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Sn-protoporphyrin inhibits both heme degradation and hemozoin formation in *Rhodnius prolixus* midgut

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ABSTRACT

Hematophagy is a feeding habit that involves the ingestion of huge amounts of heme. The hematophagous hemipteran *Rhodnius prolixus* evolved many genetic resources to protect cells against heme toxicity. The primary barrier against the deleterious effects of heme is the aggregation of heme into hemozoin in the midgut lumen. Hemozoin formation is followed by the enzymatic degradation of heme by means of a unique pathway whose end product is dicysteinyl-biliverdin IX-γ (*Rhodnius prolixus* biliverdin, RpBv). These mechanisms are complemented by a heme-binding protein (RHBP) in the hemolymph that attenuates the pro-oxidant effects of heme. In this work, we show that when insects are fed with blood enriched with a heme analog, Sn-protoporphyrin (SnPP-IX), both hemozoin synthesis and RpBv production are inhibited in a dose-dependent manner. These effects are accompanied by increased oxidative damage to the midgut epithelium and inhibition of oviposition, indicating that hemozoin formation and heme degradation are protective mechanisms that work together and contributed to the adaptation of this insect to successfully feed on vertebrate blood.

1. Introduction

Hematophagy arose independently in many insect orders, and during the course of evolution, different groups of blood-feeding insects acquired specific ways to manage and digest blood (Lehane, 1991; Mans and Neitz, 2004). Hematophagous insects can ingest in a single meal a huge amount of vertebrate blood, which contains about 10 mM of heme bound to hemoglobin. Heme is a potentially harmful molecule, exerting its toxicity either by promoting the formation of reactive oxygen species or alternatively, by insertion of free heme into phospholipid membranes eventually leading to cellular lysis (Kumar and Bandyopadhyay, 2005; Ryter and Tyrrell, 2000; Schmitt et al., 1993; Tappel, 1955; Wijayanti et al., 2004). The metabolic adaptations required for a blood diet should maintain cellular homeostasis in a pro-oxidant environment created by an increased heme concentration (Graca-Souza et al., 2006). One of the preventive mechanisms of *Rhodnius prolixus* against free heme injury is to promote the formation of a crystalline aggregate called hemozoin (Oliveira et al., 1999), a protective mechanism also found in the malaria parasite, *Plasmodium falciparum* (Slater et al., 1991), and in the blood fluke, *Schistosoma mansoni* (Oliveira et al., 2000a). In *R. prolixus*, this type of defense can account for most of the heme found in the midgut lumen and therefore it has been suggested that it has a protective role (Oliveira et al., 2000b). However, a need for other complementary mechanisms, such as antioxidant enzymes, has already been pointed (Paes et al., 2001). Another important defense mechanism against heme toxicity is achieved through the presence of heme-binding proteins in the hemolymph of *R. prolixus*. The *Rhodnius* heme-binding protein (RHBP) is a monomeric heme-binding protein in the range of 12 KDa that binds heme as a prosthetic group (Oliveira et al., 1995). RHBP controls free heme reactivity and acts as a transporter of heme to the ovaries (Dansa-Petretski et al., 1995; Machado et al., 1998).

From bacteria to men, heme disposal is accomplished by the action of heme oxygenase, an enzyme that oxidatively cleaves the porphyrin ring of the heme, releasing iron, carbon monoxide and biliverdins (Montellano, 2000; Tenhunen et al., 1969; Wilks, 2002). Therefore, it is conceivable that enzymatic degradation by the microsomal heme oxygenase should be a key aspect of heme homeostasis and an important defense against heme toxicity in blood-feeding insects. Sir Vincent Wigglesworth, in a pioneer study (Wigglesworth, 1943), described the presence of green pigments in

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R. prolixus intestinal epithelium and heart pericardial cells. In fact, Paiva-Silva et al. (2006) showed that the heart and the digestive tract have a green color due the presence of a distinctive bilin pigment, RpBv. The heme catabolism in *Rhodnius* heart proceeds through a five-step mechanism that until now has been described only in this organism. Initially, the molecule of heme is modified, receiving two consecutive additions of cysteinyl-glycine dipeptides. Only after that the porphyrin ring is cleaved, forming dicysteinyl-glycine biliverdin IX γ. Finally, the peptide linkages between cystein and glycin are hydrolysed by a dipeptidase to generate dicysteinyl-biliverdin IX γ, the *R. prolixus* biliverdin (RpBv) (Paiva-Silva et al., 2006)

In this work, we have studied the role of this unique heme degradation pathway in the midgut of *R. prolixus*, using Sn-protoporphyrin IX, a heme analogous described as a classical inhibitor of heme oxygenase activity. We found that this compound not only inhibits heme degradation, but also blocks heme aggregation into hemozoin. Blocking these primary anti-oxidant defenses leads to oxidative stress and impairment of reproductive capacity.

2. Materials and methods

2.1. Animals, hemolymph and organ extracts

The *R. prolixus* colony was maintained at 28 °C and 80% relative humidity at the Instituto de Bioquímica Médica – UFRJ. Insects were fed on rabbit blood at 3 week intervals and were normal mated females. Four days after the second meal as an adult, hemolymph was collected by cutting a leg and applying a gentle pressure to the insect abdomen. Hemolymph was diluted 1:1 with a cocktail of protease inhibitors (0.05 mg/ml of soybean trypsin inhibitor, 0.05 mg/ml leupeptin, 1 mM benzamidine and 0.01% PMSF, purchased from Sigma Chem. Company), 3–13 mg/ml phenylthiourea, 0.15 M NaCl and 50 mM sodium phosphate buffer pH7.4 (PBS) and 1 mM EDTA. The samples were centrifuged at 11,000×*g* for 5 min and the supernatants were kept at −20 °C until use. Insect midguts were dissected in PBS and transferred to distilled water, which induced a vigorous contraction of the gut, immediately expelling the luminal content. Midgut content and epithelium were collected and homogenized in PBS (100 μl).

2.2. SnPP-IX-globin

A protocol adapted from (Teale, 1959) was used to extract heme from hemoglobin. A solution of 150 mg/ml of bovine hemoglobin (SIGMA) was extensively dialyzed against deionized water and the pH was adjusted to 2.0 with NaOH 0.1 M. The solution was kept in a tube in ice, together with another tube containing ice-cold methyl-ethyl-ketone. When both tubes reached 4 °C they were gently mixed and then were kept in ice until separation of two phases. The organic phase was discarded and the aqueous phase was collected and dialyzed extensively against water at 4 °C. The final globin product was mixed with an equimolar amount of 5 mM SnPP-IX (Frontier Scientific, Logan, UT) diluted in DMSO to make the complex metalloporphyrin-globin, hereafter called SnPP-IX globin.

To get rid of the DMSO, the solution was applied onto a G-25 column (10 cm × 3 cm) equilibrated in PBS. Fractions of the flow-through containing SnPP-IX were pooled and stored at 4 °C. Protein concentration was determined by the Bradford method. Using an artificial feeding apparatus (Garcia et al., 1975), insects were fed with meals containing 33% heparinized rabbit blood, together with different concentrations of SnPP-IX globin (33, 100 or 330 μM). The final volume was adjusted with PBS.

2.3. HPLC fractionation and biliverdin (RpBv) measurement

HPLC was carried out on a LC-10 AT device (Shimadzu, Tokyo) equipped with a diode array detector (SPD-M10A). Samples were mixed with 5% acetonitrile, 0.05% TFA, pH 2.0 (1:2,v:v), centrifuged for 15 min at 12,000×*g* and then applied onto a Shimadzu CLC-ODS C18 column (15 mm × 22 cm) equilibrated with the same buffer, using a flow rate of 0.4 ml/min. After 10 min, a 40 min acetonitrile linear gradient (5–80%) was applied, followed by 20 min of 80% acetonitrile with 0.05% TFA, pH 2.0. A standard curve of biliverdin IX-α (Frontier Scientific; Logan, UT) was made and the peak area was used to estimate the amount of RpBv in samples from *Rhodnius* midgut.

2.4. Hemozoin extraction

Midguts were dissected under PBS and the luminal contents were collected, homogenized and centrifuged at 11,000×*g* for 10 min. The pellet was resuspended in 0.1 M sodium bicarbonate buffer, pH 9.1 and 2.5% SDS. Samples were centrifuged at 11,000×*g* in a microcentrifuge for 10 min and the pellet was washed 5× with the same buffer, followed by 2× with deionized water. Hz quantification was carried out by adding 1 ml of 0.1 M NaOH, vortexing the samples for 30 min, followed by determination of heme content at 400 nm in a GBC-UV/Vis-920 spectrophotometer, using a standard curve made with hemin (Frontier Scientific; Logan, UT) dissolved in NaOH 0.1 M.

2.5. Hemozoin formation in vitro assay

R. prolixus adult females were fed with rabbit plasma and the posterior midgut was dissected in PBS 4 days after feeding. The intestinal contents were isolated and stored in 0.15 M NaCl at −70 °C. Before use, the material was centrifuged at 20,000×*g* for 20 min at −4 °C and the amount of protein the pellet was estimated and used to promote heme aggregation *in vitro*. A sample corresponding to 100 μg of protein was incubated for 24 h at 28 °C in 0.5 M sodium acetate, pH 4.8, with 100 μM hemin. After incubation, this reaction mixture was centrifuged at 15,000×*g* for 15 min at room temperature. The pellet was washed five times with 1 ml of 0.1 M $NaHCO_3$ + 2.5% sodium dodecyl sulfate, pH 9.1, and twice with deionised water.

2.6. Thiobarbituric acid reactive substances assay (TBARS)

R. prolixus midgut was dissected under PBS and luminal content was washed out. Tissue was homogenized in 0.1 M sodium phosphate buffer, pH 7.2 and TBARS were measured by adding 0.2 ml of thiobarbituric acid (1% w/v), followed by incubation at 98 °C for 60 min, followed by 1 h at 4 °C. Samples were centrifuged at 11,000×*g* for 10 min and the absorbance at 532 nm was determined using a GBC 920 UV–VIS spectrophotometer (Victoria, Australia).

2.7. Statistical analyses

Comparisons between groups were done by the one-way analysis of variance and a posteriori Tukey's test for pairwisecomparisons, using the software GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Differences of $P < 0.05$ were considered to be significant. All experiments were repeated at least twice.

3. Results

Paiva-Silva et al. (2006) have previously shown that RpBv is accumulated in *R. prolixus* hearts after injection of heme into the