A SALIVARY NITROPHORIN (NITRIC-OXIDE-CARRYING HEMOPROTEIN) IN THE BEDBUG CIMEX LECTULARIUS

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Introduction

Platelet aggregation, blood clotting and vasoconstriction constitute the vertebrate hemostatic response that blood-sucking arthropods face when they attempt to feed on their vertebrate hosts. In turn, hematophagous arthropods have developed salivary anti-platelet, anti-clotting and vasodilatory factors to disarm the hemostatic apparatus of their host to facilitate the extraction of a blood meal (Ribeiro, 1987; Law et al. 1992).

A great diversity of vasodilators has been found in the salivary gland of blood-sucking arthropods (Law et al. 1992). For example, ixodid ticks possess prostaglandins (Ribeiro, 1987; Ribeiro et al. 1991), while the sand fly Lutzomyia longipalpis (Ribeiro et al. 1989; Lerner et al. 1991), the mosquito Aedes aegypti (Champagne and Ribeiro, 1994) and the black fly Simulium vittatum (Cupp et al. 1994) contain different peptidic vasodilators. The triatomine bug Rhodnius prolixus has a salivary hemoprotein that carries the anti-platelet and vasodilatory compound nitric oxide (Ribeiro et al. 1990, 1993). This hemoprotein is abundantly expressed in Rhodnius prolixus salivary glands, resulting in the deep red color of the organ (Wigglesworth, 1942/43). This unique ferric hemoprotein binds reversibly with NO in a pH-dependent manner and is responsible for the storage and transport of NO, a volatile gas, from the salivary glands of the insect to its host tissues (Ribeiro et al. 1993). Interestingly, other triatomine bugs of the genera Triatoma (such as T. rubida and T. infestans) and Panstrongylus (P. megistus) have translucent glands and do not contain nitrovasodilators (J. M. C. Ribeiro, unpublished observations), suggesting that NO and NO-carrying hemoproteins have evolved uniquely in the genus Rhodnius.

The bedbug Cimex lectularius is a hematophagous hemipteran of the family Cimicidae. Its salivary glands are of an orange/red color (J. M. C. Ribeiro, unpublished results), suggesting the presence of hemoproteins. Interestingly, Cimex lectularius and Rhodnius prolixus nitrophorin may be a case of convergent evolution.

Summary

Salivary gland homogenate of the bedbug Cimex lectularius caused vasodilation of the preconstricted rabbit aortic ring in the absence of endothelium. Vasodilation was augmented in the presence of superoxide dismutase and inhibited in the presence of Methylene Blue. Utilization of the Griess reaction indicated the presence of reactive nitrogen equivalents of the order of 337±57 pg equivalent NO2− per pair of salivary glands (mean ± s.e.m.; N=3). Salivary gland homogenates have a nitrosyl-hemoprotein that releases nitric oxide in a pH-dependent manner. The fraction containing the NO-carrying hemoprotein, when separated by HPLC, caused vasodilation of the preconstricted rabbit aortic strip. Furthermore, the presence of a nitrosyl-hemoprotein in Cimex lectularius salivary gland was verified by electron paramagnetic resonance spectroscopy. It is proposed that, as in the case of Rhodnius prolixus (Triatominae), Cimex lectularius salivary glands contain a hemoprotein (nitrophorin) that carries NO from the glands to the host tissues. However, because Cimex lectularius and Rhodnius prolixus belong to different hemipteran families (Cimicidae and Reduviidae) and evolved independently to blood feeding, Cimex lectularius and Rhodnius prolixus nitrophorin may be a case of convergent evolution.

Key words: vasodilator, salivary glands, feeding, hematophagy, bedbug, Cimex lectularius, nitric oxide, electron paramagnetic resonance, hemoproteins, nitrophorin.

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bugs evolved independently. Indeed, if one traces the hemipteran branch of the evolutionary tree from the Triatominae to the Cimicidae, a discontinuity on the blood-feeding mode is apparent. It is possible that the Cimicidae have evolved, independently of triatomines, a NO/nitrophorin system to assist hematophagy. The goals of this study were to look for the presence of salivary vasodilatory activity in *Cimex lectularius* and to investigate its possible association with salivary hemoproteins and nitric oxide.

**Materials and methods**

*Cimex lectularius* (L.) colonies were maintained in the insectary of the Center for Insect Science at the University of Arizona. Colonies were kept at 27 °C and 65 % humidity. Insects were fed every 10 days by exposing them to the shaved abdomen of an anesthetized rabbit. Salivary glands of insects at 8–10 days after feeding were dissected and stored in two different buffers: phosphate-buffered saline (PBS), 10 mmol l\(^{-1}\) sodium phosphate at pH 7.0 with 150 mmol l\(^{-1}\) NaCl; or sodium acetate 10 mmol l\(^{-1}\) at pH 5.0 with 150 mmol l\(^{-1}\) NaCl, at −75 °C until needed. Before use, salivary glands were thawed, disrupted with a dissection needle and the homogenate centrifuged in the cold (4 °C) at 14,000 revs min\(^{-1}\) for 5 min. The supernatant was recovered and used in the various experiments.

Rabbit aortic ring bioassays were performed as previously described (Ribeiro *et al.* 1990). The only modifications were the use of continuous bubbling of the Krebs solution with air instead of 95 % O\(_2\) and 5 % CO\(_2\) in order to increase the sensitivity of the aortic ring to NO and the addition 10 mmol l\(^{-1}\) Hapes buffer, pH 7.4, to the Krebs Ringer’s solution. All the assays were performed with endothelium-free preparations, as demonstrated by their insensitivity to 1 μg ml\(^{-1}\) acetylcholine. Rabbit aortic ring constriction was achieved by using 200 ng ml\(^{-1}\) norepinephrine.

HPLC bioassays of rabbit aortic strips were performed as shown in Fig. 1. Briefly, an HPLC CM400 pump from Thermoseparation Products (Riviera Beach, Florida, USA) containing solution A (148 mmol l\(^{-1}\) NaCl, 6.6 mmol l\(^{-1}\) KCl, 2.8 mmol l\(^{-1}\) CaCl\(_2\), 1.3 mmol l\(^{-1}\) MgCl\(_2\), 11.1 mmol l\(^{-1}\) sodium acetate, pH 5.0, and with 1.1 g l\(^{-1}\) glucose) at 1 ml min\(^{-1}\). A was connected to a TSK gel column (C) and the column was connected to a dual-wavelength detector (D) set at 280 nm and 438 nm with the exit directed to a three-way connector (Y). The HPLC pump delivering solution B (100 mmol l\(^{-1}\) Hapes, at pH 7.7, with 6 mmol l\(^{-1}\) Na\(_2\)HPO\(_4\), 6 mmol l\(^{-1}\) NaH\(_2\)PO\(_4\) and 2 μg ml\(^{-1}\) norepinephrine) at 0.1 l min\(^{-1}\). B was directly connected to the three-way connector (Y). The outlet of the three-way connector directed the mixture of A and B to the aortic strip (AO). The aortic strip was kept at 37 °C with a glass jacket surrounding the tissue (not shown). HPLC eluent readings and aortic tension measurements were simultaneously collected using two different recording devices (R).

Fig. 1. HPLC bioassay. The HPLC pump (P) delivered solution A (148 mmol l\(^{-1}\) NaCl, 6.6 mmol l\(^{-1}\) KCl, 2.8 mmol l\(^{-1}\) CaCl\(_2\), 1.3 mmol l\(^{-1}\) MgCl\(_2\) and 11.1 mmol l\(^{-1}\) sodium acetate, at pH 5.0, and with 1.1 g l\(^{-1}\) glucose) at 1 ml min\(^{-1}\). A was connected to a TSK gel column (C) and the column was connected to a dual-wavelength detector (D) set at 280 nm and 438 nm with the exit directed to a three-way connector (Y). The HPLC pump delivering solution B (100 mmol l\(^{-1}\) Hapes, at pH 7.7, with 6 mmol l\(^{-1}\) Na\(_2\)HPO\(_4\), 6 mmol l\(^{-1}\) NaH\(_2\)PO\(_4\) and 2 μg ml\(^{-1}\) norepinephrine) at 0.1 l min\(^{-1}\). B was directly connected to the three-way connector (Y). The outlet of the three-way connector directed the mixture of A and B to the aortic strip (AO).
homogenate from *Cimex lectularius* was exposed to argon, to remove any volatile ligand, until a total shift in the Soret band from 438 to 388 nm was observed. Exposures to argon for longer than 1 h failed to change the spectrum any further. The Soret band is a well-defined spectral property of hemoprotein, the maximum absorption of this band ranges from 375 to 450 nm (Antonini and Brunori, 1971). In addition to being a characteristic property of hemoproteins, the changes in the absorption intensity and the wavelength of the maximum absorbance of the Soret band also provide information about the hemoprotein–ligand interactions. For example, in the presence of argon, a shift from a higher wavelength to a lower wavelength indicates dissociation of a volatile ligand from a hemoprotein containing Fe$^{3+}$ in its heme moiety (Romberg and Kassner, 1979). After exposure to argon, salivary gland homogenate was reacted with dithionite (0.1 mmol l$^{-1}$), to reduce the heme iron, and exposed to carbon monoxide for 10 min. Optical measurements were again performed.

For nitric oxide binding, salivary gland homogenate at pH 7.0 was exposed to argon for 1 h or until a shift in the Soret band from 438 to 388 nm was detected spectrophotometrically. 100 mmol l$^{-1}$ citrate buffer at pH 5.0 was then added in small amounts to the homogenate until pH 5.0 was reached. 50 µl of this solution (requiring three pairs of salivary glands) was then transferred to a 50 µl cuvette previously exposed to argon, and the mixture was exposed to argon for a further 2 h. To this solution, 0.2 µl of a saturated solution of nitric oxide (10 ml of water exposed to argon for 1 h then to nitric oxide for 2 min) was added using a 10 µl Hamilton syringe, and the spectrum was measured immediately after the addition of NO.

Determination of reactive nitrogen groups (NO$_2^-$) was carried out using the Griess reaction, as previously described (Bell et al. 1963; Ignarro et al. 1987) but with further modifications. Briefly, 8 µl of 40% trichloroacetic acid (v/v) and 5 µl of sulfanilic acid (2 mg ml$^{-1}$) were added to 42 µl of sample containing 10 pairs of salivary glands in phosphate-buffered saline. The tube was vortexed, kept on ice for 10 min and centrifuged at 10,000 g for 1 min. To 50 µl of the clear supernatant was added 9.4 µl of N-(1-naphthyl)-ethylenediamine (NED, 1 mg ml$^{-1}$), and the spectrum of the samples was measured after 10 min at room temperature (25°C). Nitrite concentration was determined from the absorbance at 550 nm minus the absorbance at 625 nm. Controls lacking the salivary homogenate or NED showed no absorbance at 550 nm.

For electron paramagnetic resonance assays (EPR), salivary gland homogenates of *Cimex lectularius* (100 pairs in 20 µl of 10 mmol l$^{-1}$ sodium acetate, at pH 5.0, plus 150 mmol l$^{-1}$ NaCl) were diluted in PBS, pH 7.0, in the presence or absence of dithionite. The solution was then transferred to an EPR quartz tube previously exposed to argon, and stored at −75°C before analysis. Measurements were performed on a Bruker ESP-300E X-band spectrometer equipped with a helium cryostat (Oxford Instruments). Power attenuation was 15 dB; temperature 4.2 K; modulation frequency 93 kHz, modulation amplitude 2.83×10$^{-4}$ T (2.85 G), receiver gain 1×10$^5$, resolution 1024 points, time constant 164 ms, and sweep width 0.177 T (1700 G).

For further information regarding heme and NO analysis, see Antonini and Brunori (1971) and Feelish (1991).

All animals used in these experiments were treated according to approved protocols reviewed by the University of Arizona Institutional Animal Care and Use Committee.

**Results**

When salivary gland homogenate from *Cimex lectularius* was added to the rabbit aortic ring preconstricted with 200 ng ml$^{-1}$ norepinephrine, a reversible vasodilation was observed (Fig. 2A). The vasodilation was endothelium-independent since the endothelium was removed from the aortic ring preparations before the bioassays. The vasodilation was augmented when the aortic rings were treated with superoxide dismutase (30 units ml$^{-1}$) (1 unit of superoxide dismutase will inhibit the rate of reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 at

![Fig. 2](image-url)

Fig. 2. (A) Vasodilatory activity of salivary gland homogenate (SGH) from five pairs of glands of *Cimex lectularius* added to a rabbit aortic ring previously contracted with 200 ng ml$^{-1}$ norepinephrine in a 2.0 ml organ bath. SOD represents the addition of superoxide dismutase (30 units ml$^{-1}$) (1 unit of superoxide dismutase will inhibit the rate of reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 at 25°C) before the addition of the salivary gland. (B) The experimental trace marked MB represents salivary gland homogenate from five pairs of glands of *Cimex lectularius* in an aortic ring previously treated with 200 ng ml$^{-1}$ norepinephrine and 50 µmol l$^{-1}$ Methylene Blue.
25 °C) prior to the addition of the salivary glands (Fig. 2A). Adding salivary gland homogenate to an aortic ring in the presence of 50 μmol l⁻¹ Methylene Blue resulted in no detectable vasodilation (Fig. 2B). These observations suggest that salivary gland homogenate contains a vasodilatory compound that behaves like nitric oxide or as an endothelium-derived relaxing factor (Ignarro et al. 1987). In addition, when a Griess reaction was performed in the salivary gland homogenate, it detected 337±57 pg NO₂⁻ equivalent per pair of salivary glands (mean ± S.E.M., N=3).

Spectrophotometric analysis of the salivary gland homogenate at pH 5.0 revealed an absorbance band with a maximum at 438 nm (Fig. 3). Changing the pH from 5.0 to 7.0 resulted in a shift of the maximum from 438 to 388 nm with a shoulder at 438 nm (Fig. 3), suggesting dissociation of a ligand at neutral pH. In addition, when the spectra were taken at different times (8 min intervals) after adjusting the solution pH to 7.0, we observed a time-dependent shift in the absorbance from 438 to 388 nm, suggesting a relatively slow dissociation of a ligand at pH 7.0 (Fig. 3, inset A). Furthermore, the differential spectrum obtained by subtracting the spectrum from the homogenate at pH 5.0 from the spectrum of the homogenate at pH 7.0 resulted in a maximum at 388 nm and a minimum at 438 nm (Fig. 3, inset B). Dissociation of the putative ligand over time (40 min) at pH 5.0 accounted for only a 10% loss of the 438 nm shoulder observed at pH 7.0 (data not shown).

To study further the dependency of the absorbance maximum on the pH of the medium, salivary gland homogenates were measured spectrophotometrically at different pH values, and the ratio of absorbance at 388 nm to absorbance at 438 nm was plotted as a function of pH. The results showed a curve with a pKa of approximately 6.5 (Fig. 4), suggesting the presence of histidine as the ionizable group.

Because the absorption spectrum of the salivary gland homogenate is similar to that of an Fe(III) hemoprotein, where there is a shift of the Soret maximum from higher to lower wavelength when a ligand dissociates (Romberg and Kassner, 1979), we wanted to test for the presence of a hemoprotein in the salivary gland homogenate of Cimex lectularius. For this, the salivary homogenate at pH 7.0 was exposed to argon to remove volatile ligands; it was then reacted with dithionite in order to produce an Fe(II) heme species and finally reacted with carbon monoxide. When the salivary gland homogenate
was reacted with dithionite, a shift in the spectrum from 388 to 400 nm was observed (Fig. 5). In the presence of carbon monoxide, the maximum absorbance band shifted from 400 to 422 nm (Fig. 5, dashed line), indicating that the protein binds to carbon monoxide and behaves as a hemoprotein.

In order to evaluate whether the vasodilatory activity was associated with the salivary hemoprotein, we ran 100 pairs of homogenized salivary glands in a molecular sieving column eluted with 10 mmol l$^{-1}$ sodium acetate and 150 mmol l$^{-1}$ NaCl, at pH 5.0, and tested each fraction in a rabbit aortic ring bioassay. No vasodilatory activity was found (results not shown). We interpreted this negative result, which showed no detectable vasodilatory activity, to be due to the dilution effect produced by the restricted volume of the fraction sample that can be added to the chamber where the aortic ring is submerged. If the volume of the sample is too large, it can alter the dilution of norepinephrine and it can also increase the amount of salt in the solution. In addition, column fraction samples could be degraded between the time of elution and the time of testing in the rabbit aortic ring bioassay. To eliminate the dilution effect and to preserve the vasodilatory activity of the homogenate, we performed the column chromatography at

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**Fig. 5.** Effect of CO on the light absorbance of the salivary gland homogenate from *Cimex lectularius* exposed to argon for 1 h and then reacted with dithionite. The continuous line represents the spectrum of argon-treated homogenate treated with dithionite and the dashed line represents the spectrum of salivary homogenate hemoprotein loaded with CO. The inset represents the difference spectrum of homogenate exposed to argon then treated with dithionite minus the spectrum of homogenate exposed to argon, dithionite and CO.

**Fig. 6.** Vasodilatory activity of the eluent of three pairs of salivary glands from *Cimex lectularius* separated by HPLC-TSK gel column. Salivary gland homogenate was injected into a TSK gel column and the eluent was directed to a norepinephrine- (200 ng ml$^{-1}$) constricted aortic strip, as described in Fig. 1. (A) HPLC chromatogram of salivary gland homogenate detected at 280 nm (dashed line) and 438 nm (solid line). The inset shows the standard curve of the molecular mass markers (BA, $\beta$-amylase; BSA, bovine serum albumin; CA, carbonic anhydrase; MYO, myoglobin, CYT, cytochrome c; APR, aprotinin). (B) Percentage inhibition of norepinephrine constriction of an aortic strip by the eluent of the TSK gel column homogenate from three pairs of salivary gland from *Cimex lectularius*. Constriction of the aortic strip due to norepinephrine was 1.8 g above resting tension.
pH 5.0 and the bioassay on-line immediately after neutralization of the column effluent.

When salivary gland homogenate (from three pairs of glands) was separated by HPLC using a molecular sieving column (Fig. 6A) and the eluent was directly tested on a rabbit aortic strip, the eluent containing the most abundant hemoprotein (Fig. 6A, elution time 16.2 min) detected at 438 nm caused inhibition of vasoconstriction, of the rabbit aortic strip, induced by norepinephrine (Fig. 6B). There was a significant decrease in the inhibition of vasoconstriction when salivary gland homogenate was exposed to PBS at pH 7.0 for 30 min before the HPLC bioassay (results not shown). When salivary gland homogenate (100 pairs) was separated by HPLC using a molecular sieving column as described in Fig. 6A and the fractions collected were scanned in a spectrophotometer, the fraction containing the main protein peak with a retention time at 16.2 min had an absorption spectrum the same as that shown in Fig. 3 at pH 5.0 (not shown). In addition, this hemoprotein had an apparent relative molecular mass of 28,684 when calibrated against molecular mass standards (Fig. 6A, inset). We conclude that the salivary vasodilator of *Cimex lectularius* is a molecule unstable at neutral pH and co-eluting with a hemoprotein having an apparent relative molecular mass of 28,684.

Since the ligand of *Cimex lectularius* hemoprotein was suspected of being a volatile compound and the gas nitric oxide has been shown to be involved in vasodilation, we tested whether the ligand behaved like authentic nitric oxide and could be displaced from the head space of the protein solution by a noble gas, which is unable to react with the protein but is able to change the equilibrium towards the unliganded form. For this, salivary gland homogenate at pH 7.0 was exposed to argon, resulting in a shift in the Soret band from 438 to 388 nm (Fig. 7, continuous line). This supports the hypothesis that the ligand of the salivary hemoprotein is a volatile compound. Exposure of the homogenate at pH 5.0 to argon leads to a slower, but complete, conversion of the Soret band from 438 to 406 nm. The same spectrum was observed when the homogenate at pH 7.0 was exposed to argon and then shifted to pH 5.0 (Fig. 7, dotted line). When nitric oxide was added to the salivary homogenate at pH 5.0 that had previously been exposed to argon, the Soret band shifted from 406 to 438 nm (Fig. 7, dashed line). However, when a difference spectrum was taken from the homogenate exposed to nitric oxide and the homogenate at pH 5.0 exposed to argon, it resulted in an absorption minimum at 388 nm and an absorption maximum at 438 nm (Fig. 7, inset A). The visible spectrum also showed the appearance of typical α and β bands at 580 nm and 545 nm, respectively (Fig. 7B). The absorption spectrum of the protein charged with NO was identical to the spectrum of the native salivary homogenate at pH 5.0, suggesting that nitric oxide is the ligand of this hemoprotein.

In order to test for the presence of nitric oxide in the salivary gland of *Cimex lectularius*, we measured the electron paramagnetic resonance spectrum (EPR) of 100 pairs of salivary glands in the absence and presence of dithionite (Fig. 8). The rationale for using dithionite was that Fe(III) hemoproteins have an odd number of electrons that exist in a high-spin form when pentacoordinated and, typically, give rise to EPR spectral features at about 0.11 T (1100 G) and 0.33 T (3300 G) (spectroscopic g-factors of 6.0 and 2.0, respectively) (Walker et al. 1984). Since nitric oxide is also an odd-electron...
Nitric oxide is a vasodilator that can be used by blood-sucking insects during feeding to avoid one of the hemostatic mechanisms of their host (vasoconstriction). *Cimex lectularius* and *Rhodnius prolixus* have in their saliva a hemoprotein with similar spectroscopic characteristics and both proteins carry nitric oxide. Interestingly, *Rhodnius prolixus* and *Cimex lectularius* are from different families of Heteroptera and have evolved independently to a blood-feeding mode (Sweet, 1979; Reisberg et al., 1993).
Cobben, 1979). Thus, their nitrophorins may have arisen by convergent evolution. Comparison of the structure and physical characteristics of the nitrophorins from these two species should yield insights into the interactions of ferric hemoproteins with nitric oxide.

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References


