Insect Biochemistry and Molecular Biology 43 (2013) 229–236

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

The interaction between *Trypanosoma rangeli* and the nitrophorins in the salivary glands of the triatomine *Rhodnius prolixus* (Hemiptera; Reduviidae)

Rafaela M.M. Paim [a], Marcos H. Pereira [a], Ricardo N. Araújo [a], Nelder F. Gontijo [a], Alessandra A. Guarneri [b,*]

[a] Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Bloco I4, Sala 177, Av. Antônio Carlos 6627, Belo Horizonte, MG, Brazil
[b] Centro de Pesquisas René Rachou, FIOCRUZ, Avenida Augusto de Lima, 1715, CEP 30190-002, Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 7 November 2012
Received in revised form
21 December 2012
Accepted 24 December 2012

Keywords:
Trypanosoma rangeli
Rhodnius prolixus
Salivary glands
Nitrophorins
RNA interference

ABSTRACT

The parasite *Trypanosoma rangeli* develops in the intestinal tract of triatomines and, particularly in species of the genus *Rhodnius*, invades the hemolymph and salivary glands, where subsequent metacyclogenesis takes place. Many aspects of the interaction between *T. rangeli* and triatomines are still unclear, especially concerning the development of the parasite in the salivary glands and how the parasite interacts with the saliva. In this work, we describe new findings on the process of *T. rangeli* infection of the salivary glands and the impact of infection on the saliva composition. To ensure a complete infection (intestinal tract, hemolymph and salivary glands), 3rd instar *Rhodnius prolixus* nymphs were fed on blood containing *T. rangeli* epimastigotes using an artificial feeder. After molt to the 4th instar, the nymphs were inoculated with epimastigotes in the hemolymph. The results showed that the flagellates started to invade the salivary glands by the 7th day after the injection. The percentage of trypomastigotes inside the salivary glands continuously increased until the 25th day, at which time the trypomastigotes were more than 95% of the *T. rangeli* forms present. The salivary contents from *T. rangeli*-infected insects showed a pH that was significantly more acidic (<6.0) and had a lower total protein and hemeprotein contents compared with non-infected insects. However, the ratio of hemeprotein to total protein was similar in both control and infected insects. qPCR demonstrated that the expression levels of three housekeeping genes (18S rRNA, β-actin and α-tubulin) and nitrophorins 1–4 were not altered in the salivary glands after an infection with *T. rangeli*. In addition, the four major nitrophorins (NPs 1–4) were knocked down using RNAi and their suppression impacted *T. rangeli* survival in the salivary glands to the point that the parasite burden inside the *R. prolixus* salivary glands was reduced by more than 3-fold. These results indicated that these parasites most likely non-specifically incorporated the proteins that were present in *R. prolixus* saliva as nutrients, without impairing the biosynthesis of the antihemostatic molecules.

1. Introduction

Triatomines (Hemiptera; Reduviidae) are obligate hematophagous insects that have medical importance because they transmit *Trypanosoma cruzi,* the etiologic agent of Chagas disease in the Americas. In addition, they are also vectors of the protozoan parasite *Trypanosoma rangeli*, which commonly develops in triatomines of the genus *Rhodnius* and a variety of mammalian species, including humans and domestic and wild animals in Central and South America (Grisard et al., 2010). Although *T. rangeli* infection is harmless for the vertebrate hosts, this parasite can be pathogenic to its insect vectors by promoting defective or lethal effects in both molting and feeding processes (Brecher and Wigglesworth, 1944; Lake and Friend, 1968; D'Alessandro-Bacigalupo and Saraiva, 1992; Ferreira et al., 2010). In addition, the coexistence of *T. rangeli* and *T. cruzi* in the same area and sharing the same vertebrate and invertebrate hosts can compromise the correct diagnosis of *T. cruzi* infections due to the occurrence of crossed serological reactions (Guhl and Vallejo, 2003).

Unlike *T. cruzi*, which develops specifically within the intestinal tract of the insect vector, the *T. rangeli* life cycle has several distinct features. *T. rangeli* flagellates also develop in the intestinal tract of *Rhodnius* triatomines as epimastigotes; however, they are able to

* Corresponding author. Tel.: +55 31 33497801; fax: +55 31 32953115.
E-mail addresses: guarneri@cpqrr.fiocruz.br, aaguarneri@gmail.com (A.A. Guarneri).

0965-1748/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibmb.2012.12.011

invade the insect hemolymph and then penetrate the salivary glands where metacyclogenesis occurs. Here, the parasite assumes the trypomastigote form, which is transmitted to the vertebrate host during the bite.

The saliva of *Rhodnius prolixus*, as with other blood-feeding arthropods, has proven to be a source of a wide variety of anti-hemostatic molecules that play an important role during hematophagy (Ribeiro et al., 1990; Champagne, 2005). Among these salivary molecules, the most abundant are hemeproteins known as nitrophorins (NPs). Seven different NPs have been described (NPs 1–7) (Moreira et al., 2003; Champagne, 2005; Knipp et al., 2007), comprising approximately 50% of the salivary protein content. Among them, the most studied are NPs 1–4. These nitrophorins are multifunctional molecules that are responsible for the reddish color of the *R. prolixus* salivary glands due to the presence of a heme group in the nitrophorin molecule, which carries nitric oxide (NO) (Champagne et al., 1995). Most of the biological activities of NPs are related to NO, which is released into the host tissue and can act as both a vasodilator and an inhibitor of platelet aggregation (Andersen et al., 2005). In vertebrate host skin, the nitrophorins can also bind to the histamine that is released by mast cells, which prevents swelling and pain at the bite and can generate a defensive response by the host (Andersen et al., 2005). In addition, nitrophorin 2 (NP2) is a powerful anticoagulant that acts by binding directly to Factor Xa of the blood coagulation cascade (Ribeiro et al., 1995). Araujo et al. (2009b) demonstrated that *R. prolixus* that have low levels of salivary NPs have poorer blood-ingestion rates when feeding from vessels that have small diameters such as venules and arterioles.

Many aspects of the life cycle of *T. rangeli* in triatomine insects are still unclear, especially the processes of invasion and metacyclogenesis of the parasite in the salivary glands, as well as how the presence of the parasite interferes with the physiology and behavior of the insect vector. It has been suggested that *T. rangeli* might induce changes in the biosynthesis of salivary compounds and thereby affect the antihemostatic properties of *R. prolixus* saliva (Garcia et al., 1994). In this work, we describe new findings on the process of *T. rangeli* colonization of the salivary glands and on the impact of a *T. rangeli* salivary gland infection on the saliva composition. We also evaluated a possible role of these abundant molecules in the development and survival of the parasites into *R. prolixus* salivary glands using RNAi to knockdown NPs expression levels.

2. Materials and methods

2.1. Insects and parasites

R. prolixus were reared under controlled conditions of temperature (26 ± 2 °C) and humidity (65 ± 5.0%) in a 12/12 h light/dark cycle and fed weekly on chickens or mice according to the FIOCRUZ guidelines on animal experimentation. The use of these insects was approved by the Ethical Committee on Animal Use (L-058/08).

T. rangeli of the CHOACHI strain isolated from naturally infected *R. prolixus* from Colombia (Schottelius, 1987) was used in the present study. The epimastigote forms were cultured at 27 °C in liver-infusion tryptose (LIT) medium supplemented with 15% fetal bovine serum, 100 μg/mL streptomycin and 100 units/mL penicillin. The strain infectivity was maintained through a cyclical transmission in mice and triatomines every two months.

For all of the assays, the epimastigotes were obtained from 10-day-old culture medium, washed and resuspended in sterile PBS (for hemolymph inoculation) or inactivated blood (for infective feeding).

2.2. Rhodnius prolixus infection

Because the parasite crossing from the intestinal lumen to the hemolymph does not occur in all infections (Ferreira et al., 2010), a complete infection (intestinal tract, hemolymph and salivary glands) was ensured by first infecting *R. prolixus* nymphs through feeding them with blood containing epimastigotes and then inoculating the same nymphs intracoelomically with the parasite. For intestinal infection, epimastigotes were added to heat-inactivated (56 °C for 30 min) rabbit blood at a concentration of 10^5 flagellates/mL. Third instar nymphs were allowed to feed on this blood through a membrane feeder (control insects were fed on non-infected blood). Seven days after the nymphs molted to the 4th instar, 1 μL of PBS (0.15 M NaCl in 0.01 M Na_3PO_4, pH 7.4) containing 100 parasites was injected directly into the insect hemolymph using a thin needle (13 × 3.30 G, ½") that was attached to a Hamilton syringe. A control group was inoculated with PBS alone. Twenty-four hours after inoculation, the nymphs were fed on anesthetized healthy mice.

2.3. Monitoring the T. rangeli invasion and differentiation within the salivary glands

The invasion process of *R. prolixus* salivary glands by *T. rangeli* epimastigotes was evaluated through the daily examination of 20 nymphs starting from the 5th day after the inoculation. The confirmation of infection was made through examination of the salivary glands in a drop of saline solution on a microscope slide. To identify the developmental forms of the parasite (epimastigotes, intermediates and trypomastigotes), the salivary glands were disrupted and examined through Giemsa-stained smears by counting at least 100 parasites in each slide. Intermediate forms were defined as those parasites that had the kinetoplast and nucleus in a transitional position within the cell.

2.4. Salivary features of T. rangeli infected and non-infected insects

The salivary glands from nymphs that had been infected as 3rd and 4th instars (oral and coelomatic infections, respectively) and their respective controls were examined on the 40th day after the inoculum was given. By this time, the nymphs had molted to the 5th instar stage and a chronic infection was already established.

2.4.1. Determination of pH in salivary gland contents

The salivary gland contents of 5th instar nymphs (*T. rangeli*-infected and non-infected ones) were collected in capillary tubes that were created by stretching out microhematocrit glass tubes in a Bunsen flame. The capillaries were pre-filled with 30 μL of an aqueous solution containing pH indicator dyes at 0.05% (bromothymol blue at pH 7.5 or bromocresol purple at pH 7.0). To measure the pH of the salivary contents, the salivary glands were dissected out of the insects, transferred to a slide and washed rapidly in 0.9% saline solution. After mopping up the excess saline with filter paper, the glands were pierced using the tip of the capillaries. The color at the interface of the two liquids (saliva-dye solution) in the capillary was compared with buffered standard color solutions (0.1 M) that had been prepared with these dyes at different pH values (5.0–8.0 at intervals of 0.5 pH units) (Soares et al., 2006).

2.4.2. Estimation of total protein and hemeprotein content in the salivary glands

T. rangeli-infected and non-infected salivary glands of *R. prolixus* 5th instar nymphs were dissected and transferred to ice-cold microcentrifuge tubes containing 70 μL of PBS. The glands were mechanically disrupted and centrifuged at 12,000 g for 10 min at 4 °C to pellet the gland tissue (and the parasites in the infected