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Jaburetox-induced toxic effects on the hemocytes of *Rhodnius prolixus* (Hemiptera: Reduviidae)

Natalia R. Moyetta[a], Valquiria Broll[b], Ana Paula A. Perin[b], Augusto F. Uberti[a], Matheus V. Coste Grahl[a], Fernanda Staniscuaski[b, c], Celia R. Carlini[a, c], Leonardo L. Fruttero[a, *]

[a] Brain Institute (Instituto do Cérebro-INSCER), Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga 6690, prédio 63, CEP 90610-000 Porto Alegre, RS, Brazil
[b] Center of Biotechnology, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, prédio 43431, CEP 91501-970 Porto Alegre, RS, Brazil
[c] Department of Molecular Biology and Biotechnology, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, prédio 43431, CEP 91501-970 Porto Alegre, RS, Brazil

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ABSTRACT

Jaburetox is a recombinant peptide derived from a *Canavalia ensiformis* urease that presents toxic effects upon several species of insects, phytopathogenic fungi and yeasts of medical importance. So far, no toxicity of Jaburetox to mammals has been shown. Previous reports have identified biochemical targets of this toxic peptide in insect models, although its mechanism of action is not completely understood. In this work, we aimed to characterize the effects of Jaburetox in hemolymphatic insect cells. For this purpose, the model insect and Chagas' disease vector *Rhodnius prolixus* was used. *In vivo* and *in vitro* experiments indicated that Jaburetox interacts with a subset of hemocytes and it can be found in various subcellular compartments. In insects injected with Jaburetox there was an increase in the gene expression of the enzymes UDP-*N*-acetylglucosamine pyrophosphorylase (UAP), chitin synthase and nitric oxide synthase (NOS). Nevertheless, the expression of NOS protein, the enzyme activities of UAP and acid phosphatase (a possible link between UAP and NOS) as well as the phosphorylation state of proteins remained unchanged upon the *in vivo* Jaburetox treatment. Nitric oxide (NO) imaging using fluorescent probes showed that Jaburetox augmented NO production in the hemocyte aggregates when compared to controls. Even though Jaburetox activated the hemocytes, as demonstrated by wheat germ agglutinin binding assays, the peptide did not lead to an increase of their phagocytic behavior. Taken together, these findings contribute to our understanding of toxic effects of Jaburetox, a peptide with biotechnological applications and a prospective tool for rational insect control.

1. Introduction

Jaburetox is a recombinant peptide of ~ 11 kDa derived from one of the isoforms of urease from *Canavalia ensiformis*, a leguminous plant commonly known as jack bean (Mulinari et al., 2007). The various biological effects of urease isoforms, independent of their enzymatic activity, have been reviewed elsewhere (Real-Guerra et al., 2013; Carlini and Ligabue-Braun, 2016) and include entomo- and fungitoxicity, as well as exocytosis induction in several cell models. Jaburetox was also reported as toxic for several species of phytopathogenic filamentous fungi and yeasts of medical importance (Postal et al., 2012) and to insect species from the orders Lepidoptera (Mulinari et al., 2007), Blattodea (Mulinari et al., 2007) and Hemiptera (Defferrari et al., 2011; Martinelli et al., 2014; Galvani et al., 2015). Nevertheless, high doses of Jaburetox are not lethal nor caused symptoms of acute toxicity in mice or neonate rats when given orally or *via* injection, indicating that the peptide has potential as an option of a safe insecticide (Mulinari et al., 2007). Moreover, preliminary results with maize, sugarcane and soybean transgenic plants expressing Jaburetox indicated that those crops present higher resistance to the attack of insect pests, making this peptide a prospective tool for rational insect control (Carlini and Ligabue-Braun, 2016).

Due to its physiological characteristics and ease of raising, triatomines (Hemiptera: Reduviidae) have been used as models for understanding the toxic mode of action of ureases and derived peptides, including Jaburetox (Stanisçuaski and Carlini, 2012). Galvani et al. (2015) demonstrated in *Triatoma infestans* that the central nervous system (CNS) is a target of the peptide. These authors observed that soon after injection, the insects showed signs of neurotoxicity such as proboscis extension, abnormal movements of the antennae and leg paralysis. Immunohistochemical and western blot analyses confirmed the localization of the injected peptide in the CNS. The effects of Jaburetox on enzymatic pathways of the CNS were described in *T. infestans* (Galvani et al., 2015) as well as in the related triatomine *R. prolixus* (Fruttero et al., 2017). The treatment with Jaburetox by means of injections or feeding

Abbreviations: CNS, central nervous system; NOS, nitric oxide synthase; UAP, UDP-*N*-acetylglucosamine pyrophosphorylase; WGA, wheat germ agglutinin; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein, diaminofluorescein-FM; PB, sodium phosphate buffer; FBS, fetal bovine serum; DTT, dithiothreitol; AP, acid phosphatase; JBU, jack bean urease; NO, nitric oxide; PAMP, pathogen associated molecular pattern; SG, salivary glands; Jbtx, Jaburetox.

* Corresponding author at: Instituto do Cérebro (InsCer), Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga 6690, prédio 63, CEP 90610-000 Porto Alegre, RS, Brazil.
Email addresses: valbroll@ufrgs.br (V. Broll); fernanda.staniscuaski@ufrgs.br (F. Staniscuaski); celia.carlini@pucrs.br (C.R. Carlini); leofruttero@cbiot.ufrgs.br (L.L. Fruttero)

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led to the modulation of the enzymatic activities of nitric oxide synthase (NOS), involved in nitrinergic signaling and UDP-*N*-acetylglucosamine pyrophosphorylase (UAP), implicated in glycosylation pathways as well as in glycoinositolphospholipids and chitin synthesis (Galvani et al., 2015; Fruttero et al., 2017).

Insects lack adaptive immunity but they do possess an efficient and well-developed innate immune response (Lavine and Strand, 2002). Insects' immune system is broadly subdivided in humoral and cellular defenses. Humoral immunity includes the production of antimicrobial peptides, reactive intermediates of oxygen and nitrogen as well as the enzymatic complex cascades that regulate clotting or melanization of the hemolymph (Gillespie et al., 1997; Marmaras and Lampropoulou, 2009). On the other hand, cellular immunity encompasses hemocyte-mediated responses such as phagocytosis, nodulation and encapsulation (Jiravanichpaisal et al., 2006).

Our group recently described that Jaburetox affects the immune response of *R. prolixus*, triggering a cellular response, as shown by *in vivo* and *in vitro* aggregation of hemocytes as well as the increase in the activity of phenoloxidase, a key component of the humoral immunity (Fruttero et al., 2016). Moreover, we were able to demonstrate that this immune induction does not protect the insect against subsequent bacterial challenges, thus suggesting a Jaburetox-induced immunosuppressive effect on the insects (Fruttero et al., 2016).

Apart from being vital components of the defense system, hemocytes have multiple functions including synthesis and transport of nutrients and hormones for proper growth, and wound healing by connective tissue formation (Pandey and Tiwari, 2012). Taking into account the central role of these hemolymphatic cells in the insect's physiology and considering our previous findings pointing them as targets of Jaburetox, in this work we have explored biochemical, cellular and molecular biology aspects of the toxic effect induced by the peptide in the model insect *R. prolixus*. Our findings are discussed in the context of triatomine physiology and the current knowledge on the peptide's mode of toxic action.

2. Materials and methods

2.1. Chemicals

Goat anti-mouse IgG conjugated to Alexa 594 and anti-rabbit IgG conjugated to Alexa 488 antibodies, wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR, USA); mouse anti-α-tubulin monoclonal antibody and 4′,6-diamidino-2-phenylindole (DAPI) (Cell Signaling Technology, Danvers, MA, USA); Fluorsave (Merck Millipore, Darmstadt, Germany); PureLink® RNA Mini Kit; rabbit anti-uNOS polyclonal antibody and Phosphoprotein Phosphate Estimation Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA); High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA); GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) were purchased from the indicated commercial sources. The protease inhibitor cocktail, fluorescein isothiocyanate (FITC), 4-amino-5-methylamino-2′,7′-difluorofluorescein, diaminofluorescein-FM (DAF-FM) reagent, the anti-actin antibody and all the remaining reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Insects

All the assays were conducted with fifth instar nymphs, approximately seven days after ecdysis. The insects were provided by Dra. Alessandra Aparecida Guarnieri and Dr. José Rodrigues Coura (Oswaldo Cruz Institute, MG and RJ, Brazil, respectively). The insects were kept under controlled conditions of light (L:D = 12:12, lights on at 7.00 a.m.), temperature (27 ± 1 °C) and relative humidity (60%) in the facilities of UFRGS and fed at regular intervals of three weeks on human blood as described (Fruttero et al., 2016). All animal care and experimental protocols were conducted following the guidelines of the Committee for Ethics of Animal Use of the Universidade Federal do Rio Grande do Sul (CEUA-UFRGS) in compliance with resolutions of the National Council of Control in Animal Experimentation (CONCEA). All human blood donors gave their informed formal consent according to protocol C.A.A.E. 12574513.5.0000.5347, as approved by National Commission on Ethics in Research - CONEP.

2.3. Jaburetox and anti-Jaburetox antibody

The recombinant peptide Jaburetox was expressed and purified as described by Lopes et al., 2015. The anti-Jaburetox polyclonal antibody raised in rabbits was developed by Célula B – Serviço de Produção de Anticorpos, Porto Alegre, Brazil (http://www.ufrgs.br/celulab/index.htm).

2.4. In vivo treatment with Jaburetox

For all *in vivo* experiments, fifth-instar nymphs, weighing an average of 35 mg were employed. The insects were injected with Jaburetox (dose of 2 μg/insect) in 20 mM sodium phosphate buffer (PB, pH 7.4) or only PB for the controls as previously described (Fruttero et al., 2016). Hemolymph was collected with a micropipette from a cut in one of the legs, after previous surface sterilization of the insects with ethanol. The hemolymph from 5 insects was sampled 1, 3, 6 or 18 h after injections, pooled and diluted in anticoagulant solution (10 mM Na_2EDTA, 100 mM glucose, 62 mM NaCl, 30 mM sodium citrate, 26 mM citric acid, pH 4.6) at a ratio of 1:5 (anticoagulant:hemolymph) (Azambuja et al., 1991) unless stated otherwise. A few crystals of phenylthiourea were added to hemolymph to avoid melanization. Finally, the hemocytes were attached to slides by incubating them in a humid chamber for 1 h at room temperature. For this and the remaining experiments, cell viability was assessed by the Trypan Blue dye exclusion method (Boyse et al., 1964).

2.5. Cell culture

The hemocytes culture was establish as Defferrari et al. (2014) with minor modifications (Fruttero et al., 2016). Hemocytes were incubated with 100 nM Jaburetox for 5 and 24 h. The cultures were kept inside an incubator at 24 °C without CO_2 addition. Cells were viable for at least 10 days with the addition of fresh culture medium every 48 h.

2.6. Immunocytochemistry

Cells were washed with *Rhodnius* saline (Lane et al., 1975) and fixed with 4% paraformaldehyde for 20 min. All incubation steps were done in PB at room temperature. After the fixation, hemocytes were washed three times with PB (5 min each) and permeabilized/blocked with 0.1% Triton X100, 5% fetal bovine serum (FBS) in PB for 30 min. Subsequently, cells were incubated with 1% FBS plus anti-α-tubulin and anti-Jaburetox (1:1000 each) as primary antibodies, in PB for 1 h. The cells were washed again and a solution with 1% FBS containing the secondary antibodies conjugated to Alexa (dilution 1:400 each) was added. Controls were performed without the addition of primary or secondary antibodies as well as with cells not treated with Jaburetox (data not shown). After 1 h, the cells were washed and the nuclei stained with 0.1 μg/ml DAPI for 5 min. One final wash was done with distilled water; the slides were air-dried and mounted with Fluorsave. The slides were analysed with a Zeiss Axiovert 200 inverted fluorescence microscope equipped with an Axiocam MRc camera and appropriate filters for Alexa 488/DAF-FM (excitation 450–490 nm/emission 515–565 nm), Alexa 594 (excitation 540–552 nm/emission 575–640 nm) and DAPI (excitation 365 nm/emission 445–450 nm) (Carl Zeiss, Jena, Germany) and the images were acquired using AxioVision Rel 4.8 Software. For this and the remaining experiments, a magnification of 1000 × was employed in the analysis of the cells.

2.7. Jaburetox-induced changes on gene expression

Hemolymph from control and Jaburetox-treated insects (pools of 12 insects each) was collected 6 h post-injection in cold sterile microtubes containing *Rhodnius* saline in a proportion 1:4. The samples were centrifuged at

1000 × *g*, 5 min, room temperature, the supernatant discarded and the pelleted hemocytes employed for RNA extraction as described in Fruttero et al. (2017). Total RNA was extracted using the commercial PureLink® RNA Mini Kit, following manufacturer's instructions. cDNA was synthetized using the High-Capacity cDNA Reverse Transcription Kit and the resulting product was diluted 4 times and stored at − 20 °C. Gene expression experiments were carried out in the equipment Eco Real-Time PCR System using software Eco (Illumina Inc., San Diego, CA, USA), using the GoTaq® qPCR Master Mix. All samples were analysed in quadruplicate and the reaction parameters were: initial denaturation at 95 °C, for 2 min, followed by 40 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 15 s) and extension (72 °C, 15 s), finishing in a melting curve (55–95 °C, with 0.1 °C increments/s). The primers employed were 5′-GCACGATGTTTGATGTCCTG-3′ and 5′-GCAAAGCGAACCATTTCAAG-3′ for *NOS*, 5′-GCGGAGCTAAAGTTGTGGAG-3′ and 5′-CATCGTTGTTACGCATTTCAGC-3′ for *UAP* and 5′-CATGAAAGCAATGGTGGATGTTGTAGCAATTG-3′ and 5′- ACTGAATGTTTAGAGACATAGTTCTCCCACC-3′ for *chitin synthase*. Primers for the normalizer *elongation factor 1* (5′-GATTCCACTGAACCGCCTTA-3′ and 5′-GCCGGGTTATATCCGATTTT-3′) were based on Majerowicz et al. (2011). The results were analysed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. Jaburetox-induced changes on NOS protein expression

Hemolymph from control and Jaburetox-injected insects (pools of 5 insects each) was collected 6 h post-injection in PB and protease inhibitors, homogenized and centrifuged at 10,000 × *g* for 5 min at 4 °C. Forty micrograms of protein samples were subjected to 8% SDS-PAGE and then transferred to nitrocellulose membranes, blocked and incubated with the anti-uNOS antibody overnight (1:1000) followed by an anti-rabbit HRP-conjugated antibody (1:5000) (Galvani et al., 2015). The bands were detected by enhanced chemiluminescence (ECL) using a commercial kit. Loading control was carried out using a polyclonal anti-actin antibody (1:500).

2.9. Enzymatic activities and phosphate content estimation

For measurement of UAP activity, insects were injected with Jaburetox in 50 mM Tris–HCl (pH 7.5). The hemolymph was collected and pooled from 5 insects in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) with protease inhibitors, homogenized and centrifuged (10,000 × *g*, 10 min, 4 °C). UAP activity was determined by spectrophotometric techniques in the supernatant following the methodology described by Mio et al. (1998) with slight modifications (Fruttero et al., 2017). Specific activity was expressed as the absorbance (A_{655}) per mg of protein.

For determination of acid phosphatase (AP) activity, hemolymph was collected 6 h after injection, pooled from 5 insects in 20 mM sodium acetate buffer pH 4.0 containing 10 mM DTT, 10 mM Na_2EDTA and protease inhibitors, homogenized and centrifuged at 14,000 × *g* for 5 min at 4 °C. Acid phosphatase activity was measured by spectrophotometric techniques as described previously (Leyria et al., 2015; Fruttero et al., 2017). Specific activity was expressed as nmol of *p*NP/mg protein/min.

The phosphate content of hemolymph from control and Jaburetox-treated insects was assessed employing the Phosphoprotein Phosphate Estimation Assay Kit. The insects were injected with Jaburetox in 50 mM Tris–HCl (pH 7.5) or with buffer alone (controls), the hemolymph from 3 insects was pooled and collected 6 h afterwards in *Rhodnius* saline with a few crystals of phenylthiourea. The samples were homogenized, centrifuged (10,000 × *g*, 10 min, 4 °C) and the phosphate content (expressed as μg of phosphorus/μg of protein) of the supernatants was estimated according to the manufacturer's instructions.

2.10. NO imaging assay

The DAF-FM probe was used to obtain information regarding NO production (Gazos-Lopes et al., 2012) by hemocytes treated with Jaburetox *in vivo*. The hemolymph of control and Jaburetox-treated insects was collected 6 h post-injection, the hemocytes were adhered to poly-L-lysine-coated slides for 1 h at room temperature and thereafter incubated with 1 mM DAF-FM in *Rhodnius* saline for 1 h. The cells were washed with saline and immediately analysed upon fluorescence microscopy as stated in Section 2.6.

The relative intensity of fluorescence of the images was determined by establishing the mean grey value of pixels in manually defined areas of the cells using ImageJ software. The background was subtracted by taking a representative area as reference using the plugin "BG subtraction from ROI". The threshold for the signal was established considering the fluorescence detected in the control experiments. The assay was repeated three times and the digital data of 50 individual cells for each treatment was recorded.

2.11. Effect of in vivo Jaburetox treatment on phagocytosis

The phagocytic activity was assessed by incubating control and *in vivo* Jaburetox-treated hemocytes, previously adhered to slides, with *Saccharomyces cerevisiae* cells labelled with FITC as described in Figueiredo et al. (2006, 2008) with slight modifications (Fruttero et al., 2016). The number of yeasts associated and internalized by the hemocytes was counted; the experiment was repeated 4 times, examining 50 hemocytes in each one.

2.12. Hemocytes activation by WGA staining and fluorescence quantitation

The hemolymph from controls and Jaburetox-treated insects was collected 6 h after the injections and the hemocytes adhered to poly-L-lysine-coated slides. Cells were fixed and permeabilized/blocked as in Section 2.6, incubated with 2.5 μg/ml of WGA-Alexa Fluor 488 (30 min, 37 °C), with 0.1 μg/ml DAPI and finally washed with distilled water. Slides were processed for fluorescence microscopy.

The relative intensity of cell fluorescence was determined as described in Section 2.10.

2.13. Statistical analysis

Student's *t*-test or one-way ANOVA were used for comparisons and a P value < 0.05 was considered statistically significant. Graphs and statistical tests were performed using the software programs GraphPad Prism 5 and GraphPad Instat 3.0 (San Diego, CA, USA). Unless otherwise stated, results were expressed as mean ± standard error of the mean (SEM) and all the experiments were performed at least in triplicates.

3. Results

In order to study the interaction of Jaburetox with hemocytes, immunofluorescence assays were conducted after *in vivo* and *in vitro* treatments with the peptide. As can be seen in Figs. 1 and 2, upon injection, the Jaburetox distribution in the cells is variable, since the signal for the peptide was found in the nuclei, in the perinuclear region and dispersed throughout the cytosol and the cell surface. The Jaburetox label was observed either as a punctate pattern in the cytoplasm and/or the nucleus (Figs. 1 and 2) or as a diffused signal (Fig. 2), suggesting an interaction with the cell surface (probably including the cytoskeleton). The variety of fluorescence patterns was visualized at all times investigated: 1 and 3 h post-injection (Fig. 1), 6 h and 18 h post-injection (Fig. 2). Not all cells exhibited Jaburetox-associated fluorescence. There was, however, an increase in the cells with Jaburetox label when we compared the longer time (18 h post-injection, ~ 35%) *vs* the shorter ones (1 and 3 h post-injection, ~ 5–10%). The finding of isolated nuclei (very likely caused by the breakdown of the cytoplasm as explained in Azambuja et al., 1991) stained

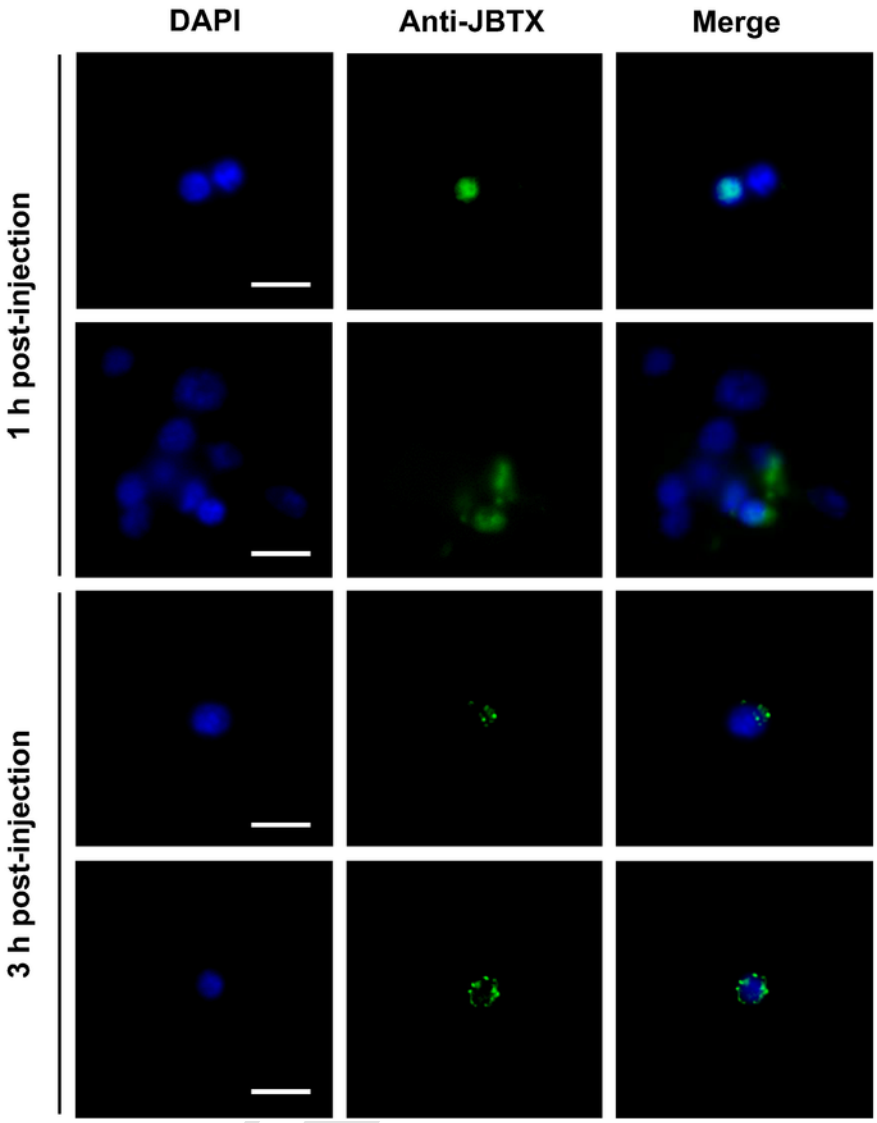

Fig. 1. Jaburetox localization in hemocytes from injected insects. The hemolymph was collected 1 and 3 h after injection and the hemocytes adhered to the slides as described in Materials and methods. In the images, the fluorescence pattern for DAPI is shown in blue and that of the anti-Jaburetox/Alexa 488 is shown in green. Bars: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with Jaburetox (Fig. 2), strengthens the hypothesis that this organelle is a cell target of the toxic peptide, thus indicating a possible effect on gene expression. The formation of Jaburetox-positive aggregates was seen as early as 1 h post-injection (Fig. 1) and maintained throughout the times analysed.

Similar to the *in vivo*-treated cells, positive signaling for Jaburetox was observed in cultured cells after the incubation with 100 nM of the peptide (Fig. 3). Also in line with the above findings, a variety of fluorescence patterns was observed at shorter and longer times post-incubation, including nuclear, cytoplasmatic and cell surface labelling. Moreover, in some cells, the Jaburetox label coincided with cells undergoing chromatin condensation (Figs. 3, 5 h incubation). Again, most of the fluorescence patterns associated with Jaburetox (punctate, diffuse, in the nucleus, in the cytoplasm, *etc.*) were visualized in all assayed times.

For the remaining experiments, we chose to focus on *in vivo* approaches to facilitate comparisons to our previous works (Fruttero et al., 2016, 2017). Taking into account that Jaburetox induces modifications in gene expression (Fruttero et al., 2017) and that the toxic peptide was found in the nucleus, we decided to study the gene expression of target enzymes for Jaburetox. As showed in the Fig. 4a, the *in vivo* treatment with the peptide elicited a modification in the gene expression of all assayed genes, being the increment of NOS gene expression remarkably high. Notwithstanding, the Jaburetox injection did not modify NOS protein expression, as depicted in Fig. 4b. Likewise, when the UAP activity was measured in the hemolymph of control and treated insects, no changes were observed (Fig. S1a). No significant differences were seen in the activity of acid phosphatase, a possible link between NOS and UAP (Fig. S1b) nor in the phosphate content (indicative of the phosphorylation state of proteins) of control and Jaburetox-treated hemolymph samples (Fig. S1c).

A series of cellular approaches were undertaken to explore the Jaburetox-induced responses in hemocytes. By means of NO imaging, it was demonstrated that the *in vivo* Jaburetox treatment caused a local increase of NO production (visualized as higher fluorescent signal) coincident with aggregates (Fig. 5). That fluorescence intensification was not found in the control aggregates. Moreover, no significant changes in the signal were observed after fluorescence quantification of free cells in both treatments (Fig. 5).

On the other hand, the phagocytic behavior of hemocytes obtained from injected insects with Jaburetox was studied following the methodology of Figueiredo et al. (2006, 2008). No significant differences were detected in the number of fluorescently-labelled yeast cells associated or internalized by the hemocytes when controls and Jaburetox-treated cells were compared (Fig. 6)

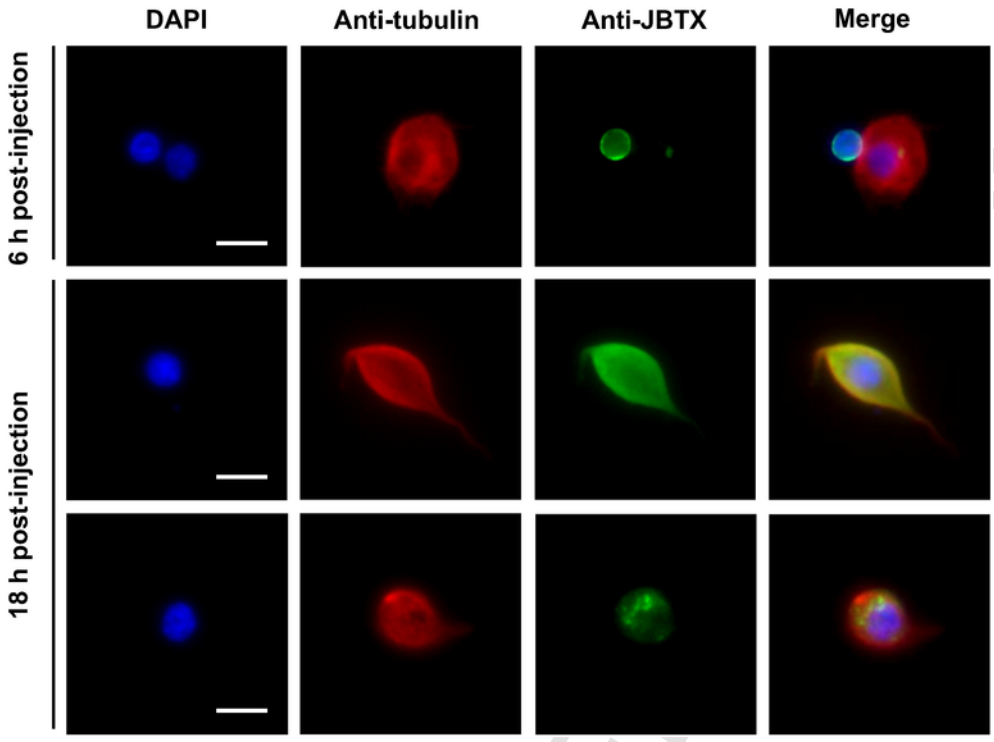

Fig. 2. Jaburetox localization in hemocytes from injected insects. The hemolymph was collected 6 and 18 h after injection and the hemocytes adhered to the slides as described in Materials and methods. In the images, the fluorescence pattern for DAPI is shown in blue, for the anti-α-tubulin/Alexa 594 in red and for the anti-Jaburetox/Alexa 488 is shown in green. Bars: 20 μm.

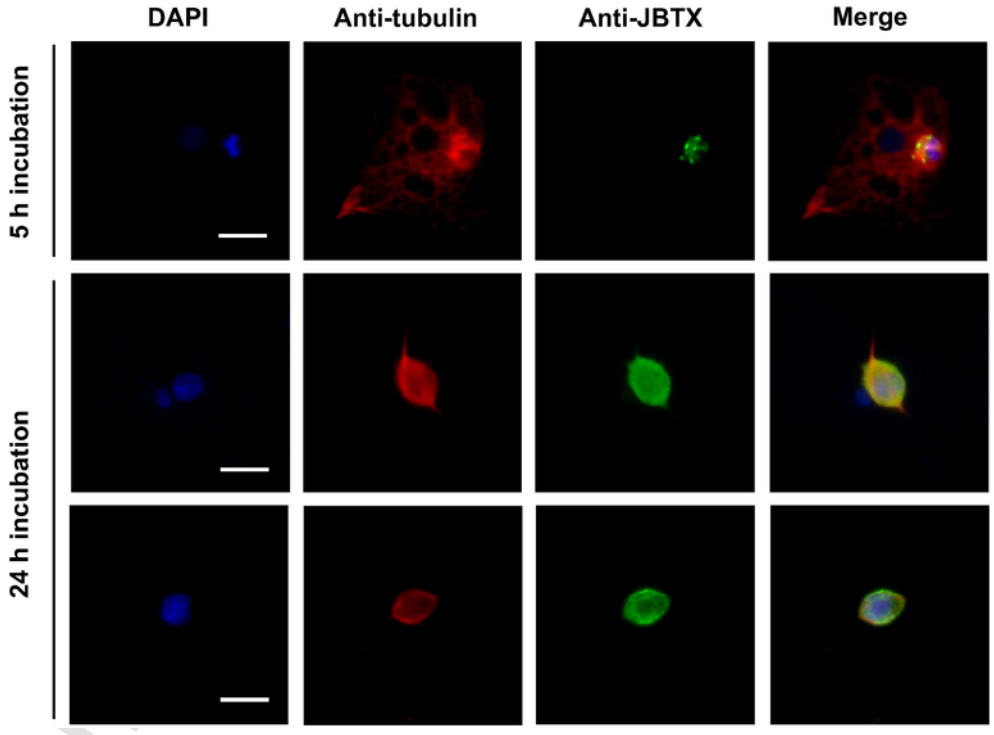

Fig. 3. Jaburetox localization in cultured hemocytes. The hemolymph was collected in sterility and the hemocytes were cultured in a special insect medium as described in Materials and methods. Cells were incubated for 5 and 24 h with 100 nM Jaburetox and processed for fluorescence microscopy. In the images, the fluorescence pattern for DAPI is shown in blue, the anti-α-tubulin/Alexa 594 in red and the anti-Jaburetox/Alexa 488 in green. Bars: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

although a trend of Jaburetox-treated cells to internalize 5 or more yeast cells was seen.

The capacity of the hemocytes to interact with WGA lectin was also explored in cells treated *in vivo* with Jaburetox. As observed in Fig. 7, Jaburetox activates the hemolymphatic cells since the WGA binding activity was higher

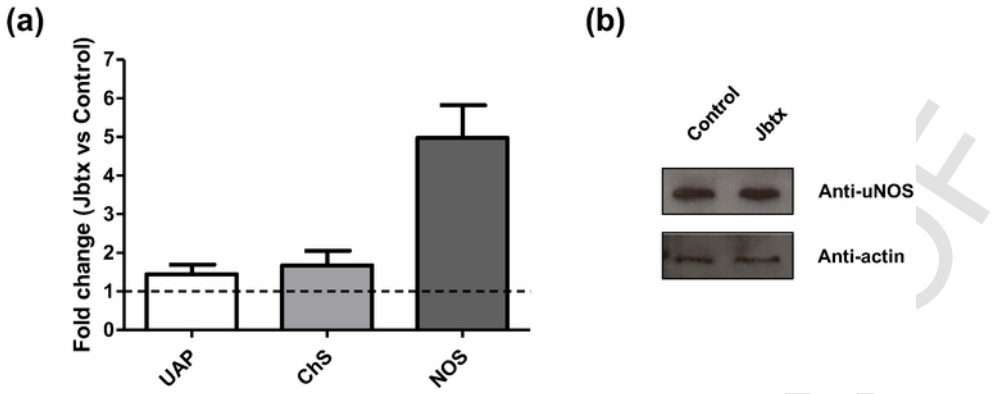

Fig. 4. Effect of the *in vivo* Jaburetox treatment on mRNA and protein expression. Insects were injected with Jaburetox (2 μg/insect) in PB or with PB alone (control) and the hemolymph collected 6 h afterwards. Samples were processed as described in Materials and methods. **(a)** UAP, chitin synthase and NOS mRNA expression as measured by qPCR. Elongation factor 1 was employed as normalizer and the results are expressed in relation to control insects. Results are expressed as fold change and are means ± SEM (n = 4) of a typical experiment. **(b)** Effect of the *in vivo* Jaburetox treatment on NOS protein expression as determined by Western blot. The samples were processed as described in Materials and methods and subjected to SDS-PAGE and Western blot employing an anti-uNOS antibody and an anti-actin antibody as loading control. The immunoreactive bands for NOS and actin presented the expected molecular weights (~ 132 kDa and ~ 40 kDa, respectively).

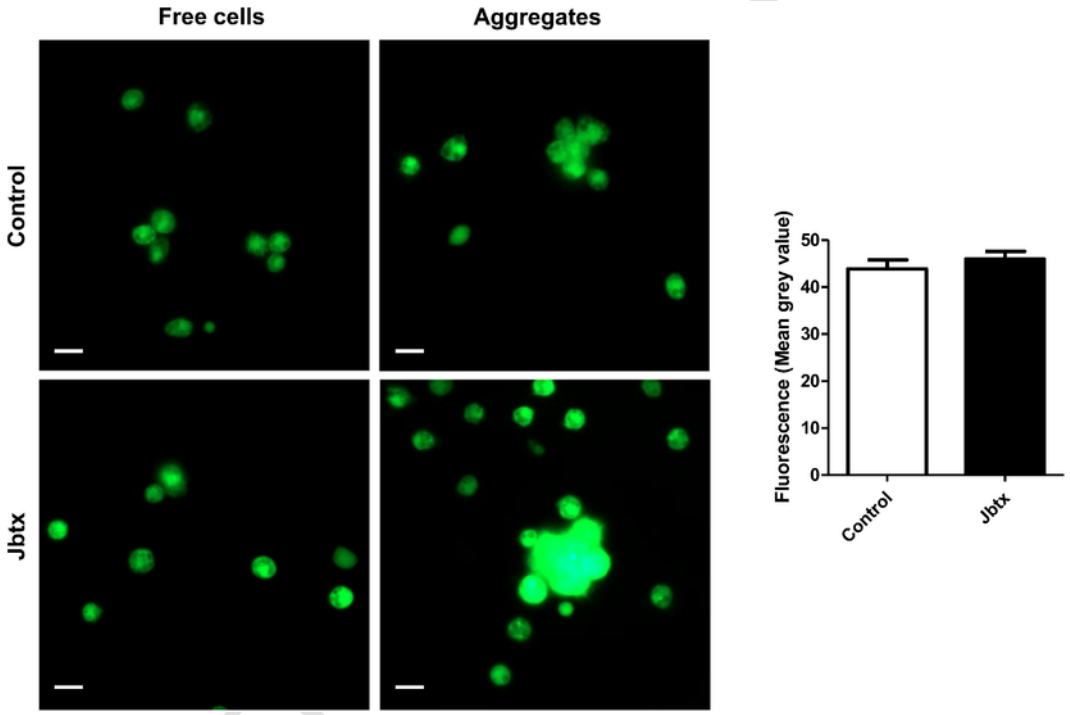

Fig. 5. NO imaging in hemocytes from injected insects. The hemolymph was collected 6 h after injection and the hemocytes adhered to the slides as described in Materials and methods. The hemocytes were incubated with the NO fluorescent probe DAF-FM (green) for 1 h and analysed by fluorescence microscopy. Bars: 20 μm. Right panel: fluorescence quantitation of free cells using ImageJ software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the cells treated with the peptide than in controls. Fluorescence quantification of the images confirmed this finding.

4. Discussion

Jaburetox presents several characteristics of a promising tool for rational pest control. First, the peptide is an insecticide that kills a variety of species, including not only crop pests as the fall armyworm *Spodoptera frugiperda* or the cotton stainer bug *Dysdercus peruvianus* but also the milkweed bug *Oncopeltus fasciatus*, the German cockroach *Blattella germanica* and the kissing bugs *R. prolixus* and *T. infestans* (Reviewed in Stanisçuaski and Carlini, 2012). Second, Jaburetox seems to be safe for mammals since the administration of the peptide did not cause acute toxicity to rodents (Mulinari et al., 2007) and mammalian cell lines incubated with Jaburetox did not show evidences of cyto- or genotoxicity (Portugal, 2017). Thus, ideally, the employment of Jaburetox as a transgene in commercial crops would diminish the need for chemical insecticides, and under the control of tissue-specific or damage-associated promoters, it could decrease environmental damage by mainly affecting pest species and leaving beneficial ones unharmed. Preliminary findings with Jaburetox-expressing crops are already showing auspicious results (Carlini and Ligabue-Braun, 2016).

To facilitate the biotechnological use of Jaburetox, it would be desirable to understand comprehensively its mode of action. Several reports from our group have shed light on various aspects of the peptide's toxicity, including its disruptive effect on lipid membranes (Barros et al., 2009), the alteration of the transmembrane potential and the inhibition of diuresis in Malpighian tubules of *R. prolixus* (Stanisçuaski et al., 2009). On the other hand, Martinelli et al. (2014) reported that Jaburetox affects the neuromuscular junction of the cockroach *Nauphoeta cinerea* reducing the strength of the muscle contraction. More recently, Galvani et al. (2015) established that the insect's CNS is a tar-

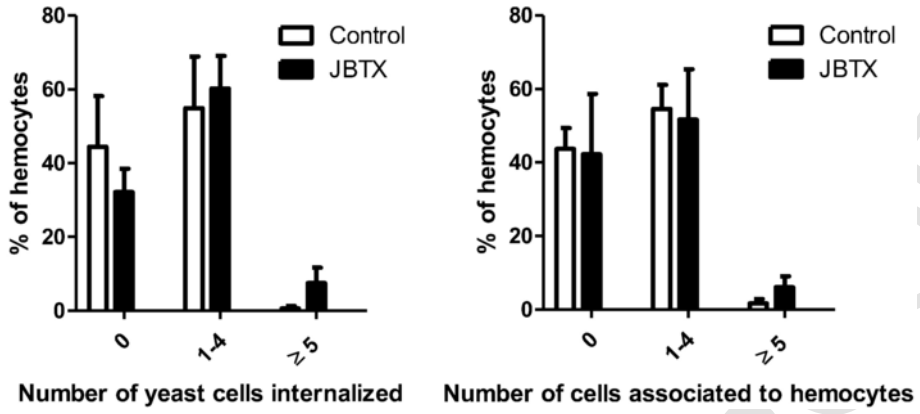

Fig. 6. Phagocytosis assay in hemocytes from insects injected with Jaburetox. Hemocytes were collected 6 h after injection, adhered to slides as described in Materials and methods and incubated with 1×10^7 FITC-conjugated yeast cells/ml for 1 h. The yeast cells internalized by the hemocytes and associated to them were counted and the values represent the mean ± SEM (n = 4).

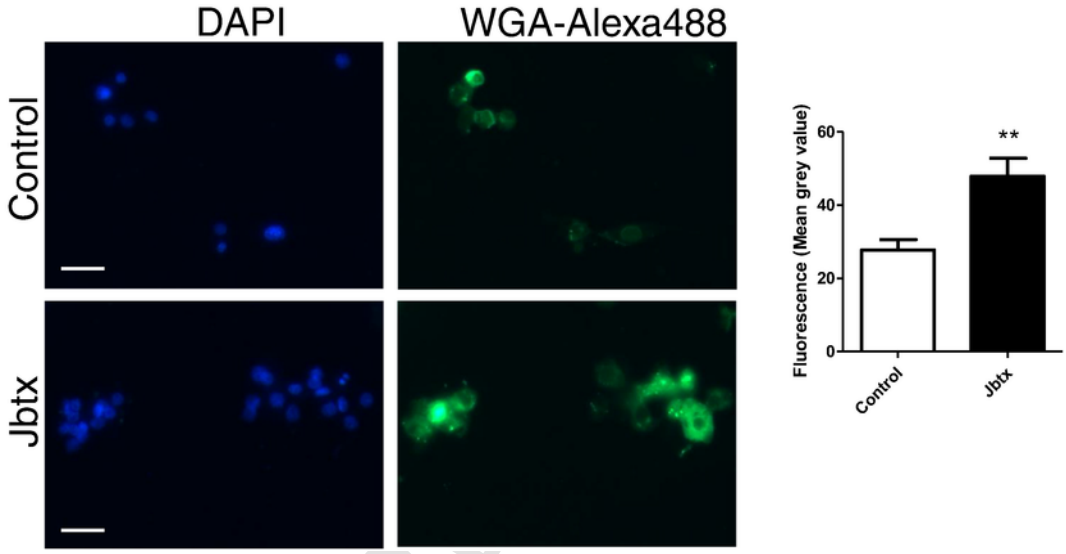

Fig. 7. Hemocyte activation. The hemolymph was collected 6 h after injection and the hemocytes adhered to the slides as described in Materials and methods. The hemocytes were incubated with the wheat germ agglutinin-Alexa 488 probe (green) for 1 h and processed for fluorescence microscopy. Bars: 20 μm. Right panel: Fluorescence quantitation using ImageJ software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

get of the toxic peptide, while Fruttero et al. (2017), using *R. prolixus*, corroborated the relevance of this organ in the Jaburetox's mechanism of action and added the salivary glands as another Jaburetox-affected organ.

In this work, our results indicated that the hemocytes are also a direct target of Jaburetox since, upon injection or incubation, several immunolabelled cells were detected (Figs. 1–3). Interestingly, the occurrence of the diverse peptide localizations (nucleus, cytoplasm and cell surface) does not seem to depend on the time post-incubation or post-injection because the different patterns were observed for all assayed times (Figs. 1–3). In similar *in vitro* incubation experiments employing cultured *R. prolixus* hemocytes exposed to the jack bean urease (JBU), Defferrari et al. (2014) also found a variable distribution of JBU in the cells, as the urease was spotted in the cytosol as well as at the cell surface. It is worth to mention that Jaburetox is a part of the JBU molecule and it is well exposed at the protein's surface (Piovesan et al., 2014), which might explain the similar subcellular distribution of the molecules seen in the hemocytes. Expectably, in our model, the percentage of Jaburetox positive cells increased after longer times post-injection, although not all hemocytes were immunolabelled. This is in line with our previous results in cultured hemocytes incubated with Jaburetox, in which only a subset of treated cells presented chromatin condensation and apoptosis (Fruttero et al., 2016). The affinity of the Jaburetox for lipid membranes and its preference for interaction with certain types of lipids is well described (Barros et al., 2009; Piovesan et al., 2014; Micheletto et al., 2016). Because there are several hemocyte classes defined in *R. prolixus* (Azambuja et al., 1991), it is possible that each type presents different lipid composition of their plasma membrane, thus rendering more or less favorable interactions with the peptide. Future approaches, employing specific markers and cell sorting, will be directed to identify the specific hemocyte type(s) that interact with the peptide.

The gaseous transmitter nitric oxide (NO) is generated by the activity of NOS by converting L-arginine to L-citrulline. In insects, NO signaling has been implicated in several functions including learning and memory, axonal guidance, chemosensory, mechanosensory and visual processing (Davies, 2000). In *R. prolixus*, salivary NO acts as a vasodilator and platelet anti-aggregating agent during blood-feeding (Ribeiro and Nussenzveig, 1993). Moreover, NO participates in invertebrate immunity, increasing its levels in response to parasite challenges (Dimopoulos et al., 1998; Luckhart et al., 1998; Hahn et al., 2001). NO can cause oxidation of heme groups and nitrosylation of amino acids, thus affecting the secondary structure of pathogen proteins (Radi, 2004). Our results from this and other works (Fruttero et al., 2016, 2017) indicate that the Jaburetox effects on the nitrinergic system are complex. Jaburetox injections led to a marked increase in NOS mRNA expression of hemocytes (Fig. 4a), although the protein expression (Fig. 4b) and the enzyme activity levels remained unchanged when compared with those of vehicle-injected controls (Fruttero et al., 2016). Nevertheless, local increments in

NO production were observed in Jaburetox-induced aggregates but not in aggregates of controls (Fig. 5). This profile, in which Jaburetox increases NOS mRNA expression but does not modify the enzyme activity, was previously observed in the salivary glands of *R. prolixus* nymphs fed with Jaburetox (Fruttero et al., 2017). A different pattern was observed in the CNS of nymphs fed with the peptide in which NOS mRNA remained unchanged but the enzyme activity was significantly decreased (Fruttero et al., 2017). Galvani et al. (2015) reported in *T. infestans* that Jaburetox injections triggered a diminution in NOS enzyme activity accompanied by a decrease in NO levels, without changing the expression of NOS protein. These findings reinforce the views that: a) Jaburetox affects the nitrinergic system; b) the peptide's effects are tissue-specific and c) some effects can be triggered irrespective of the administration mode (injection or feeding).

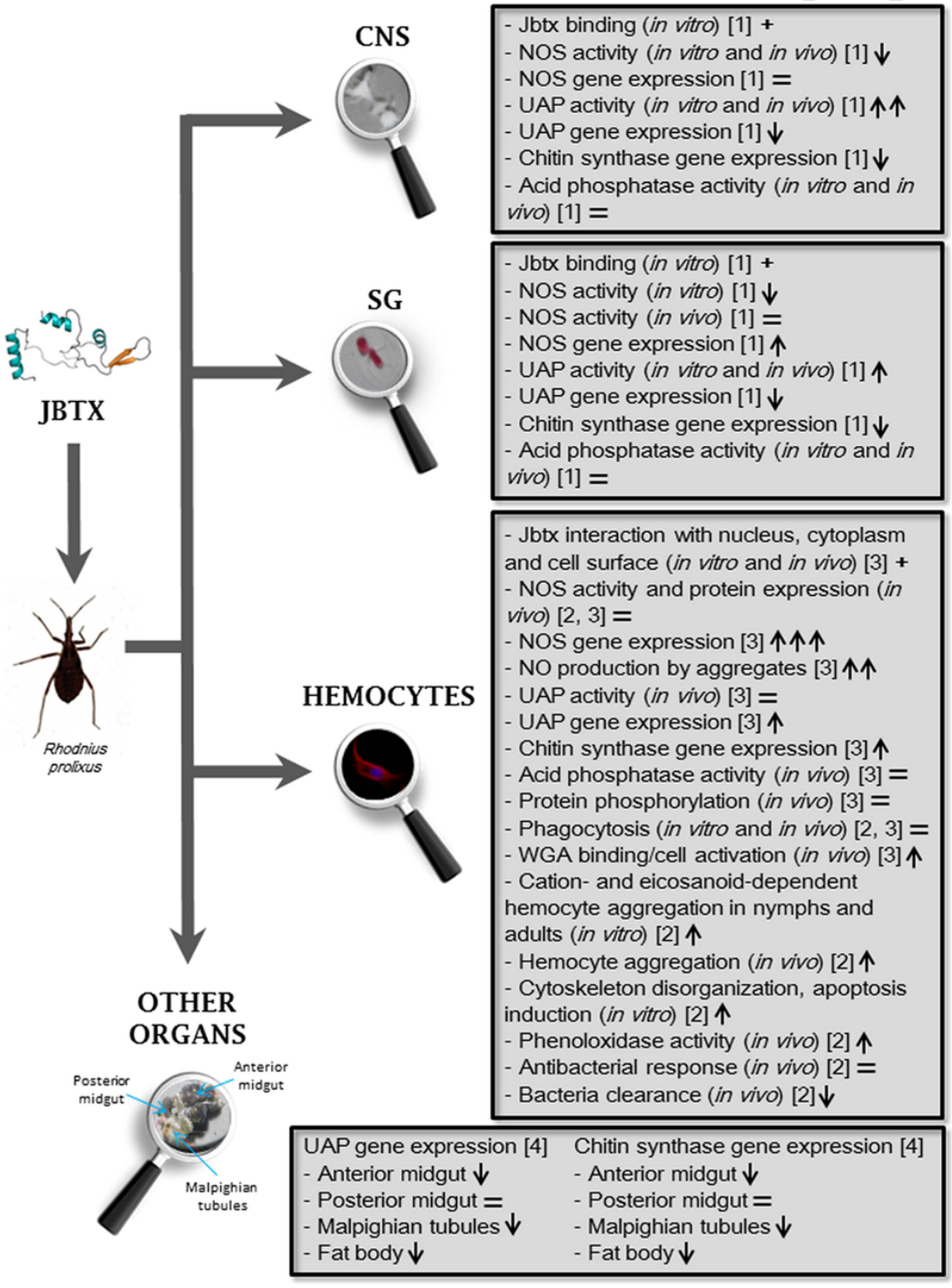

Fig. 8. Jaburetox-induced effects on *R. prolixus*. The number and direction of arrows indicates the type of effect (increase or decrease) exerted by the peptide in that parameter. Plus signs indicate presence and equal signs represent no significant changes. CNS: central nervous system; SG: salivary glands; Jbtx: Jaburetox. The *in vivo* Jaburetox administration was made by injection in the case of hemocytes and by feeding in the other organs. [1] Fruttero et al., 2017; [2] Fruttero et al., 2016; [3] present work; [4] unpublished results.

UDP-*N*-acetylglucosamine is an essential, widely distributed metabolite that plays a major role in several metabolic processes (Moussian, 2008). UDP-*N*-acetylglucosamine is the substrate of chitin synthase and is the precursor of *N*-linked glycosylation and GPI-anchors of cellular proteins, among others (Merzendorfer, 2006, 2011). Chitin in insects has been reported as component of the cuticle, salivary glands, trachea, peritrophic matrix (Merzendorfer, 2006, 2011) and perimicrovillar membrane (Alvarenga et al., 2016). UDP-*N*-acetylglucosamine pyrophosphorylase is the enzyme involved in the generation of UDP-*N*-acetylglucosamine (Mio et al., 1998; Kramer and Muthukrishnan, 2005). Galvani et al. (2015) established in *T. infestans* that UAP physically interacted with the injected Jaburetox in the CNS and that the peptide injections led to an increment in UAP's enzymatic activity. Likewise, Fruttero et al. (2017) corroborated the Jaburetox-induced increment in UAP activity in the CNS and salivary glands of *R. prolixus* nymphs fed with the peptide, although the treatment also triggered a decrease in the UAP and chitin synthase mRNA expression. Our results in the hemocytes of Jaburetox-injected insects showed an increment in UAP (and chitin synthase) mRNA (Fig. 4a) without a modification in the UAP enzyme levels (Fig. S1a), stressing again the organ-specific effect of the toxic peptide. Gazos-Lopes et al. (2012) reported that, in the salivary glands of *R. prolixus*, an increase in glycoinositolphospholipids led to a decrease in NOS and acid phosphatase activity. Taking into account that finding and the fact that phosphorylation-dephosphorylation can regulate NOS activity (Alderton et al., 2001); we investigated whether Jaburetox could induce changes in the acid phosphatase activity of hemocytes. Nonetheless, no differences were observed in the hemolymph of injected insects when compared to control ones (Fig. S1b). The phosphorylation state of proteins also appears to be not affected by the toxic peptide because the phosphate content of hemolymphatic proteins did not significantly change between control and Jaburetox-treated samples (Fig. S1c).

Previous experiments of our group have shown that cultured hemocytes incubated 24 h with Jaburetox did not modify their phagocytic behavior against FITC-labelled yeast (Fruttero et al., 2016). Similar results were obtained here, since hemocytes derived from Jaburetox-injected insects did not show significant changes in the number of yeast internalized or associated to them (Fig. 6), highlighting the fact that, in our experimental conditions, not all hemocytes are affected by the Jaburetox treatment.

Drosophila melanogaster larvae infection by larvae of parasitic wasps is an established model for hemocyte activation. The parasitic challenge is characterized by cell aggregation, increasing WGA lectin binding and Ras-mitogen activated protein kinase (MAPK) signaling (Mortimer et al., 2012). The process of blood feeding in the mosquito *Anopheles gambiae*, also considered an immune challenge, similarly activates the hemocytes, leading to higher WGA binding (Bryant and Michel, 2014). In our model, the hemocytes from Jaburetox-injected nymphs demonstrated an increase in WGA binding (Fig. 7), indicating that the treatment changed the glycoconjugates content at the cell surface.

5. Conclusion

In this work, we confirmed that hemocytes are a target of Jaburetox and described for the first time the subcellular distribution of the toxic peptide in these cells. It was also reinforced the view that the Jaburetox effect is tissue- and organ-specific and that some responses can be elicited administrating the peptide orally, *via* injection or even *in vitro* by incubation (Fruttero et al., 2017). We corroborated the involvement of the nitrinergic system as well as the complexity of Jaburetox effect on that pathway, affecting differentially NO production, NOS mRNA and protein expression and NOS activity in a tissue-specific manner (Fruttero et al., 2016). Jaburetox appears to act on a subset of hemocytes, activating them and leading to an increase in NO production by cell aggregates, resembling a response to an infection. This is in line with our previous findings that Jaburetox triggers a cellular and humoral immune response (Fruttero et al., 2016) and strengthens the hypothesis that the insect immune machinery recognizes the peptide as a pathogen associated molecular pattern of the Jaburetox's toxic effects, encompassing several organ systems and affecting metabolite production, enzyme activities and gene expression.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2017.06.001.

Conflict of interests

The authors declare no conflict of interests regarding this work.

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References

Alderton, W.K., Cooper, C.E., Knowles, R.G., 2001. Nitric oxide synthases: structure, function and inhibition. Biochem. J. 357, 593–615.

Alvarenga, E.S., Mansur, J.F., Justi, S.A., Figueira-Mansur, J., Dos Santos, V.M., Lopez, S.G., Masuda, H., Lara, F.A., Melo, A.C., Moreira, M.F., 2016. Chitin is a component of the Rhodnius prolixus midgut. Insect Biochem. Mol. Biol. 69, 61–70.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A., 1991. Aspects of classification of Hemiptera hemocytes from six triatomine species. Mem. Inst. Oswaldo Cruz 86, 1–10.

Barros, P.R., Stassen, H., Freitas, M.S., Carlini, C.R., Nascimento, M.A., Follmer, C., 2009. Membrane-disruptive properties of the bioinsecticide Jaburetox-2Ec: implications to the mechanism of the action of insecticidal peptides derived from ureases. Biochim. Biophys. Acta 1794, 1848–1854.

Boyse, E.A., Old, L.J., Chouroulinkov, I., 1964. Cytotoxic test for demonstration of mouse antibody. Methods Med. Res. 10, 39–47.

Bryant, W.B., Michel, K., 2014. Blood feeding induces hemocyte proliferation and activation in the African malaria mosquito, Anopheles gambiae Giles. J. Exp. Biol. 217, 1238–1245.

Carlini, C.R., Ligabue-Braun, R., 2016. Ureases as multifunctional toxic proteins: a review. Toxicon 110, 90–109.

Davies, S., 2000. Nitric oxide signalling in insects. Insect Biochem. Mol. Biol. 12, 1123–1138.

Defferrari, M.S., Demartini, D.R., Marcelino, T.B., Pinto, P.M., Carlini, C.R., 2011. Insecticidal effect of Canavalia ensiformis major urease on nymphs of the milkweed bug Oncopeltus fasciatus and characterization of digestive peptidases. Insect Biochem. Mol. Biol. 41, 388–399.

Defferrari, M.S., Lee, D.H., Fernandes, C.L., Orchard, I., Carlini, C.R., 2014. A phospholipase A2 gene is linked to Jack bean urease toxicity in the Chagas' disease vector Rhodnius prolixus. Biochim. Biophys. Acta 1840, 396–405.

Dimopoulos, G., Seeley, D., Wolf, A., Kafatos, F.C., 1998. Malaria infection of the mosquito Anopheles gambiae activates immune-responsive genes during critical transition stages of the parasite life cycle. EMBO J. 21, 6115–6123.

Figueiredo, M.B., Castro, D.P., Nogueira, N.F.S., Garcia, E.S., Azambuja, P., 2006. Cellular immune response in Rhodnius prolixus: role of ecdysone in hemocyte phagocytosis. J. Insect Physiol. 52, 711–716.

Figueiredo, M.B., Genta, F.A., Garcia, E.S., Azambuja, P., 2008. Lipid mediators and vector infection: Trypanosoma rangeli inhibits Rhodnius prolixus hemocyte phagocytosis by modulation of phospholipase A2 and PAF-acetylhydrolase activities. J. Insect Physiol. 54, 1528–1537.

Fruttero, L.L., Moyetta, N.R., Uberti, A.F., Grahl, M.V., Lopes, F.C., Broll, V., Feder, D., Carlini, C.R., 2016. Humoral and cellular immune responses induced by the urease-derived peptide Jaburetox in the model organism Rhodnius prolixus. Parasit. Vectors 9, 412.

Fruttero, L.L., Moyetta, N.R., Krug, M.S., Broll, V., Coste Grahl, M.V., Real-Guerra, R., Stanisçuaski, F., Carlini, C.R., 2017. Jaburetox affects gene expression and enzyme activities in Rhodnius prolixus, a Chagas' disease vector. Acta Trop. 168, 54–63.

Galvani, G.L., Fruttero, L.L., Coronel, M.F., Nowicki, S., Demartini, D.R., Defferrari, M.S., Postal, M., Canavoso, L.E., Carlini, C.R., Settembrini, B.P., 2015. Effect of the urease-derived peptide Jaburetox on the central nervous system of Triatoma infestans (Insecta: Heteroptera). Biochim. Biophys. Acta 1850, 255–262.

Gazos-Lopes, F., Mesquita, R.D., Silva-Cardoso, L., Senna, R., Silveira, A.B., Jablonka, W., Cudischevitch, C.O., Carneiro, A.B., Machado, E.A., Lima, L.G., Monteiro, R.Q., Nussenzveig, R.H., Folly, E., Romeiro, A., Vanbeselaere, J., Mendonça-Previato, L., Previato, J.O., Valenzuela, J.G., Ribeiro, J.M., Atella, G.C., Silva-Neto, M.A., 2012. Glycoinositolphospholipids from Trypanosomatids subvert nitric oxide production in Rhodnius prolixus salivary glands. PLoS One 7, e47285.

Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. Annu. Rev. Entomol. 42, 611–643.

Hahn, U.K., Bender, R.C., Bayne, C.J., 2001. Involvement of nitric oxide in killing of Schistosoma mansoni sporocysts by hemocytes from resistant Biomphalaria glabrata. J. Parasitol. 87, 778–785.

Jiravanichpaisal, P., Lee, B.L., Soderhall, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213–236.

Kramer, K.J., Muthukrishnan, S., 2005. Chitin metabolism in insects. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science. 4. Elsevier, New York, pp. 111–144.

Lane, N.J., Leslie, R.A., Swales, L.S., 1975. Insect peripheral nerves: accessibility of neurohaemal regions to lanthanum. J. Cell Sci. 18, 179–197.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(− Delta Delta C(T)) method. Methods 25, 402–408.

Lopes, F.C., Dobrovolska, O., Real-Guerra, R., Broll, V., Zambelli, B., Musiani, F., Uversky, V.N., Carlini, C.R., Ciurli, S., 2015. Pliable natural biocide: Jaburetox is an intrinsically disordered insecticidal and fungicidal polypeptide derived from jack bean urease. FEBS J. 282, 1043–1064.

Luckhart, S., Vodovotz, Y., Cui, L., Rosenberg, R., 1998. The mosquito Anopheles stephensi limits malaria parasite development with inducible synthesis of nitric oxide. Proc. Natl. Acad. Sci. U. S. A. 95, 5700–5705.

Majerowicz, D., Alves-Bezerra, M., Logullo, R., Fonseca-de-Souza, A.L., Meyer-Fernandes, J.R., Braz, G.R., Gondim, K.C., 2011. Looking for reference genes for real-time quantitative PCR experiments in Rhodnius prolixus (Hemiptera: Reduviidae). Insect Mol. Biol. 20, 713–722.

Marmaras, V.J., Lampropoulou, M., 2009. Regulators and signalling in insect haemocyte immunity. Cell. Signal. 21, 186–195.

Martinelli, A.H., Kappaun, K., Ligabue-Braun, R., Defferrari, M.S., Piovesan, A.R., Stanisçuaski, F., Demartini, D.R., Dal Belo, C.A., Almeida, C.G., Follmer, C., Verli, H., Carlini, C.R., Pasquali, G., 2014. Structure-function studies on jaburetox, a recombinant insecticidal peptide derived from jack bean (Canavalia ensiformis) urease. Biochim. Biophys. Acta 1840, 935–944.

Merzendorfer, H., 2006. Insect chitin synthases: a review. J. Comp. Physiol. B. 176, 1–15.

Merzendorfer, H., 2011. The cellular basis of chitin synthesis in fungi and insects: common principles and differences. Eur. J. Cell Biol. 90, 759–769.

Micheletto, Y.M., Moro, C.F., Lopes, F.C., Ligabue-Braun, R., Martinelli, A.H., Marques, C.M., Schroder, A.P., Carlini, C.R., da Silveira, N.P., 2016. Interaction of jack bean (Canavalia ensiformis) urease and a derived peptide with lipid vesicles. Colloids Surf. B Biointerfaces 145, 576–585.

Mio, T., Yabe, T., Arisawa, M., Yamada-Okabe, H., 1998. The eukaryotic UDP-N-acetylglucosamine pyrophosphorylases. Gene cloning, protein expression and catalytic mechanism. J. Biol. Chem. 273, 14392–14397.

Mortimer, N.T., Kacsoh, B.Z., Keebaugh, E.Sm., Schlenke, T.A., 2012. Mgat1-dependent N-glycosylation of membrane components primes Drosophila melanogaster blood cells for the cellular encapsulation response. PLoS Pathog. 8, e1002819.

Moussian, B., 2008. The role of GlcNAc in formation and function of extracellular matrices. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 149, 215–226.

Mulinari, F., Stanisçuaski, F., Bertholdo-Vargas, L.R., Postal, M., Oliveira-Neto, O.B., Rigden, D.J., Grossi-de-Sa, M.F., Carlini, C.R., 2007. Jaburetox-2Ec: an insecticidal peptide derived from an isoform of urease from the plant Canavalia ensiformis. Peptides 28, 2042–2050.

Pandey, J.P., Tiwari, R.K., 2012. An overview of insect hemocyte science and its future application in applied and biomedical fields. Am. J. Biochem. Mol. Biol. 2, 82–105.

Piovesan, A.R., Martinelli, A.H., Ligabue-Braun, R., Schwartz, J.L., Carlini, C.R., 2014. Canavalia ensiformis urease, Jaburetox and derived peptides form ion channels in planar lipid bilayers. Arch. Biochem. Biophys. 547, 6–17.

Portugal, C.S., 2017. Avaliação dos efeitos do peptídeo recombinante Jaburetox em linhagens celulares e Drosophila melanogaster (Ph.D. Thesis). Universidade Luterana de Brasil, Canoas, Brazil.

Postal, M., Martinelli, A.H., Becker-Ritt, A.B., Ligabue-Braun, R., Demartini, D.R., Ribeiro, S.F., Pasquali, G., Gomes, V.M., Carlini, C.R., 2012. Antifungal properties of Canavalia ensiformis urease and derived peptides. Peptides 38, 22–32.

Radi, R., 2004. Nitric oxide, oxidants and protein tyrosine nitration. Proc. Natl. Acad. Sci. U. S. A. 101, 4003–4008.

Real-Guerra, R., Carlini, C.R., Stanisçuaski, F., 2013. Role of lysine and acidic amino acid residues on the insecticidal activity of Jackbean urease. Toxicon 71, 76–83.

Ribeiro, J.M.C., Nussenzveig, R.H., 1993. Nitric oxide synthase activity from a hematophagous insect salivary gland. FEBS Lett. 330, 165–168.

Stanisçuaski, F., Carlini, C.R., 2012. Plant ureases and related peptides: understanding their entomotoxic properties. Toxins 4, 55–67.

Stanisçuaski, F., Te Brugge, V., Carlini, C.R., Orchard, I., 2009. In vitro effect of Canavalia ensiformis urease and the derived peptide Jaburetox-2Ec on Rhodnius prolixus Malpighian tubules. J. Insect Physiol. 55, 255–263.