06 (n = 11), and for the atropine-NORF, it was 8.53 ± 0.30 (n = 7). These values are statistically different (P<0.01).

**Responses of Holtzman rat mesenteric arterial rings to NORF and bradykinin**

Relaxed mesenteric arterial rings from Holtzman rats (n = 4) exposed to NORF (100 μg/ml) failed to produce a contractile response. After washing the preparation and awaiting a few minutes, these same Holtzman rat arterial rings contracted to bradykinin (2.0 μM) added to the organ chamber.
DISCUSSION

Absence of a direct contractile effect of NORF on relaxed mesenteric arterial rings (a preparation containing adrenergic nerve endings) suggests that this venom fraction has no direct contractile action on vascular smooth muscle and does not induce neurotransmitter release in our preparation, contrarily to what was observed in isolated guinea pig atrium (2) and in isolated rat heart (3).

Our results show that no influence of NORF exists on phenylephrine-receptor interaction in endothelium-denuded rat mesenteric arterial rings. Concentration-response curves to this agonist with 1.0 μM atropine abolishes ACh-induced relaxation, while decreasing only partially (45 ± 5%) NORF-induced relaxation. Records are representative of 4–5 experiments.

Endothelium-denuded preparation, it becomes clear that some substance is released from the endothelium to induce the relaxation. Among all substances that could potentially be responsible for such an effect, we decided to start searching for the participation of prostacyclin and nitric oxide because these are the two main endothelium-derived relaxing substances described in scientific literature (17). Indomethacin, a cyclooxygenase inhibitor, showed no influence in the relaxing response induced by NORF, indicating that no prostanoids are involved in this effect. L-NAME, a substance that inhibits nitric oxide synthase, abolished the relaxing effect of NORF indicating that nitric oxide is involved in the relaxation mechanism.

It is known that acetylcholine relaxes pre-contracted blood vessels by endothelium-dependent nitric oxide liberation. Our results showing that the relaxation concentration-response curve to NORF is parallel to the relaxation concentration-response curve to acetylcholine suggested that both substrates, acetylcholine and NORF, would have the same mechanism of action. However our results show that atropine (1.0 μM) abolishes completely the relaxation induced by acetylcholine (0.3–3 μM) in phenylephrine pre-contracted rings, while the same concentration of atropine induces only partial inhibition of NORF-induced relaxation of same magnitude. Such a difference suggests that some distinct mechanism of action could exist to explain NORF-induced release of nitric oxide. The finding that the pA₂ value for atropine-acetylcholine is significantly higher than the pA₂ value for atropine-NORF reinforces the suggestion of different mechanisms of action.

Nitric oxide release induced by acetylcholine is a consequence of the binding of this agonist to a muscarinic receptor in the endothelial cell membrane, leading to intracellular biochemical reactions (18). NORF could also bind to this muscarinic receptor, as one would expect once this seems the most likely mechanism. However, in view of the above discussed results comparing the effects of atropine in acetylcholine- and NORF-induced relaxation, we are inclined to accept that an alternative mechanism or an additional mechanism besides that involving muscarinic receptors could be responsible for the NORF-induced release of nitric oxide.

One alternative hypothesis for the mechanism of action of NORF leading to nitric oxide formation could be a mediation by a tissue kallikrein-kinin system, like that shown in rabbit corpus cavernosum (9). However, the following results do not support such a hypothesis: First, HOE-140, a bradykinin B₂ receptor inhibitor, did not affect NORF-induced relaxation on phenylephrine pre-contracted mesenteric arterial rings; and second, NORF did not contract relaxed mesenteric arterial rings from Holtzman rats. It was shown that bradykinin induces an unusual response on relaxed mesenteric arterial rings from Holtzman rats.
Biochemical steps for venom purification and fractionation were resources with no financial aid from research supporting agencies. Therefore, if NORF-induced responses were through bradykinin liberation, the liberated kinin from Holtzman rat relaxed mesenteric arterial rings would probably have contracted this preparation (15). However such an effect was not observed in our results: relaxed mesenteric arterial rings from Holtzman rats failed to contract to a large dose of NORF while contracting to bradykinin, as expected. The above discussion seems to indicate that bradykinin is not involved in the relaxing responses to NORF.

In conclusion, NORF induces nitric oxide-mediated relaxation of rat mesenteric arterial rings via endothelium receptors. Muscarinic receptors might be involved but they are probably not the only ones. Some additional mechanism could exist but this additional mechanism of action remains to be investigated.

To our knowledge, there are no reports in the scientific literature demonstrating nitric oxide release by Phoneutria nigriventer venom or its fractions in blood vessels. One of the reasons for such absence of detecting nitric oxide release by the venom is probably because studies were done in the past using preparations whose endothelial layer had been mechanically removed (7) and therefore no nitric oxide release could be detected. Nitric oxide release by Phoneutria nigriventer venom was described in the rabbit corpus cavernosum (9). In that preparation, nitric oxide release was obtained with the whole venom, not a fraction as in the present paper and was demonstrated as being a consequence of kallikrein-kinin system activation. Our results show that in another animal (rat) and in another preparation, a different mechanism exists for nitric oxide release by the Phoneutria nigriventervenom fraction NORF. Because more than one mechanism exists for nitric oxide release, we wondered whether any direct effect in arterial endothelium, releasing nitric oxide, could also be responsible for erection observed in dogs, rats, mice and other animals (19).

Finally, our finding seems important to explain the “sharp fall in blood pressure” observed in dogs with small doses of intravenously injected venom (19) and similar results observed in rats (20). It is also possible that the peripheral vascular collapse, which sometimes occurs in children stung by the spider, is not only a consequence of pain but may have nitric oxide release involved in such a response.

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